Autocrine and paracrine IL-4 maintains breast cancer stem cells traits via RAS/MAPK/DUSP pathway

Background: Despite the advent of successful treatment of localized malignancies, metastatic cancer still lacks efficacious therapeutic approaches, including breast cancer. It is now well established that within malignant tumors Cancer Stem Cells (CSCs) constitute a unique cell subset that fuel and succeed at tumor growth and metastases formation. Tumor microenvironment sustains CSCs characteristics, making the molecular mechanisms driving tumor progression and recurrence more complex and difficult to elucidate. Furthermore, the interaction that occurs between CSCs and the nearby stroma has proven to enhance the aggressive behavior of several carcinomas through the secretion of microenvironmental cytokines. In this context, IL-4 has already been described to promote survival of cancer cells through the up-regulation of several anti-apoptotic factors. However, still little is known about its role in promoting breast cancer progression.

Results: Here we show for the first time that autocrine and paracrine production of IL-4 regulates breast CSCs (BCSCs) features, including cell proliferation, motility and cytoskeletal organization via RAS/MAPK pathway. Interestingly, relief from IL-4 impaired BCSCs proliferation, colony forming efficiency and in vivo tumor formation, while it fostered the expression of the dual specificity phosphatase-4 (DUSP4) in triple-negative basal-like BCSCs, leading to the decrease of CD44+/CD24- population. DUSP4 is commonly deficient in the most aggressive breast cancers, such as the basal-like subtype. Likewise, we observed that the enforced expression of DUSP4 increases the CD24+ compartment in basal-like BCSCs, determining also a dramatic decrease of their proliferation, colony forming efficiency, invasiveness and metastases formation. Contrarily, in luminal-like BCSCs, DUSP4 suppression favors BCSCs cell traits, including their tumorigenic and metastagenic properties.

Methods: Patient-derived BCSCs were obtained by digestion of breast cancer tissues and plated in serum-free media with bFGF and EGF. DUSP4 were inserted into the p-Lenti expression vector. Stable DUSP4 knockdown was produced by lentiviral transduction of the pGFP-C vector. IL-4 function was impaired by using a high affinity IL-4Ra antagonist. To assess tumorigenicity and metastases formation, BCSCs were suspended in matrigel and injected either orthotopically or intra caudal in NOD/SCID mice.

Conclusions: These findings will shed light on the molecular basis of cancer progression and on the complex crosstalk occurring between tumor and its microenvironment. The identification of tumor-related molecular events, such as the IL-4 activated signaling, might be clinically exploited as therapeutic targets in the adjuvant setting and synergize the effect of conventional chemotherapy in patients affected by breast cancers with limited therapeutic options, such as triple-negative breast cancers.

This abstract will be presented in a Poster Session entitled Stemness Properties of Breast and Ovarian Cancer on Tuesday, April 19, 2016, 8:00 am - 12:00 noon located in Poster Section 31, Poster Board 9.
Combined PI3K and AURKA inhibition are efficacious in triple negative breast cancer models

**Introduction:** Nearly half of metastatic triple negative breast cancer (TNBC) patients develop brain metastases (BM) and face a poor prognosis. There are no FDA-approved systemic therapies to treat TNBC BM, due in part to the blood-brain barrier. BCBMs exhibit both activation of the PI3K pathway and AURKA amplification/overexpression relative to primary breast cancers. In this study, we evaluate the efficacy of brain-penetrant, clinically-available inhibitors of PI3K and AURKA in TNBC cell lines that are capable of growing in the mouse brain.

**Methods:** *In vitro* characterization of the pan-PI3K inhibitor BKM120 and AURKA inhibitor MLN8237 were conducted in 2 human-derived TNBC cell lines, SUM149 and (MDA-MB-231) Br. A siRNA screen (720 kinase genes) was used to identify synthetic enhancers of lethality with BKM120 treatment. To assess the efficacy of these drugs, the IC50s of BKM120 and MLN8237 and synergy of the combination were determined. To compare the effects of BKM120 and/or MLN8237 treatment on cell cycle progression, FACS analysis was conducted at 24, 48, and 72 hours in parent cells and in cells continuously cultured in MLN8237-treated media for 12 weeks.

**Results:** The screen confirmed that combined PI3K and AURKA inhibition synthetically enhanced lethality in SUM149 and 231Br cells. SUM149 and 231Br cells and two additional TNBC cell lines (MDA-MB-468 and MDA-MB-436) exhibited similar IC50s (1.3-21 µM) to BKM120. However, there was a >2.5 fold range (26.5-69 µM) in IC50s for MLN8237, with the greatest potency in the 231Br line. Concurrent treatment with BKM120+MLN8237 was synergistic or additive in 231Brs at most doses, whereas the combination was additive to antagonistic in SUM149s. Pretreatment with MLN8237 prior to concurrent BKM120+MLN8237 improved synergy in SUM149s, while BKM120 pretreatment improved synergy in 231Brs.

FACS analysis of BKM120 in the SUM149 and 231Br cells induced a slight G1 arrest from 24 to 72 hours, while MLN8237 initially induced a G2 arrest at 24 hours, polyploidy at 48 hours, and a mixed polyploid/G2 arrested population at 72 hours. These effects were more pronounced in the 231Brs than the SUM149s. Combined BKM120+MLN8237 in both cell lines yielded results similar to MLN8237 alone. Cells continuously exposed to increasing MLN8237 concentrations from 50 nM to 300 nM for 12 weeks were resistant to MLN8237-induced cell cycle changes as compared to passage-matched controls.

**Conclusions:** Combined PI3K+AURKA inhibition using brain-penetrant compounds is a promising strategy for a patient population with few options. *In vivo* studies evaluating the efficacy of BKM120+MLN8237 in intracranial TNBC mouse models to provide the translational foundation for future clinical studies are warranted.

This abstract will be presented in a [Poster Session](https://www.aacr.org/meetings/aacr-conferences) entitled **Targeting Protein Kinases, Death Pathways, and the Tumor Microenvironment** on **Tuesday, April 19, 2016**, **1:00 pm - 5:00 pm** located in Poster Section 19, Poster Board 29.
Glycoprotein non-metastatic melanoma B (GPNMB) is a transmembrane protein overexpressed in 30 - 40% of triple negative breast cancer (TNBC) and has shown to be associated with metastasis and disease recurrence. An anti-GPNMB antibody drug conjugate, Glembatumumab Vedotin (CDX-011), is currently in Phase II clinical trials for the treatment of metastatic TNBC patients, with promising outcomes. Positron Emission Tomography using radiolabeled antibodies could be advantageous in stratifying patients who may benefit from CDX-011, tracking the biodistribution of CDX-011, and assessing GPNMB expression in vivo. To this end, we radiolabeled the naked antibody, Glembatumumab (CR011), with the positron-emitting 89Zr (half life = 3.3 days). We characterized the stability, affinity, rate of cellular internalization, and specificity of 89Zr-CR011 using various cell-binding assays in human TNBC cell lines. We determined that 89Zr-CR011 is stable in serum solution for up to 5 days, binds specifically to GPNMB+ TNBC cells with high affinity (KD = 16 nM), and internalizes rapidly (50% within 30 - 60 min). We conducted a biodistribution study from 1 - 12 days post administration via tail vein in GPNMB+ MDA-MB-468 xenografts to determine the time point at which we achieve the optimal tumor-to-nontarget ratios. A subset of mice was administered a blocking dose of unlabeled CR011 (100-fold excess, 1 mg/mouse), where we observed a 2.5-fold reduction in 89Zr-CR011 tumor uptake, confirming the specificity of 89Zr-CR011 for GPNMB+ TNBC tumors. PET imaging studies and dosimetry calculations are currently in progress. This preliminary study demonstrates that 89Zr-CR011 may be an excellent companion diagnostic agent for CDX-011 therapy and an essential tool to assess the function of GPNMB in vivo.

This abstract will be presented in a Poster Session entitled Molecular and Cellular Imaging of Cancer 1 on Tuesday, April 19, 2016, 1:00 pm - 5:00 pm located in Poster Section 33, Poster Board 17.
In vivo efficacy of the PAK4 allosteric modulator KPT-9274 against a triple-negative breast cancer model

The p21-activated kinases (PAK) belong to a family of serine threonine kinases that promote cell survival and play an important role in cell proliferation, cell cycle regulation and cell shape determination. There are six mammalian PAK proteins which can be subdivided into two groups by sequence homology and mode of activation - Group A PAKs consisting of PAK 1, 2 and 3 and Group B PAKs consisting of PAK 4, 5 and 6. We have found that PAK4 protein levels are elevated in breast cancer, including Her2 positive and triple negative breast cancers, while it is expressed at low levels in normal mammary tissue, making it an attractive drug target. PAK inhibitors are being tested for effectiveness against solid tumors, but generation of highly specific PAK4 inhibitors has been a challenge. Furthermore, PAK4 has been reported to have kinase-independent functions. Therefore inhibiting its kinase activity alone might not be sufficient in blocking its tumorigenic potential. Our lab has previously reported the effectiveness of PAK4 allosteric modulators (PAM; KPT-8752 and KPT-9274) against multiple breast cancer cell lines. These novel PAK4 inhibitors reduce steady state protein levels and were able to block cell growth, cell migration and induce apoptosis in breast cancer cell lines, without affecting the control cells. Here, we tested the efficacy of the orally bioavailable PAM, KPT-9274 against tumors formed by the triple negative breast cancer cell line, MDA-MB-231. Following six weeks of treatment with orally administered KPT-9274 (150mg/kg bid×4), there was almost a five-fold reduction in tumor volume and tumor weight in the treatment group as compared to the control group. The treatment did not significantly affect mice body weight. After six weeks of treatment, the tumors were excised and analyzed for PAK4 levels. We observed a significant decrease in PAK4 levels in excised tumors from the treatment group as compared to those from the control group. PAK1 levels were monitored to see any off-target effects, but their levels were unchanged. Our results indicate that PAK4 plays a key functional role in triple negative breast cancer and treatment with an orally administered KPT-9274 was capable of specifically binding and inhibiting PAK4, and consequently reducing tumor growth. Future studies analyzing the effects of KPT-9274 in blocking PAK4 mediated functions that promote tumorigenesis are ongoing. Additional studies of the effectiveness of KPT-9274 on mammary fat pad tumors formed by MDA-MB-231 and the ER positive cell line, MCF7 are under investigation.

This abstract will be presented in a Poster Session entitled GTPase, RAF, and Growth Factor Pathways on Monday, April 18, 2016, 1:00 pm - 5:00 pm located in Poster Section 4, Poster Board 2.
Metastasis suppressors regulate the tumor microenvironment by blocking recruitment of pro-metastatic TAMs

Triple-negative breast cancer (TNBC) patients have the highest risk of recurrence and metastasis. Because they cannot be treated with targeted therapies, and many do not respond to chemotherapy, they represent a clinically underserved group. While physiological inhibitors of metastasis (metastasis suppressors) play key roles in regulating tumor growth, invasion and metastasis, their role in regulating the tumor microenvironment and immune system is unknown. We hypothesized that the metastasis suppressor Raf Kinase Inhibitory Protein (RKIP) regulates stromal cells, which then affect tumor invasiveness.

Using species-specific RNaseq we determined that expression of RKIP in tumors markedly reduces the number and metastatic potential of infiltrating TAMs. While TAMs isolated from TNBC xenografts drive in vitro invasion, RKIP+ derived TAMs did not drive invasion and had decreased secretion of pro-metastatic factors including SLPI, OPN, MMP-12, Galectin-3, VEGF-A, VEGF-D, TNFR2, and PGRN. We determined that RKIP regulates TAM recruitment by blocking HMGA2, which activates CCL5 expression. CCL5 rescued pro-metastatic TAM infiltration as well as tumor intravasation. We additionally showed that factors decreased in RKIP-derived TAMs were restored in CCL5-derived TAMs. CCL5 derived TAMs were also able to promote metastasis when co-injected with MDA-MB-231 tumors. These tumor cells demonstrated permanent increases in both growth and invasive potential after co-injection with highly pro-metastatic CCL5 derived TAMs.

To determine the clinical utility of these TAM genes we combined their expression with RKIP signaling in the tumor to create a signature that strikingly separates TNBC patients based on outcome.

Our results demonstrate for the first time that metastasis suppressors can regulate the microenvironment, regulating invasion through TAMs. Our results also suggest aggressive triple negative breast cancers could be controlled by attacking CCL5 derived TAMs crucial for promoting metastasis.

Funded by: GM087630, CA184494, and CA192780

This abstract will be presented in a Poster Session entitled Cellular and Molecular Mediators of Metastasis on Monday, April 18, 2016, 8:00 am - 12:00 noon located in Poster Section 29, Poster Board 4.
Identifying and targeting chemoresistant subclones in triple negative breast cancer

Triple-negative breast cancer (TNBC) is an aggressively metastatic subtype that can only be treated by chemotherapy. Nearly 50% of patients have residual disease after neoadjuvant chemotherapy (NACT) and have extremely poor prognoses. Recent genome sequencing studies have revealed extensive intratumoral heterogeneity (ITH) in treatment-naïve TNBC. However, the functional contribution of ITH to chemoresistance in TNBC is unknown. To understand how this occurs, we are generating patient derived xenograft (PDX) models from treatment-naïve TNBC in order to identify and characterize tumor cell populations that are responsible for chemoresistance with the ultimate goal of selectively targeting resistant tumor clones.

Through an IRB-approved clinical trial using standard techniques, samples (primary tumor, skin metastasis, and blood for germline reference) were obtained from a patient with newly diagnosed, untreated metastatic TNBC. Subsequently, this patient was found to have disease resistant to chemotherapy. Tumor cells were implanted into the humanized mammary fat pad of NOD/SCID mice to establish PDX models of the primary (PIM1-P) and metastatic (PIM1-M) tumors. RNA sequencing and whole-exome sequencing (~300X) were performed on the patient’s primary and metastatic tumors and the first and third passage PDX tumors. Mouse sequences were computationally subtracted from the PDX data and which was then processed according to Genome Analysis Toolkit best practices workflow. Variants were called using MuTect and copy number alterations were estimated by ExomeCN. This revealed high concordance between the genomic profiles of the patient and PDX models. While there were 81 somatic non-silent mutations shared in the patient and PDX, only a few fell in known cancer genes, including TP53 (V143fs), ELF4 (L593H), and ARID3A (R351P). Modeling of clonal clusters with ABSOLUTE revealed ITH in the patient tumor that is preserved in the PDX.

Both the patient and PDX model (PIM1-P) exhibited progressive disease when treated with paclitaxel. Only partial responses were observed in mice treated with doxorubicin plus cyclophosphamide (AC), consistent with residual disease after standard NACT for patients with TNBC. AC treatment resulted in an ~60% reduction in tumor volume which was not enhanced by repeated cycles. Tumors rapidly re-grew when treatment was halted or if treated tumor cells were engrafted into the mammary fat pads of new recipient mice. This suggests that a subpopulation of AC-resistant tumor cells is present in PIM1-P tumors. To dissect this subpopulation, we established conditions for simultaneous tracking of thousands of PIM1-P tumor clones in vivo using a high-complexity library of up to 30 million unique DNA barcodes. Mice engrafted with barcoded PIM-1P tumors are being treated with AC to identify and characterize AC-resistant tumor cells present in PIM1.

This abstract will be presented in a Poster Session entitled Intratumor Heterogeneity and Treatment Responses on Monday, April 18, 2016, 1:00 pm - 5:00 pm located in Poster Section 29, Poster Board 8.
Feasibility and efficacy of a precision treatment approach for triple-negative breast cancer in mouse models

15-20% of human breast tumors are triple negative breast cancer (TNBC), an aggressive and deadly subtype of breast cancer that currently lack targeted therapies, leaving chemotherapy as the only systemic treatment option. There is a pressing need for a better understanding of disease mechanisms of TNBC, and for the development of new treatment options. While activating mutations of PIK3CA are frequently found in ER-positive and Her2-amplified breast cancer, inactivation of lipid phosphatases is more frequent in TNBC. Analysis of large human genomics data in TCGA reveals that heterozygous-loss of INPP4B, a lipid phosphates in the PI3K signaling pathway, is enriched in TNBC subtype, and strongly correlates with loss of ER expression. Guided by human genomics information, we have crossed INPP4B phosphatase deletion mice into TNBC mouse model to determine whether INPP4B loss cooperates with loss-of-function of p53 and/or Brca1 to promote tumorigenesis in vivo. Our results show dose-dependent increase in tumor development frequency in K14cre; Brca1$^{\text{flox/flox}}$; p53$^{\text{flox/flox}}$ mice carrying INPP4B Phosphatase KO allele, HET allele compared to INPP4B WT allele. Importantly, these tumors resemble human TNBC in their pathology, histological patterns and gene expression patterns, providing a valuable platform to test their responsiveness to various therapeutic drugs, including PI3K-inhibitors. Our goal is to generate data that form the basis for a phase I/II clinical study that lead to substantially improved treatment strategies for basal-like breast cancer. Toward this goal, we have banked the endogenous tumors in a way allowing transplantation in nude mice, and have performed randomized drug treatment studies. We found that tumors that are heterozygous or deletion for INPP4B are more sensitive to PI3K-inhibition, suggesting these tumors are more dependent on PI3K activation to thrive. Although tumors show partial response early on, they inevitably relapsed, begging for an understanding of innate and selected drug-resistant mechanisms. To this end, we have completed large-scale RNAseq and whole exome sequencing (WES), and have performed in-depth analyses on the mouse genomics data to identify genetic alternations including chromosomal number variations (CNVs), mutations and gene fusions. Focusing on gene-fusions, we found that by targeting these fusions, we can achieve better treatment outcomes and in some cases, even complete tumor remission. Significantly TCGA data analyses revealed the presence of similar fusions in human TNBC patients and we are in the process of investigating whether human cells carrying these fusions respond equally well compared to their mouse counterparts. Our approach of precision-medicine guided treatment optimization has lead to substantially improved treatment outcomes.

This abstract will be presented in a Poster Session entitled Targeting Protein Kinases, Death Pathways, and the Tumor Microenvironment on Tuesday, April 19, 2016, 1:00 pm - 5:00 pm located in Poster Section 19, Poster Board 20.
BET protein inhibition blocks growth of triple-negative breast cancer by inducing mitotic and cytokinetic dysfunction

Bromodomain and Extra Terminal (BET) proteins are epigenetic “readers” that recognize acetylated histones and mark areas of the genome for transcription. BRD4, a BET family member protein, has been implicated in a number of types of cancer. It has been recently found to associate with super-enhancers, and elevated levels of BRD4 have been linked to increased expression of MYC as well as other oncogenes. BET protein inhibitors are currently being tested for their potential use in the treatment of HIV, heart failure, and cancer, all of which are diseases of aberrant transcription. However, little is known regarding their efficacy in triple-negative breast cancer (TNBC). We found JQ1, a prototypical BET inhibitor, impedes growth of seven TNBC cell lines in a dose-dependent manner within 72 hours of treatment. JQ1 also suppresses growth of three different TNBC xenografts, including a patient-derived model of basal-like breast cancer. Growth arrest, in vitro, is followed by either apoptosis or senescence. However, prior to the induction of these two terminal responses, prolonged treatment results in polyploidy in many of the cell lines, suggesting BETi disrupts mitosis/cytokinesis. Live-cell imaging revealed JQ1 significantly increased the duration of mitosis, and microarray analyses showed a significant JQ1-mediated downregulation of genes critical for cell cycle progression, mitosis, and cytokinesis. In all TNBC cell lines tested, Aurora kinases, proteins critical for proper progression through mitosis and cytokinesis, are suppressed in response to JQ1. Treatment with AZD1152, an Aurora kinase B inhibitor, elicits the same cellular responses as BET inhibition, indicating that the suppression of Aurora kinases plays a key role in the response of TNBC cells to BET inhibitors. These findings reveal that BET inhibitors block the growth of highly aggressive TNBC cells by inducing mitotic dysfunction and that these drugs are promising potential therapeutics for the treatment of TNBC.

This abstract will be presented in a Poster Session entitled Cellular Responses to Anticancer Drugs on Wednesday, April 20, 2016, 8:00 am - 12:00 noon located in Poster Section 14, Poster Board 18.
Jessica M. Wagner, BS  
Fox Chase Cancer Center: Temple College of Medicine, Philadelphia, PA  
Abstract # 3000

Intra-tumoral accumulation of NK1.1/CD3+ cells and anti-metastasis effects of dose-intensified ONC201 in tumor-bearing mice

ONC201, a novel first-in-class, orally active anti-tumor agent that upregulates the cytotoxic ligand TRAIL (Allen et al., Sci. Trans. Med., 2013; Wagner et al., Oncotarget, 2014), activates the integrated stress response leading to tumor cell upregulation of TRAIL death receptor 5 (Kline et al., Sci. Sig., in press). ONC201 is active against bulk tumor and cancer stem cells (Prabhu et al., Can. Res., 2015). ONC201 is under clinical development by Oncoceutics, and is being evaluated in multiple phase I/II clinical trials. Results of the first-in-human ONC201 study presented at the 2015 AACR-NCI-EORTC meeting (Stein et al., Abstract C138) demonstrated ONC201 to be safe in humans, to exhibit predicted PK and sustained PD characteristics, and revealed a preliminary efficacy signal. As patients were dosed on an every 3-week schedule, based on supportive preclinical data, we investigated dose-intensification of ONC201 to determine whether a higher dose/frequency schedule might impact efficacy with limited toxicity. We hypothesized that ONC201 may be effective in dose-intensified schedule and may inhibit metastases. We tested a range of ONC201 doses including 25, 50, and 100 mg/kg and frequencies including every 4, 3, 2, 1 week as well as twice a week dosing. In colon and triple-negative breast cancer we observed that ONC201 exerts a dose- and schedule-dependent effect on tumor progression in vivo. Frequency effects were more pronounced at lower doses and dose-dependency was more impactful with less frequent schedules. We noted a potent anti-metastasis effect of ONC201 in vivo, not previously reported, as a function of both increased ONC201 dose and frequency of administration. ONC201 inhibits cancer cell migration and invasion in vitro in a TRAIL-dependent manner. We found ONC201 more potently suppresses Akt and ERK in tumors in vivo in a dose- and frequency-dependent manner, whereas its effect on TRAIL serum levels appeared to be impacted by frequency. We observed accumulation of CD3+/NK1.1+ cells within ONC201-treated tumors in athymic nude mice that lack T-cells. Accumulation of CD3+/NK1.1 cells within ONC201-treated tumors was more pronounced with dose intensification that correlated with superior efficacy. In summary, we have uncovered a potent anti-metastasis effect of ONC201 coupled with the appearance of CD3+/NK1.1+ cells within ONC201-treated tumors. We are further evaluating the biomarker characteristics and immune function of the CD3+/NK1.1+ cells and the relationship of their intra-tumoral accumulation to observed anti-tumor effects of ONC201, including in fully immunocompetent mice. Our results suggest that clinical investigation of both dose and frequency of ONC201 administration is warranted and is being evaluated in an ongoing clinical trial (NCT02609230).

This abstract will be presented in a Poster Session entitled New Mechanisms of Anticancer Drug Action on Tuesday, April 19, 2016, 8:00 am - 12:00 noon located in Poster Section 16, Poster Board 20.
Fibroblast growth factor-2-derived from cancer-associated fibroblasts stimulates proliferation and migration of human breast cancer cells

Cancer-associated fibroblasts (CAFs) constitute a major compartment of the tumor microenvironment. CAFs produce a variety of cytokines, growth factors and extracellular matrix proteins, thereby stimulating tumor progression. CAFs are distinct from normal fibroblasts for their overexpression of α-smooth muscle actin and fibroblast specific protein-1. Recent studies suggest that CAFs play an important role in proliferation and migration of cancer cells through crosstalk with them. In the present study, we investigated the role for CAFs in proliferation and migration of breast cancer cells and underlying molecular mechanisms. When triple-negative human mammary MDA-MB-231 cells were treated with the conditioned medium (CM) collected from cultured CAFs, the cell viability and migration were significantly elevated. Furthermore, these cells manifest a more proliferative phenotype, exhibiting enhanced mRNA expression of cycinD1, c-Myc and PCNA as well as increased phosphorylation of Akt and STAT3. In addition, MDA-MB-231 cells exhibited elevated expression of proliferative and invasive genes including MMP2 and MMP9 when incubated with CAFs in the indirect co-culture system. Notably, mRNA levels of fibroblast growth factor-2 (FGF2), stromal-derived factor-1, interleukin-6 and interleukin-8 detected in CAFs were higher than those in normal fibroblasts of the same patients. In contrast, FGF2 was expressed at a relatively low level in breast cancer cells. FGF2 exerts its biological effects by binding to and activating FGF receptor (FGFR), a subfamily of cell surface receptor tyrosine kinases. Notably, the expression of FGFR1 was up-regulated in triple-negative breast cancer cells including MDA-MB-231 cells while another major FGF2-corresponding receptor FGFR2 was rarely expressed. Therefore, we focused on FGF2-FGFR1 signaling in the context of paracrine communication between breast cancer cells and CAFs. CAF-stimulated MDA-MB-231 cell migration as well as FGFR1 expression was abolished when FGF2-neutralizing antibody was added to the CAF-CM. In addition, treatment of MDA-MB-231 cells with FGF2 induced the phosphorylation of FRS2 and Akt. FGF2-induced cell migration and up-regulation of cyclinD1 expression were abrogated by siRNA-mediated FGFR1 silencing. Furthermore, FGF2 promotes nuclear localization of FGFR1. Taken together, above findings suggest that secretion of FGF2 by CAFs stimulates proliferation and migration of breast cancer cells through interaction with FGFR1 which may contribute to human breast cancer progression.

This abstract will be presented in a Poster Session entitled Host-Tumor Interactions on Tuesday, April 19, 2016, 1:00 pm - 5:00 pm located in Poster Section 29, Poster Board 23.
Macrophages increase the expression of RhoC in inflammatory breast cancer leading to increased migration

Inflammatory breast cancer (IBC) is considered the most lethal form of breast cancer due to its ability to progress quickly and the frequent presence of metastasis at diagnosis. African Americans are disproportionately diagnosed with IBC and often have worse outcomes than Caucasians. By investigating IBC in both African American and Caucasian cell lines we seek to understand the differences in IBC progression and help address disparities by providing new anti-IBC strategies. RhoC GTPase is overexpressed in 90% of IBC tumors and is known to increase cell motility. Sites of inflammation, as seen in IBC, attract tumor associated macrophages (TAMs), which have been found to facilitate the movement and invasion of many breast cancers. We hypothesize that TAMs play a role in increasing RhoC expression in IBC cell lines, consequently leading to IBC’s severe migratory and metastatic potential.

A novel microfluidic device created by our team was used to measure the migratory phenotype of IBC cell lines in response to macrophage conditioned media (CM) and cytokine stimulation. IBC cell lines were treated with CM, cytokines, or pathway inhibitors then Western blotting was used to determine protein expression and phosphorylation to identify important signaling pathways.

We found the expression of RhoC significantly increased in two different IBC cell lines, SUM149 (African American) and SUM190 (Caucasian), after culturing with conditioned media from the macrophage-differentiated U937 monocytic cell line. This increase was not detected in either the normal-like MCF-10A breast epithelial cell line or the non-IBC MDA-MB-231 triple negative breast cancer cell line. CM caused a significant increase in the migration distance and frequency of both SUM149 and SUM190 cell lines. Analysis of the CM determined CCL2, CCL5, and IL-8 to be the key mediators in the macrophage CM. Western blotting proposes that CCL2, CCL5, and IL-8 stimulation causes twice as much RhoC expression compared to the control. Further analysis suggests a role for the MAPK pathway in controlling RhoC expression and migration.

Macrophage conditioned media causes an increase in RhoC expression in IBC cell lines and stimulates migration. Individual cytokines can lead to an increase in RhoC possibly through the MAPK pathway. Studies involving RhoC inhibitors are ongoing and could yield promising therapies for the prevention of metastasis in IBC. By understanding the specific mechanism of TAMs’ effects on IBC, we hope to learn how to control the lethal metastatic nature of IBC and improve outcomes for patients of all ethnicities.

This abstract will be presented in a Poster Session entitled Innate Immune System, Myeloid Cells, and Tumorigenesis on Tuesday, April 19, 2016, 8:00 am - 12:00 noon located in Poster Section 26, Poster Board 9.
Mary Kathryn Pitner, PhD
The University of Texas MD Anderson Cancer Center, Houston, TX
Abstract # 1624

Silencing of ERK2 reverses EMT and suppresses the CSC phenotype, inhibiting lung metastasis in triple-negative breast cancer

Background: Triple-negative breast cancer (TNBC) is an aggressive subtype lacking estrogen receptor, progesterone receptor, and HER2 overexpression. Patients with TNBC have a generally poor prognosis due to metastasis, high rates of recurrence, and lack of FDA-approved targeted therapies. We previously showed using functional proteomics that patients with high-ERK2-expressing TNBC tumors had a higher risk of death than those with low-ERK2-expressing tumors. Moreover, ERK2 but not ERK1 plays an important role in epithelial-mesenchymal transition (EMT) and is required for acquisition of stem cell-like characteristics. Compared to other breast cancer subtypes, TNBC has a higher proportion of cancer stem cells (CSCs) and is linked to EMT, two critical features associated with breast cancer progression, metastasis, and recurrence in patients. The MAPK signaling pathway is activated in TNBC, but the roles of ERK isoforms in tumor progression and metastasis are not well defined. We hypothesized that ERK2 but not ERK1 promotes EMT, the CSC phenotype, and metastasis in TNBC.

Methods and Results: Knockdown of ERK2 in SUM149 and BT549 TNBC cells significantly inhibited anchorage-independent colony formation (p<0.0001), inhibited formation of mammospheres (p=0.003), and reduced the CSC population (CD44+/CD24-) (p=0.002) in vitro. This effect correlated with a reduction in migration (p=0.0004) and invasion (p<0.0001). SCID-beige mice injected via the tail vein with SUM149 shERK2 cells had a significantly lower lung metastatic burden than control mice (p=0.0034) or mice injected with shERK1 cells (p=0.0012), suggesting that ERK2 is a mediator of metastatic burden. To determine the mechanism by which ERK2 mediates this phenotype, we performed an Affymetrix Human Genome U133 Plus 2.0 array and compared the gene expression levels between SUM149 cells with ERK2 or ERK1 knockdown or transfection with control shRNA. Analysis of microarray data revealed that global gene expression changes associated with ERK2 knockdown predominantly altered regulation of the EMT pathway. Among the genes with ERK2-knockdown-associated expression change was EGR1, an immediate early response transcription factor whose downstream targets affect cell growth and differentiation. EGR1 is down-regulated 6-fold (p=0.00013) compared to control and shERK1 cells. Knockdown of ERK2, but not ERK1, resulted in significantly lower EGR1 at both the mRNA and protein levels, validating our microarray data.

Conclusions and Future Directions: Our findings support our hypothesis, indicating that ERK2 promotes EMT and the CSC phenotype through EGR1 and mediates metastasis in TNBC. Future studies will determine ERK activity and pathway engagement using a novel peptide sensor based on the Sox fluorophore. We will pursue a therapeutic approach using siRNA against ERK2 incorporated in a DOTAP:cholesterol liposome.

This abstract will be presented in a Poster Session entitled Metastasis-Promoting and -Suppressing Genes on Monday, April 18, 2016, 8:00 am - 12:00 noon located in Poster Section 31, Poster Board 11.
Roman Camarda, BSc
University of California, San Francisco, San Francisco, CA
Abstract # 2673

Inhibition of fatty-acid oxidation as a therapy for MYC-overexpressing triple-negative breast cancer

Expression of the oncogenic transcription factor MYC is disproportionately elevated in triple-negative breast cancer (TNBC) compared to estrogen, progesterone and/or human epidermal growth factor 2 receptor-positive (RP) breast tumors. We and others have shown that MYC alters metabolism during tumorigenesis. However, the role of MYC in TNBC metabolism remains largely unexplored. We hypothesized that pharmacologic inhibition of MYC-driven metabolic pathways may serve as a therapeutic strategy for this clinically challenging subtype of breast cancer. Using a targeted metabolomics approach, we identified fatty-acid oxidation (FAO) intermediates as dramatically upregulated in a MYC-driven model of TNBC. A lipid metabolism gene signature was identified in patients with TNBC in the TCGA and multiple other clinical datasets, implicating FAO as a dysregulated pathway critical for TNBC metabolism. We find that MYC-overexpressing TNBC, including a transgenic model and patient-derived xenograft (PDX), display increased bioenergetic reliance upon FAO. Pharmacologic inhibition of FAO catastrophically decreases energy metabolism of MYC-overexpressing breast cancer, blocks growth of a MYC-driven transgenic TNBC model and MYC-overexpressing PDX. Our results demonstrate that inhibition of FAO is a novel therapeutic strategy against TNBCs that overexpress MYC.

This abstract will be presented in a Minisymposium entitled Mechanisms and Vulnerabilities of Metabolic Reprogramming on Monday, April 18, 2016, 3:00 pm - 5:00 pm located in Room 265, Morial Convention Center.
Benzyl isothiocyanate mediates glucose uptake through AKT activation in breast cancer cells

We have shown previously that benzyl isothiocyanate (BITC) administration retards the development of mouse mammary tumors driven by Her-2 oncogene proving in vivo evidence for its chemopreventive efficacy. However, we also observed an increase in glucose uptake by tumor in BITC treatment group compared with control mice. Proteomics using tumors from control and BITC-treated mice revealed changes in proteins related to metabolism. Thus, we studied the mechanism by which BITC increases glucose uptake using four cell lines with different genetic backgrounds (e.g., ER+, ER-, HER-2+, triple negative). Consistent with in vivo findings, BITC treatment enhanced the protein levels of glucose transporters (GLUT1 and GLUT4) regardless of ER, HER-2 or p53 status. On the other hand, lactate dehydrogenase (LDH) was downregulated in response to BITC treatment. Pyruvate kinase (PKM2) as well as tricarboxylic cycle (TCA cycle) related enzymes (IDH1, PDH, MDH) were upregulated in BITC treated cells compared with control. In agreement with these results, BITC treatment increased glucose, pyruvate, and acetyl-CoA levels but decreases lactate level. These results suggested that elevated glucose after BITC treatment was largely utilized through TCA cycle. Because AKT is known to regulate tumor metabolism including glycolysis and oxidative phosphorylation, we explored its involvement in BITC-mediated metabolic alterations. To our surprise, BITC treatment resulted in a marked increase in activation of AKT. Pharmacological inhibition of AKT antagonized BITC-mediated increase in GLUT1 expression as well as increase in glucose and pyruvate levels. In addition, AKT inhibition augmented BITC-mediated inhibition of cell survival (colony formation), induction of apoptosis, and suppression of migration in human breast cancer cells. Based on these findings, we propose that mammary cancer chemopreventive efficacy of BITC may be augmented by a combination regimen involving an AKT inhibitor. This study was supported by the grantCA129347 awarded by the National Cancer Institute.

This abstract will be presented in a Poster Session entitled Models and Mechanisms in Cancer Prevention on Sunday, April 17, 2016, 1:00 pm - 5:00 pm located in Poster Section 37, Poster Board 13.
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Abstract # 4275

Exploring the metastatic potential of exosomal NM23 signaling using a triple negative breast cancer model in mice

Decades of research has established the idea that cancer cells of the primary tumor prime the metastatic microenvironment prior to establishing metastases at distant sites in the body. While this process is thought to facilitate neocolonization of dislodged primary tumor cells, the mechanisms by which cancer cells communicate to their remote targets remain largely unknown. Our lab has previously shown that triple negative breast cancer cells release exosomes carrying NM23, a nucleoside diphosphate kinase (eNDPK) implicated in promoting angiogenesis and pro-metastatic events extracellularly. To further elucidate the role of eNDPK in breast cancer signaling, we are developing a novel murine model that will allow us to examine its effects in vivo, as well as establish a timeline for the occurrence of metastases.

To establish our metastasis model in mice, we first showed that injecting human MDA-MB-231 cells into the mammary fat pad results in the formation of a primary tumor and subsequent development of metastases in the lung. Treating mice with inhibitors of NDPK activity reduces the size of the primary tumor and prevents secondary tumor formation in the lung. To mimic the pulmonary tumor microenvironment, we have isolated endothelial cells from the lungs of 6-8 day old mouse pups using magnetic beads conjugated to the endothelial cell markers CD54, CD102, or CD106. Purity of endothelial cells was confirmed by immunofluorescence staining. In preparation for in vivo experiments that demonstrate eNDPK targeting to lung endothelial cells, we have 3D printed biocompatible polylactic acid (PLA) tubular inserts that are capable of supporting lung endothelial cells encompassed in a gelatinous cell scaffold. Inserts containing lung endothelial cells are surgically introduced into the subcutaneous tissue of adult SCID mice. Human MDA-MB-231 cells expressing GFP-tagged tetraspanin are then injected orthotopically into the mammary fat pads of mice. After a period of 7-21 days, the inserts are excised and analyzed for the presence of GFP-labeled exosomes containing eNDPK and reorganization of the insert microenvironment.

Using this model for metastasis, we will be able to confirm the involvement of eNDPK in cell-cell communication between primary tumor cells and their targets in vivo. The significance of uncovering the mechanism(s) by which cancer cells metastasize is emphasized by the fact that recurrence of cancer at distant sites is associated with the most negative outcome in women diagnosed with breast cancer. Implication of eNDPK signaling in metastasis will lead to future research in developing novel small molecule inhibitors. Further, eNDPK can be used as a biomarker for the beginning stages of metastatic breast cancer, replacing current unreliable early detection methods and improving long-term survival outcomes.

This abstract will be presented in a Poster Session entitled New Cell Lines and 3D Models on Tuesday, April 19, 2016, 1:00 pm - 5:00 pm located in Poster Section 35, Poster Board 25.
Phosphorylation regulates EZH2 neoplastic functions in triple-negative breast cancer

Background: Triple negative (estrogen receptor, progesterone receptor, HER2-neu negative) breast cancers (TNBC) comprise 15% of all breast cancers but are responsible for a disproportionately high number of deaths. Overexpression of the histone methyltransferase EZH2 (Enhancer of Zeste Homolog 2) is an independent prognostic biomarker significantly associated with poorly-differentiated TNBCs and poor patient outcome. We previously identified a novel link between EZH2 and the p38 mitogen-activated protein kinase, an important mediator of progression and metastasis of TNBC. We found that EZH2 binds to p38, and that EZH2 and activated p38 are concordantly expressed in the metastases of breast cancer patients. Based on these data and previous in vitro studies, we hypothesized that p38 MAPK may also regulate EZH2 through phosphorylation in breast cancer. We further hypothesized that this phosphorylation event may be important for EZH2 contribution to malignancy.

Methods: In order to test this hypothesis, we performed knockdown rescue experiments in triple-negative breast cancer cell lines MDA-MB-231 and MDA-MB-436. Stable knockdown of EZH2 was achieved using shRNA targeting the 3’UTR. Knockdown of EZH2 was rescued by reintroduction of myc-EZH2 (WT) or a T367A-EZH2 phosphorylation-deficient mutant. Cell lines were then used in functional assays of proliferation, migration, and invasion. In order to further interrogate the importance of this phosphorylation event, we developed a phospho-specific EZH2 T367 antibody.

Results: p38-mediated phosphorylation of EZH2 at T367 contributes to the migratory and invasive properties of TNBC. Mechanistically, phosphorylation by p38 does not affect binding to other PRC2 members but may affect EZH2 activity. We are currently investigating the relevance of pEZH2 as a biomarker of breast cancer survival with our new antibody.

Conclusions: We provide evidence that p38 phosphorylation of EZH2 at T367 contributes to malignancy of triple-negative breast cancers. Our data suggest a new mechanism by which EZH2 is regulated and may offer an additional mechanism by which EZH2 contributes to TNBC progression.

This abstract will be presented in a Poster Session entitled Epigenetic Biomarkers and Therapies on Wednesday, April 20, 2016, 8:00 am - 12:00 noon located in Poster Section 4, Poster Board 25.
PKCα mediates FOXC2 transcriptional repression of p120-catenin in breast cancer

Background: Protein kinase C alpha (PKCα) has been studied as a predictive biomarker for breast cancer aggressiveness and resistance to therapy. In this study, we identified a novel signaling pathway regulated by PKCα in breast cancer cells that involves FOXC2 and p120-catenin (p120), a prominent member of adherens junctions (AJs). We report here that PKCα causes dissolution of AJs, a mechanism which contributes to cancer cells becoming more migratory and invasive, two key features of metastasizing cells. Interestingly, we found that this mechanism is relevant in both estrogen receptor (ER)-positive, endocrine resistant as well as triple negative breast cancer (TNBC) (lack of ER, progesterone receptor, and HER2/neu amplification).

Methods: ER+, endocrine resistant (T47D/PKCα and T47D:A18-TAM1) and TNBC cell lines (HCC1143 and HCC1937) were cultured according to previous publication and ATCC guidelines. Migration and invasion assays were done in Transwell® polycarbonate membranes (Corning). Promoter activity of p120 was measured by luciferase assay using p120 reporter construct obtained from Dr. Fariborz Mortazavi (Division of Hematology/Oncology, UCLA). Chromatin immunoprecipitation (ChIP) to assess binding of FOXC2 on p120 promoter was done using ChIP-grade FOXC2 antibody (Abcam). Lipofectamine®2000 (Invitrogen) was used for transfection of PKCα (Sigma) and FOXC2 (IDT) siRNAs

Results and Conclusions: We found that FOXC2 is a downstream target of PKCα; activation of PKCα by a phorbol ester significantly up-regulated FOXC2 mRNA and knockdown of PKCα by siRNA reduced expression of FOXC2 at both the mRNA and protein level. Immunofluorescence staining showed that depletion of PKCα rescued E-cadherin, secondary to the recovery of p120 expression at the AJ. We demonstrated by ChIP that FOXC2 binds to the promoter region and represses transcription of p120, indicating a novel finding that FOXC2 is a transcriptional repressor of p120 in breast cancer. Therefore, through FOXC2, PKCα can negatively regulate the AJ complex integrity. Correspondingly, knockdown of either PKCα or FOXC2 led to a reduction in the migration and invasion, concomitant with an increase of p120 at the AJ. Data obtained from the Cancer Genome Atlas (TCGA) indicates that high co-expression of PKCα and FOXC2 along with low expression level of p120 are characteristic of TNBC patients, confirming the relevance of this signaling axis in clinical samples. In conclusion, we have identified a new mechanism for migration and invasion in breast cancer cells: in the presence of PKCα, transcriptional repression of p120 by FOXC2 results in down-regulation of E-cadherin and dissociation of the AJs. Notably, we demonstrated that this mechanism is relevant in two distinct molecular subtypes of breast cancer: ER+, endocrine resistant and TNBC. Targeting the PKCα-FOXC2-p120 axis has the potential to reduce the metastatic capacity of these cancer cells.

This abstract will be presented in a Poster Session entitled Molecular Regulation of Tumor Invasion on Sunday, April 17, 2016, 1:00 pm - 5:00 pm located in Poster Section 31, Poster Board 1.