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The molecular therapeutics currently available for the treatment of breast cancer (BC) do not effectively treat metastatic disease. Recent work suggests FGFR plays critical roles in BC progression, but therapeutic targeting of this signaling pathway is limited by the currently available small molecule inhibitors and the lack of effective diagnostics. Herein, we identify β3 integrin and FGFR1 as part of an epithelial-mesenchymal transition program, both of which are required to facilitate cellular growth in response to fibroblast growth factor-2 (FGF2). Mechanistically, β3 integrin physically disrupts an interaction between FGFR1 and E-cadherin, leading to enhanced FGF2 signaling and increased three dimensional outgrowth of metastatic BC cells. Consistent with these mechanistic data, we demonstrate that FGFR, β3 integrin and focal adhesion kinase (FAK) constitute a molecular signature capable of predicting decreased patient survival. Importantly, covalent targeting of a conserved cysteine in the P-loop of FGFR1,2,3 and 4 with a newly developed small molecule, FIIN-4, blocks β3 integrin/FGFR signaling. In vivo application of FIIN-4 potently inhibited the growth of metastatic, patient-derived BC xenografts and murine-derived metastases growing within the pulmonary microenvironment. Overall, our data provide novel strategies to identify and treat BC patients with the most aggressive forms of the disease.
INTRODUCTION: Patients with Triple-Negative Breast Cancer (TNBC) who have residual disease (RD) after neoadjuvant chemotherapy (NAC) are at an increased risk of relapse and death. These patients display a marked diversity in outcome, thus markers that can predict prognosis would be of benefit. BRE09-146 was a Phase II post-neoadjuvant clinical trial testing Cisplatin + Rucaparib versus Cisplatin alone in TNBC patients with RD post-NAC. TP53 is mutated in 70-80% of TNBCs, but its prognostic potential has yet to be elucidated. Herein, we seek to comprehensively determine the prognostic capability of mutated TP53 to determine outcome with post-neoadjuvant Cisplatin in BRE09-146.

METHODS: We performed sequence and copy number analysis of 134 genes in 76 tumors from BRE09-146 using the Oncomine Research Panel along with Ion Proton Next Generation Sequencing. All patients included had RD. Somatic mutations were called by identifying mutations that were present in the tumor that were not present in the germ line DNA from a normal blood sample. Mutations were annotated using the IARC TP53 somatic mutation database. Gene copy numbers in tumors were identified using the Ion Reporter System and called as copy number loss, normal, or gain based upon a comparison to a reference range established from the normal blood samples. Survival analyses were generated using Log-Rank and Kaplan-Meier statistics.

RESULTS: TP53 was highly mutated in this patient cohort (92% incidence). TP53 copy loss was independently associated with disease-free survival (DFS) and overall survival (OS); DFS events = 50% vs. 34%; median = 19.09 mos. vs. NR (Not Reached); p=0.03; HR=2.27 (95% C.I.: 1.13-7.28); OS events = 40% vs. 37%; median = 24.02 mos. vs. NR; p=0.02; HR=2.64 (95% C.I.: 1.28-11.35). Cancers without a TP53 mutation or subclonal TP53 mutation (present in a fraction of cells) demonstrated superior OS; events = 40% vs. 10%; median = 29.14 mos. vs. NR; p=0.005; HR=6.00 (95% C.I.: 1.46-7.96). Likewise, cancers bearing compound mutations (point mutation plus loss-of-heterozygosity/copy loss) were correlated with inferior DFS and OS; DFS events = 50% vs. 26%; median = 19.09 mos. vs. NR; p=0.009; HR=2.64 (95% C.I.: 1.30-5.67); OS events = 45% vs. 16%; median = 24.02 mos. vs. NR; p=0.0007; HR=4.26 (95% C.I.: 1.89-10.01).

CONCLUSIONS: It is well-known that TP53 is heavily mutated in TNBC, yet to-date, realizing the prognostic potential for this marker has not been accomplished. We have demonstrated the prognostic significance of TP53 mutations for TNBC patients with residual disease after NAC. Additionally, we have uncovered p53 dysfunction as a continuum in this patient population in which cancers with higher degrees of dysfunction are correlated with a poorer prognosis and cancers with less dysfunction a superior prognosis.

Anticancer drug target identification and/or validation Research Technician
NOVEL BIOMARKERS AND MOLECULAR ALTERATIONS FOR BREAST CANCER INITIATION AND SUSCEPTIBILITY

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Background: Despite significant advances in diagnosis and treatment, breast cancer remains the leading cause of cancer-related death in women worldwide. Therefore, there is a critical need to identify molecular mechanisms responsible for cancer initiation and progression. To date, there have been attempts to identify biomarkers that can predict progression of pre-malignant lesions (i.e. DCIS) to invasive carcinoma. Our study aims to identify earliest markers of breast cancer initiation.

Methods: We evaluated local (breast tissue) and systemic (serum/plasma) molecular alterations associated with breast cancer susceptibility using the unique resources available at the Komen Normal Tissue Bank at IUSCC. Human specimens (serum/plasma and breast tissue) donated by women before their cancer was clinically detectable were used to identify circulating biomarkers that are associated with breast cancer risk. Specimens from age-matched controls were used for comparison. Sera/plasma were analyzed for circulating miRNAs and telomeric level in the cell-free circulating DNA (cfDNA). We employed next generation sequencing to obtain transcriptome in the “normal” breast of women who eventually developed breast cancer and age-matched control normal breast.

Results/Discussion: Serum/plasma of women who developed breast cancer showed variation in circulating miRNAs as well as telomeric cfDNA levels as compared to the healthy control group. Among 385 microRNAs detected in circulation, 10 miRNAs were present at different levels in the susceptible group. In addition, susceptible group displayed reduced levels of plasma telomeric cfDNA and telomere shortening in breast epithelium. Transcriptome analysis of microdissected breast epithelium and stroma revealed molecular alterations in the “normal” breast tissue associated with cancer development. Preliminary data suggest inflammatory response, transcription regulation and lipid metabolism as major mechanisms associated with breast cancer initiation.

Conclusion/Impact: Functional analysis of biomarkers identified in this study may help in cancer risk assessment and improvement of preventive therapy.
STAT3 MEDIATES APC-DRIVEN CHEMORESISTANCE IN BREAST CANCER

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Introduction: Breast tumors frequently develop resistance to treatment with a variety of standard (i.e. cisplatin or doxorubicin) or targeted chemotherapeutic agents, resulting in tumor recurrence and increased patient mortality. The Adenomatous Polyposis Coli (APC) tumor suppressor is lost by hypermethylation or mutation in up to 70% of sporadic breast cancers; however, the downstream effects of APC loss have not been well explored in breast cancer. Heterozygous \textit{Apc} mutation using the \textit{Apc\textsuperscript{Min/+}} mouse model enhanced tumorigenesis in the mouse mammary tumor virus – Polyoma Middle T transgenic model (MMTV-PyMT). Cells isolated from MMTV-PyMT;Apc\textsuperscript{Min/+} mice are resistant to chemotherapy, express higher levels of multidrug resistance protein 1 (MDR1), and have a greater population of tumor initiating cells (TICs) compared to controls.

Methods: Using western blot and flow cytometric analysis, we have investigated the role of signal transducer and activator of transcription 3 (STAT3) in regulation of APC-mediated chemoresistance.

Results: Here we demonstrate that APC loss-of-function cells have increased activation of STAT3, and the anti-apoptotic proteins Bcl-2 and Mcl-1. STAT3 over-expression is common in breast cancer, leads to poor prognosis, and up-regulates MDR1 expression leading to chemotherapeutic resistance. We have made the novel observation that chemotherapy treatment has a time-dependent effect on the activation of STAT3 and expression of Mcl-1 specific to \textit{Apc}-mutant cells. To explore the functional role of STAT3 in mediating therapeutic resistance in APC loss-of-function models, we examined the impact of STAT3 inhibition on MDR1 and the TIC population to cause therapeutic resistance in the MMTV-PyMT;Apc\textsuperscript{Min/+} vs MMTV-PyMT;Apc\textsuperscript{+/+} murine breast cancer cells.

Conclusions: Future studies will use STAT3 inhibitors in combination with standard chemotherapy to alleviate APC-mediated resistance. Combined these data suggest that loss of APC activates STAT3-driven pathways resulting in the development of chemotherapeutic resistance.
Amplification of epidermal growth factor receptor (EGFR) is capable of transforming mammary epithelial cells, and contributes to several early events in tumor invasion and dissemination. However, during the later stages of metastasis the function of EGFR paradoxically shifts to an inhibitor of cell growth. Herein, we demonstrate that EGFR overexpression results in a strict localization to the plasma membrane in mammary epithelial cells. However, following EMT and metastasis these same cells displayed enhanced nuclear trafficking of EGFR (nEGFR). Importantly, even though these metastatic breast cancer cells express reduced overall levels of EGFR they display an enhanced ability to activate signaling transducer and activator of transcription 1 and 3 (Stat1 and 3) in response to EGF stimulation. To further understand the role of EGFR:Stat signaling we pharmacologically inhibited nEGFR function using our recently described, gefitinib analogue that is chemically conjugated to an SV40 nuclear localization sequence. Nuclear targeting of EGFR results in specific inhibition of EGF:Stat signaling while EGF-mediated activation of extracellular regulated kinase 1/2 (Erk1/2) remained unaffected in several different breast cancer models. Importantly, blockade of nEGFR signaling inhibited EGF-induced apoptosis and/or EGF-mediated growth arrest. Moreover, specific blockade of EGF:Erk1/2 signaling using Trametinib lead to a dramatic increase in EGF-induced apoptosis in metastatic breast cancer cells. Overall, our studies demonstrate that the paradoxical function of EGFR through breast cancer metastasis results due to an imbalance in MAPK versus Stat signaling that is governed by changes in the subcellular trafficking of the receptor following ligand engagement.
The overwhelming majority of breast cancer deaths are caused by the metastasis of cancer cells from the primary tumor to distant sites in the body. For cancer cells to successfully metastasize, they must: detach from the primary tumor, move out of the original tissue into the circulatory or the lymphatic system, travel to a new site followed by arresting their movement, and then extravasate to begin colonizing a secondary site. When normal epithelial cells detach from the extracellular matrix (ECM), they induce caspase-dependent cell death which is known as anoikis. Anoikis can therefore serve as a critical barrier to metastasis as cancer cells are exposed to limited and variable matrix conditions during each step of the metastatic cascade. However, our previous studies suggest that anoikis evasion is not sufficient to protect ECM-detached cells from cell death. Using MCF10A mammary epithelial cells, we have previously shown that ECM-detachment induced metabolic changes can compromise the survival of detached cells, although the precise mechanism controlling cell death remains unclear. Here, we present data suggesting that ECM-detached cells can also be eliminated by regulated necrosis (RN), a genetically programmed, caspase-independent form of necrosis that is morphologically indistinguishable from classical necrosis. In general, the molecular mechanisms involved in RN are poorly understood. With this in mind, the current most appreciated subtype of RN, termed necroptosis, is dependent upon TNFα, RIP1K, RIP3K, MLKL, and PGAM5 to execute cell death. Our data in ECM-detached cells suggest that cells are being eliminated by a mechanism that is dependent on the kinase activity of RIP1K. Further studies have shown an increase in the expression of CYLD, a deubiquitinating enzyme that specifically removes ubiquitin chains from RIP1K, in turn stabilizing both RIP1K expression and activation. Upon analyzing other dependent executioners of necroptosis, we have strikingly found that TNFα, RIP3K, and MLKL are all dispensable for RN to occur in ECM-detachment. Furthermore, using a variety of molecular tools, such as 3D cell culture, we have found that PGAM5 is necessary for the execution of ECM-detachment induced RN. These findings uncover a novel and distinct RN pathway and highlight the need to more thoroughly understand RN as well as the mechanisms employed by ECM-detached cells to antagonize RN and promote their survival in detachment.
In order for cancer cells to survive during metastasis, they must overcome anoikis, a caspase-dependent cell death process triggered by extracellular matrix (ECM) detachment, and rectify detachment-induced metabolic defects that compromise cell survival. However, the precise signals utilized by cancer cells to facilitate their survival during metastasis remain poorly understood. We have discovered that oncogenic Ras facilitates the survival of ECM-detached cancer cells by utilizing distinct effector pathways to regulate metabolism and block anoikis. Surprisingly, we find that while Ras-mediated PI(3)K signaling is critical for rectifying ECM-detachment-induced metabolic deficiencies, the critical downstream effector is SGK-1 rather than Akt. Our data also indicate that oncogenic Ras blocks anoikis by diminishing expression of the phosphatase PHLPP1, which promotes anoikis through activation of p38 MAPK. Thus, our study represents a novel paradigm whereby oncogene-initiated signal transduction can promote the survival of ECM-detached cells through divergent downstream effectors.
FIBRONECTIN MEDIATES EPITHELIAL-MESENCHYMAL TRANSITION IN METASTATIC BREAST CANCER

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Introduction: During the process of epithelial-mesenchymal transition (EMT), breast cancer cells demonstrate reduced expression of epithelial cell markers such as E-cadherin (Ecad), and increased expression of mesenchymal cell markers such as vimentin and fibronectin (FN). Recent studies indicate that both epithelial and mesenchymal cell types contribute to the metastatic progression of breast cancer, but how these two cell types work in concert to drive metastasis remains undefined. Therefore, we created mosaic tumors of both epithelial and mesenchymal breast cancer cells. Using differential cell labeling we demonstrate that mesenchymal cells themselves are not metastatic but they support the metastasis of epithelial cells when grown within the same tumor. The current study is addressing the role of FN in facilitating this paracrine effect of EMT-driven metastasis.

Methods: In this study, we utilized cells from the MCF-10A progression series. Ca1h cells are extremely mesenchymal, Ecadlo and FNhi. In contrast, the Ca1a cells are epithelial, Ecadhi and FNlo. To determine what role FN plays in Ca1h paracrine-mediated metastasis of the Ca1a cells, we first used shRNA to deplete FN in Ca1h cells. We then determined FN and Ecad expression in Ca1a and Ca1h cells upon depletion of FN using qRTPCR and immunoblotting. We performed immunofluorescence to determine if Ecad expression is junctional in these cells. We determined CD24/CD44 surface expression using flow cytometry. We also performed 3D growth luminescence assays to determine if Ca1h promoted Ca1a cell growth and to what extent this was dependent on FN expression.

Results: Robust depletion of FN was successfully observed in the Ca1h cells using two different shRNA constructs. Depletion of FN resulted in an increase in Ecad mRNA and protein. Importantly, this expression of Ecad was localized to the cell-cell junctions. Also, depletion of FN in the Ca1h cells caused an increase in CD24. In contrast, Ca1a cells grown on FN coated plates did not show any phenotypic change, and CD24 and Ecad expression were not altered. Consistent with our in vivo, we found that Ca1h cells promoted Ca1a cell growth within mosaic 3D cultures, and this affect was partially reversed by depletion of FN.

Conclusions: FN is required for the mesenchymal phenotype and reduced expression of E-cad in Ca1h cells. Finally, FN may be acting in a paracrine fashion to promote mesenchymal-mediated metastatic progression of epithelial cells.

Cell and molecular biology of breast cancer
Graduate Student
Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults, and is characterized by impaired normal hematopoiesis and the accumulation of immature myeloid cells. Previous studies have shown that epigenetic abnormalities are common in AML, and more recent studies have indicated that these abnormalities can often be connected to somatic mutations in epigenetic regulatory genes. Indeed, these mutations can often arise in progenitor cell types but without being sufficient for full development of AML on their own, suggesting that they may be promising targets for clinical intervention to combat AML. However, it has also become apparent that the amount of aberrant epigenetic modification in AML cannot be explained by these somatic mutations. In some cases, genome-wide epigenetic aberrancies occur in AML cases with no known epigenetic regulators, suggesting that more work is necessary to fully understand the role of epigenetic modification in AML. One way to study the effects of epigenetics is cellular reprogramming – inducing fully differentiated cells back to a pluripotent state by the expression of a few key transcription factors. This process produces induced pluripotent stem cells (iPSCs), which lose their tissue-specific epigenetic profiles and regain profiles resembling embryonic stem cells. A recent study using chronic myeloid leukemia (CML) has shown that this epigenetic reversion is enough to restore normal differentiation potential of cells differentiated from CML-derived iPSCs, and to eliminate their ability to cause leukemia when transplanted into recipient mice. This study also revealed a strong link between a single oncogenic protein and the aberrant DNA methylation pattern, suggesting that cellular reprogramming could be a powerful tool to understand the role of epigenetics in leukemia development and progression. To this end, we reprogrammed CD34+ bone marrow cells from primary AML using non-integrative methods. The resulting AML-BM-CD34+ iPSCs were evaluated for pluripotency using qPCR and flow cytometry. Ongoing studies are evaluating the hematopoietic differentiation patterns of the AML-BM-CD34 iPSCs, as well as their genomic and epigenomic profiles relative to starting populations and normal controls. These studies will enable us to gain novel insight into the development of AML.
PARALLEL FUNCTIONS OF THE ENDOPLASMIC RETICULUM CHAPERONE PROTEIN GRP78 IN TUMORIGENESIS AND THE INDUCTION OF PLURIPOTENCY

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Accumulating evidence has suggested that cancer recurrence may be due to subpopulations within a tumor that may be more stem-like in nature, as these cells are typically more resistant to chemotherapy. Previous work done by our laboratory and others has shown that pathways regulating oncogenesis are similar to pathways important in the formation of induced pluripotent stem cells (iPSCs), a process known as reprogramming. By therefore understanding the mechanisms that govern reprogramming, we may gain insight into the methods by which cancer cells acquire and exploit stem cell characteristics, so that we may more strategically target these cell populations to prevent malignant relapse. In exploring specific parallels between oncogenesis and reprogramming, our work led us to first focus on the molecular chaperone GRP78, a protein generally restricted to the endoplasmic reticulum in normal tissues, but expressed at the cell surface in both iPSCs and many types of tumors. Although previous studies have shown that GRP78 plays an important role in tumor growth, metastasis, and patient prognosis, how GRP78 is regulating these effects is still poorly understood. Since GRP78 has been shown to be aberrantly expressed on the surface of a number of different types of cancers (e.g. breast, pancreas, lung, ovarian), any insight into the mechanisms by which GRP78 is functioning could assist in therapeutic targeting relevant to numerous types of cancers. We have discovered that GRP78 expression is induced during reprogramming, and becomes localized to the cell surface in pluripotent cells. Overexpression of GRP78 in somatic cells induced their reprogramming efficiency. We further found that a GRP78 antibody, that disrupts cell surface GRP78 binding, inhibited reprogramming. Treatment of pluripotent stem cell populations with this GRP78 antibody also decreased proliferation, but did not impact pluripotency. These combined findings suggest that GRP78 may be localized to the cell surface during reprogramming where it functions to promote proliferation and/or survival. Interestingly, overexpression of GRP78 in human breast cancer cell lines caused an increased resistance to the chemotherapeutic drug cisplatin. Furthermore, inhibiting cell surface function of GRP78 in these breast cancer lines resulted in a higher susceptibility to cisplatin treatment, demonstrating a specific function for cell surface GRP78 in cisplatin resistance. Strikingly, overexpression of GRP78 in breast cancer cells caused a previously identified stem-like subpopulation (CD24-/CD44+) to increase significantly. Using cell-surface specific protein labeling coupled with mass-spectrometry proteomics, we have identified potential binding partners of GRP78 on iPSCs and cancer cells. Ongoing studies are aimed at exploring these potential binding partners and delineating the GRP78-dependent molecular mechanisms regulating these effects. The combined stem cell functions of GRP78 parallel many of the functions of GRP78 for cancer cells, and thus provide insight into understanding how cancer cells acquire and exploit stem cell properties.

Cell and molecular biology of breast cancer  Post-Doctoral/Medical Fellow
The dependence receptors (DR) are cell surface receptors that can mediate two different intracellular signals. In the presence of ligands, DR generates a positive signal leading to cell survival, differentiation or migration. In contrast, in the absence of ligands, DR initiates a signal for cell death/apoptosis. Thus, alteration of the DR signaling pathway may play a role in tumorigenesis. In this study, we focus on the function of UNC5A, a DR, and its secreted ligand Netrin-1 (NTN1) in breast cancer and metastasis. Since estrogen (E2) and its receptor (estrogen receptor-alpha; ERα) are major signaling players in ERα-positive breast cancers, we performed all the studies in ERα-positive breast cancer cells. We observed that UNC5A and NTN1 are estrogen-inducible genes depending on cell types. In addition, RNA-seq analyses of vector control and UNC5A-knockdown cells treated with or without E2 revealed ~10-fold increase in several E2-regulated genes in UNC5A-knockdown cells. Cell proliferation and the anti-apoptotic BCL2 were significantly up-regulated in UNC5A knockdown cells. Moreover, UNC5A knockdown cells showed elevated up-regulation of βNp63, a TP53 family transcription factor that promotes breast epithelial stem cell maintenance and basal-like breast cancer. Consistent with the known role of TP63 in cancer stem cells, UNC5A-knockdown cells displayed cancer stem cell phenotype as evident from ~3-fold increase in the number of CD44+/CD24− and CD44+/EpCAM+ subpopulation compared with control cells. Furthermore, in vivo studies in mice determined that implantation of UNC5A-knockdown cells can form tumors in the mammary fat pad and are able to colonize multiple organs such as lungs, ovaries and adrenal glands. Thus, knockdown of UNC5A resulted in deregulated expression of E2-regulated genes, E2-independent and anti-estrogen-resistant growth in vitro, and E2-independent tumor formation in xenograft models. Overall, our results suggest that E2 induces UNC5A expression as a negative regulatory loop to restrict or fine tune ERα:E2 signaling and maintain luminal phenotype. Loss or mutation of UNC5A, as frequently observed in cancer, could lead to unrestricted E2:ERα signaling and anti-estrogen resistant growth while simultaneously enabling ERα-positive luminal breast cancer cells to acquire basal-like and cancer stem cell-like features.
Tumor cell invasion is a complex process that requires the molecular and physical adaptation of both the cell and its microenvironment. Increased MAPK signaling, small GTPase activation, cytoskeletal rearrangements, and the directed targeting of proteases to sites of extracellular matrix (ECM) degradation all accompany the process of tumor cell invasion. We have shown that nucleotide cycling on the ARF6 protein regulates the release of protease-loaded, plasma membrane-derived microvesicles from tumor cells into the microenvironment to promote ECM degradation. These shed vesicles are distinct from invadopodia, which are protrusions at the adherent surface of cells and mediate cell invasion. While the release of protease-loaded microvesicles potentially serves as a mechanism to bring about matrix degradation and also deposit paracrine information at distal locations creating paths of least resistance, invadopodia facilitate pericellular proteolysis of the ECM at the cell’s invasive front and appear to be linked to a mesenchymal type of tumor cell motility. We show that Rho GTPase signaling and the matrix microenvironment govern whether tumor cells produce invadopodia, or adopt an amoeboid phenotype and release microvesicles. The ability of tumor cells to switch between the aforementioned, qualitatively distinct modes of invasion may facilitate movement through the different microenvironments. Understanding the interplay between these two invasive cell programs will provide new and important insights into the progression toward pathophysiological states.
IDENTIFICATION OF CANCER-SPECIFIC SIGNALING NETWORKS: WHAT IS “NORMAL” CONTROL?

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Success of precision medicine depends on definitive identification of cancer-specific alterations in signaling pathways. However, identifying cancer-specific signaling networks is challenging because of lack of proper control tissue for differential gene expression analyses. Most studies in breast cancer utilize tumor-adjacent normal tissue or reduction mammoplasty samples as “normal” controls. We recently reported that breast epithelial cells from healthy donors as well as tumor-adjacent normal are in different differentiation states compared with tumor cells and the differences in differentiation status alone could account for major transcriptome variations between normal and tumor. To overcome these limitations, we propagated breast epithelial cells from three healthy donors (healthy-normal), two high-risk patients, two tumor-adjacent normal (HR/AD-normal) and five tumor samples of different molecular subtypes. Phenotypically defined (CD49f+/EpCAM+) luminal progenitor cells were sorted from these cultures and subjected to RNA-seq analyses. Pathway analysis revealed activation of cell-intrinsic pro-inflammatory signaling in HR/AD-normal cells compared with healthy-normal cells. This signaling network was further amplified in tumor cells. The pro-inflammatory chemokine CCL2, which is overexpressed in highly aggressive breast cancer, and the cytokine TNFRSF11B were elevated in HR/AD-normal luminal progenitor cells. Despite using phenotypically defined cells in the transcriptome analyses, cancer-specific signaling network identification was directly influenced by the type of controls used; healthy-normal or HR/AD-normal. While cancer-enriched PI3K and NF-κB activation was observed when compared to any kind of control, SRC kinase activation was noted only when cells from healthy-normal were used as a control. In general, the number of tumor signaling networks identified using healthy-normal as a control was higher than when compared with HR/AD-normal as a control. These results suggest that considerable attention should be placed on the type of tissues used as control for definitive identification of cancer-specific signaling networks and therapies to target such pathways. Additionally, these data show that non-cancer tissues of breast cancer patients acquire a cell intrinsic pro-inflammatory phenotype, which may be prerequisite for cancer development and potentially an early-detection tool.

Cell and molecular biology of breast cancer Research Technician
LUNASIN HAS POTENTIAL THERAPEUTIC ROLE AGAINST BREAST CANCER

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Lunasin, a 43-44 amino-acid soybean bioactive peptide, has both chemopreventive and chemotherapeutic activities. Previously, we observed that Lunasin has significant in vitro and in vivo anticancer activity in non-small cell lung and melanoma cells. The aim of this study was to determine the chemotherapeutic potential of Lunasin against breast cancer. We found that Lunasin has significant effects on breast cancer cell lines both in vitro and in vivo. Parental human breast cancer cell lines exhibited significant reductions in colony formation or colony size in soft agar colony-forming assays and a decrease in mammosphere formation. Previous studies have identified ALDH as a biomarker for cancer initiating cells (CICs) in several cancer models including skin, colon, and breast. Cells expressing ALDH are enriched in subpopulations displaying stem-like properties including sphere formation and tumorigenicity in immunodeficient mice. In our studies, ALDHhigh breast cancer cells displayed significant sensitivity to Lunasin. Lunasin inhibited the self-renewal capacity of ALDH expressing cells, and reduced soft agar colony and mammosphere formation in vitro. Lunasin also significantly decreased tumor burden in a mouse xenograft model using MDA-MB-231 cells. These results establish that Lunasin may have potential as a component of breast cancer treatment that targets the tumorigenicity of CICs.

Novel modalities or technologies for breast cancer diagnosis and treatment Faculty
INTRODUCTION: The multidisciplinary research team at IUPUI and IUSOM fabricated a microfluidic channel and investigated passing behaviors of normal and cancer cells through the channel. A specific question the team addressed was: Is it possible to distinguish normal vs. cancer cells by detecting their passing behaviors through a narrow channel? The hypothesis, tested in this study was: due to higher deformability, softer cancer cells may pass through the channel quicker than normal cells.

MATERIALS and METHODS: Two cell lines, employed herein, were normal breast epithelial cells (MCF-10A; 12.0 ± 2.0 μm in diameter) and metastatic breast cancer cells (MDA-MB-231; 12.5 ± 2.1 μm in diameter). The microfluidic channel was 100 μm long and linearly tapered with a width of 30 μm at an inlet and 5 μm to an outlet.

RESULTS: The result revealed that the passage time (mean ± s.d.) was 42.6 ± 41.0 sec for normal cells (N = 152), and 2.5 ± 2.6 sec for cancer cells (N = 158). It is reported that metastatic cancer cells are less stiff than normal cells. Thus, our working hypothesis is that a significant difference in passage time is likely to be caused by rigidity difference of two cell lines.

DISCUSSION: One out of 8 women in the U.S. suffers breast cancer in lifetime. Patients with breast cancer at advanced stages may die not because of primary tumors but because of secondary tumors through metastasis. Several treatment options to treat breast cancer metastasis are available, including surgery, chemotherapy, radiation therapy, hormone therapy, or targeted therapy using antibodies. However, these options are not ideal because of side effects, limited efficacy and cost. As a next step, we plan to conduct the following experiments: (a) determination of cell stiffness (Young’s modulus); (b) comparison of passage time among heterogeneous breast cancer cells; and (c) sorting and destruction of metastatic cancer cells isolated from the blood. The research team appreciates support from a local advocacy group in Indianapolis, 100 Voices of Hope.

CONCLUSION: The result herein suggests that analysis of cell passing behavior through a narrow channel can distinguish metastatic cancer cells from normal ones, supporting a possibility of diagnosing and reducing metastatic burden using a novel microfluidics-based approach.
INTRODUCTION

Incorporation of next-generation sequencing to detect plasma-derived tumor DNA (ptDNA) is emerging as a popular method for tumor genotyping and for monitoring therapeutic response. The vast majority of studies so far have focused on detecting ptDNA from patients with metastatic disease. Herein, we tested whether ptDNA could be used as a biomarker to predict relapse in triple-negative breast cancer (TNBC) patients with residual disease after neoadjuvant chemotherapy and surgery.

METHODS

BRE09-146 was a Phase II clinical trial that randomized TNBC patients with residual disease after neoadjuvant chemotherapy to Cisplatin or Cisplatin+Rucaparib. From the combination arm, 1ml of plasma was collected at four predefined time points post-surgery. In total, 39 patients with matched tumor, blood, and plasma were analyzed. Extracted DNA underwent library preparation and amplification using the Ion Ampliseq Oncomine Research Panel which consists of 134 cancer genes that are well-known to be mutated in cancer. Samples were then sequenced on an Ion Proton next-generation sequencer to at least 2500X coverage followed by bioinformatic analyses using the Torrent VariantCaller.

RESULTS

We first detected high-quality somatic mutations in primary tumors. TP53 mutations were the most prevalent (70%) followed by AKT1 (8%). Somatic mutation frequencies in our trial were congruent with publically-available mutation data of TNBCs from The Cancer Genome Atlas. Using these somatic mutations, we then analyzed the plasma-sequencing data to detect the same mutations in the circulation. Out of 39 patients, 14 patients had a clinical relapse (median follow-up for disease free survival = 24 months). Of the 14 patients, we were able to detect somatic ptDNA in 4 patients (3 TP53 mutations, 1 AKT mutation). Notably, all 4 patients had a rapid recurrence (0.3, 4.0, 5.3, and 8.9 months). ptDNA-sequencing was unable to detect distant recurrence. The combination of a paucity of ptDNA molecules in the circulation of patients who have no evidence of disease along with a limited amount of plasma available per patient are potential factors for the inability to detect distant recurrence.

Conclusions: Next-generation ptDNA-sequencing of triple-negative breast cancer patients after neoadjuvant chemotherapy and surgery can detect rapid-recurrence but sensitivity to detect distant recurrence is limited. Studies to increase sensitivity by incorporating mutation calling from ptRNA along with ptDNA are currently underway.

Novel modalities or technologies for breast cancer diagnosis and treatment Graduate Student
The advancement of personalized drug therapies in oncology could benefit from more effective methods of translation in drug development. Many drug candidates and experimental agents must be discarded, or at least are severely limited in their use, due to the necessary dosage to achieve efficacy as single agents. Methodologies that enable a systematic approach to predict enhanced efficacy using tumor genome data offer important avenues to address these gaps. "Synthetic lethal combination" in the context of cancer therapy refers to the combined loss or inhibition of two or more protein-functions leading to selective cell death. Inhibition of any single protein function of the pair is not effective. In many cancers, including breast cancer, there is evidence of dysregulation of DNA replication and repair pathways that involve the scaffold protein PCNA. However, due to universal necessity of PCNA, direct inhibition of all its functions would reduce selective targeting. The main hypothesis is that identifying disease specific dysregulation of PCNA will provide a basis for antagonist combinations to render high tumor specificities. Disease specific networks are created by mining the TCGA Data Portal for gene expression profiles of breast tumor subclasses. A PCNA network is composed of all of its primary and secondary interactors has been derived from BioGrid and HIPPIE databases. Subnetworks of the PCNA network are created based on gene ontology terms and differential expression of genes in the disease state versus the normal tissue. By investigating these subnetworks for pathway dysregulation leading to losses in redundancy and the emergence of addiction, synthetic lethal relationships are identified. Potential combinations of existing therapeutics are derived from these networks based on the detection of aberrant network connectivity. One of the most notable synthetic-lethal relationships is that of a PCNA antagonist and the PARP inhibitor, Olaparib. Based upon these network features and additional functional insights, new antagonists for PCNA protein interactions are being pursued for testing for combined effects with olaparib. This relationship was specific to both the Basal Triple Negative (TN) and the HER2+ subtypes. There is consistent upregulation of effectors of homologous recombination (HR) in both subtypes as well as dysregulation of DNA repair pathway selectors that indicate an HR bias. This bias would provide a context for PCNA antagonists to be more effective than in normal cells where other forms of DNA repair are depended upon. If proven as a viable combination it would provide a therapy with high specificity for both HER2+ tumors and TN tumors. TN tumors are ~20% more lethal than other tumors and possess fewer therapeutic options than hormone dependent breast tumors.

Metastatic microenvironments are spatially and compositionally heterogeneous. This seemingly stochastic heterogeneity provides researchers great challenges in elucidating factors that determine metastatic outgrowth. Herein, we develop and implement an integrative platform that will enable researchers to obtain novel insights from intricate metastatic landscapes. Our two-segment platform begins with whole tissue clearing, staining, and imaging to globally delineate metastatic landscape heterogeneity with spatial and molecular resolution. The second segment of our platform applies our custom-developed SMART 3D (Spatial filtering-based background removal and Multi-chAnnel forest classifiers-based 3D ReconsTruction), a multi-faceted image analysis pipeline, permitting quantitative interrogation of functional implications of heterogeneous metastatic landscape constituents, from subcellular features to multicellular structures, within our large three-dimensional (3D) image datasets. Coupling whole tissue imaging of brain metastasis animal models with SMART 3D, we demonstrate the capability of our integrative pipeline to reveal and quantify volumetric and spatial aspects of brain metastasis landscapes, including diverse tumor morphology, heterogeneous proliferative indices, metastasis-associated astrogliosis, and vasculature spatial distribution. Collectively, our study demonstrates the utility of our novel integrative platform to reveal and quantify the global spatial and volumetric characteristics of the 3D metastatic landscape with unparalleled accuracy, opening new opportunities for unbiased investigation of novel biological phenomena in situ.
This year in the United States more than 200,000 new patients will be diagnosed with breast cancer resulting in 40,000 deaths. The current gold standard for tumor removal is lumpectomy, an invasive resection technique that may result in relapse if the tumor is not fully removed. Existing imaging techniques are inadequate to evaluate the success of lumpectomy procedures; therefore, there exists a clinical need for a rapid, sensitive, and label free method for intraoperative breast tumor margin assessment that can discriminate between healthy and cancerous tissues. We use multispectral photoacoustic tomography (MPAT) to meet this need, as it uses non-ionizing pulsed near-infrared light to induce tissue specific acoustic waves that can be detected and reconstructed into relevant compositional images. MPAT specifically uses 1100nm and 1210nm light to differentiate lipid (healthy) from blood (tumor) tissue with depths up to several centimeters. In our initial studies, we developed tumor phantoms to evaluate our MPAT technique. These phantoms are comprised of mouse abdominal adipose, chicken breast fat, and rat blood, fixed in 0.1% paraformaldehyde and suspended in 1% agarose. Through the use of these phantoms, we were able to qualitatively evaluate the ability of our MPAT technique in differentiating lipid from blood and determine its effectiveness in assessing tumor structure. Preliminary results show that MPAT can accurately detect blood and lipid, as well as differentiate tumor phantom structures. We also developed MATLAB algorithms to acquire, process, and merge lipid and blood MPAT images. This script collects two wavelength-specific serial MPAT scans, and then merges the acquired images into one stack. This stack is then concatenated into a 3D matrix that can be used to visualize and discriminate blood from lipid within the tumor. These 3D stacks have also been imported to Fiji to create 3D topographical reconstructions of the phantoms. Our group is currently developing an algorithm to utilize both MATLAB and Fiji to automatically process our MPAT images and recreate 3D tumor margin maps of biopsied tissue. Future work will focus on developing our MPAT technique by optimizing optical parameters of our setup such as fiber bundle focal length and light transmission. We will continue to develop this technique in the hopes that it can one day aid clinicians in breast cancer treatment, and improve the lives of thousands of cancer patients.

**Novel modalities or technologies for breast cancer diagnosis and treatment**

**Graduate Student**
Breast cancer is one of the most common malignancies. With its progress, breast cancer causes systemic effects such as functional limitation, sarcopenia, and cachexia. These effects are manifested as muscle weakness, body pain or depletion of skeletal muscle without apparent loss of body weight. Although onset of cachexia in breast cancer is not as rapid and severe as in lung and pancreatic cancer, over a quarter of breast cancer patients experience a precachexia to cachexia syndrome. However, mechanistic studies on systemic effects of breast cancer are limited. In this project, we used myoblast cell line C2C12 and rat embryonic cardiomyocyte cell line H9C2 for in vitro assays and MMTV-Her2/Neu and MMTV-PyMT mammary tumor models for in vivo assays to illuminate key players linked to systemic effects, to identify functional biomarkers, and to develop a rational therapy for systemic complications of breast cancer. In in vitro studies, we found treatments with conditioned media from a number of mammary tumor cell lines, including PO1058 (poorly invasive tumor cells from PyMT-WapCre-mGFP+ mice in C57BL6 background), PO1059 (highly invasive cells from PyMT-WapCre-mGFP+ mice), and MMT8 (tumor cells from MMTV-Neu mice in FVB/N background) resulted in a significant decrease of miR486 in C2C12 cells but not in H9C2 cells. miR486 is a muscle-enriched microRNA, which controls differentiation of myoblasts and its deregulation is linked to musculoskeletal defects in muscular dystrophy patients. Interestingly, we also found lower circulating miR-486 in the plasma of patients with pancreatic and bladder but not lung cancer. These data are consistent with our previous report of lower circulating miR486 in plasma of breast cancer patients with metastasis compared with healthy women. In in vivo study, we observed deteriorating physical and functional conditions in PyMT+ mice with the progression of mammary tumor. Compared to wildtype mice, PyMT+ mice with mammary tumors showed decreased fat mass and decreased grip strength, both markers of advancing cachexia. In our preliminary drug intervention study, Diaminomethylparthenolide (DAMPT), a NF-κB inhibitor, partially reduced mammary tumor development in tumor numbers and sizes in PyMT+ mice and several of the systemic effects of cancer. To date, we have not found an effect of mammary tumor on cardiac functions of 12-week old PyMT+ mice. Continuous monitoring of these mice with the progression of their tumors will reveal more detailed mechanistic changes associated with systemic effects of breast cancer. In summary, mammary tumor resulted in changes in body composition and the decrease of grip strength of PyMT+ mice, which may be associated with altered NF-κB pathway. Additionally, circulating miR-486 may serve as a useful clinical indicator of progression and systemic effects of certain cancers.
GENETIC BIOMARKER FOR EARLY BREAST CANCER DETECTION: TARGETING TELOMERE REPETITIVE SEQUENCES IN PLASMA

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Introduction:
The ability to detect breast cancer at an early and often highly treatable stage is key to breast cancer prevention and control. This study focuses on developing a reliable genetic-based blood test for early breast detection. Telomeres are protective DNA structures located at the end of chromosomes, and their maintenance plays an essential role in genomic stability. Notably, excessive telomere shortening is observed in breast cancer lesions when compared to adjacent non-cancerous tissues, suggesting that telomere length may represent a key biomarker for early breast cancer detection. Because tumor-derived, cell-free DNA (cfDNA) is often released from cancer cells and circulates in the bloodstream, we hypothesize that breast cancer development is associated with changes in the amount of telomeric cfDNA that can be detected in the plasma.

Methods:
To test the hypothesis, we first devised a novel, highly sensitive and specific quantitative PCR (qPCR) assay, termed telomeric cfDNA qPCR, to quantify plasma telomeric cfDNA levels. To control for the input amount of cfDNA which is by nature highly fragmented and of unknown ploidy, we designed our internal reference primers to target a non-coding, repetitive element, LINE (Long INterspersed Element). Telomeric cfDNA levels were determined relative to LINE amount. cfDNA was extracted from frozen plasma using a sodium iodide-based method and 50–150 pg cfDNA was used for each qPCR reaction. A total of 47 plasma samples from breast cancer patients before chemo/radio treatment (32-73 y) and 42 from healthy women (27-73 y) were assessed for telomeric cfDNA levels.

Results:
The accuracy of the telomeric cfDNA qPCR assay was demonstrated by amplification with the LINE internal reference primers which correctly reflected input cfDNA amount (R\(^2\) = 0.910, \(P = 7.82 \times 10^{-52}\)). Using the new assay, we found that age impacts plasma telomeric cfDNA levels in healthy individuals (n = 42, R\(^2\) = 0.094, \(P = 0.048\)), which is a similar phenomenon to leukocyte telomere shortening with increasing age. In addition, plasma telomeric cfDNA level was significantly lower in breast cancer patients (n = 47), compared to control individuals (n = 42) (\(P=4.06 \times 10^{-8}\)). The sensitivity and specificity for the telomeric cfDNA qPCR assay was 91.49% and 76.19%, respectively. Furthermore, the telomeric cfDNA level distinguished even the Ductal Carcinoma In Situ (DCIS) group (n = 7) from the healthy group (n = 42) (\(P = 1.51 \times 10^{-3}\)).

Conclusions:
This study highlights the quantitative abnormality of plasma telomeric cfDNA observed in patients with pre-malignant DCIS. Our findings propose that decreasing plasma telomeric cfDNA levels could serve as a liquid biopsy, which would be useful for improving early cancer detection, either by itself or in combination with other diagnostic tools. Future studies are highly warranted to test the assay’s potential for clinical utility.

Novel modalities or technologies for breast cancer diagnosis and treatment Post-Doctoral/Medical Fellow
POSTER #22

DUAL PI3K AND WNT PATHWAY INHIBITION IS A SYNERGISTIC COMBINATION AGAINST TRIPLE NEGATIVE BREAST CANCER

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Introduction: Triple negative breast cancer (TNBC) accounts for 15% of all breast cancer cases in the United States, and despite its lower incidence, contributes to a disproportionately higher rate of morbidity and mortality compared to other breast cancer subtypes. Because these tumors lack expression of the estrogen, progesterone, or HER-2 receptors (“triple negative”), TNBC patients do not respond to targeted therapies that have been successfully used against tumors that over-express these proteins. Thus, there exists a critical need to improve the outcomes of TNBC patients through the implementation of novel targeted agents.

Methods: RNA-seq data from 94 TNBCs and 20 microdissected normal breast tissues (Komen Tissue Bank) were merged and imported into Partek Genomics Suite. Statistically significant genes were imported into Ingenuity Pathway Analysis (IPA) to identify therapeutic targets. For in vitro anti-tumor studies, we tested a panel of seven TNBC cell lines using Buparlisib (a PI3K pathway inhibitor) and WNT974 (a WNT pathway inhibitor), individually and in combination. Cell viability was assessed via Celltiter-Fluor. Synergy between the two drugs was calculated using the Chou-Talalay method. In vivo studies were performed using the TMD-231 cell line and a patient derived xenograft (PDX) from Jackson Laboratory. Dosing of the mice was performed using 30 mg/kg and 3 mg/kg of Buparlisib and WNT974 respectively, both in combination and individually.

Results: Using next-generation RNA sequencing data, Ingenuity Pathway Analysis identified over-expression and hyper-activation of the PI3K/AKT/mTOR. Sequencing also found a hyper-activation of the WNT pathway after treatment with Buparlisib on TNBC cell lines. When anti-tumor efficacy against these pathways was assessed, a significant reduction in cell viability using Buparlisib and WNT974 in combination was observed across the panel of cell lines. Using the Chou-Talalay method, we found for MDA-MB-231 and Hs578T, a ~50% reduction in cell viability at 100nM concentration of each drug that was highly synergistic. In our in vivo PK/PD studies there was no drug-drug interaction observed. Furthermore, the tumor portion of this study saw shrinkage of ~40% after seven days. In our in vivo PDX experiment, we observed an 80% survival using combination therapy compared to 40% using the drugs individually or vehicle after 30 days.

Conclusion: PI3K/mTOR/AKT and Wnt pathways are a vital target for treatment of TNBC. Using small molecule inhibitors that are in phase trials (Buparlisib and WNT974) we have found that there is a strong synergy when given at low nanomolar doses. In vitro studies of inhibitors of these two pathways in a panel of TNBC cell lines demonstrated significant efficacy in reducing cell viability with substantial synergy when used in combination. Furthermore, cell line and PDX mouse studies display a similar synergy both drugs when given in combination when compared to these two drugs alone.
Novel modalities or technologies for breast cancer diagnosis and treatment

Research Technician
NEW SMALL MOLECULES SUPPRESS BREAST CANCER COLONIZATION OF BONE

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Starting with the structure of previously-discovered uPAR antagonists, we designed and synthesized a new class of small-molecule derivatives, XHS32 and XHS34. Both compounds inhibited breast MDA-MB-231 and pancreatic Mia-Paca2 invasion in a Boyden Chamber assay with IC50s of 1-3 uM, an order of magnitude increase over the parental structures. The significant aspect of this discovery is that these compounds have no cytotoxicity, suggesting that their effect on invasion is strictly due to blocking pathways associated cell invasion. The compounds exhibited weaker activity against glioblastoma U87 and non-small cell lung adenocarcinoma H460. Both XHS32 and XHS34 inhibited MDA-MB-231 adhesion to vitronectin with single-digit micromolar IC50s. The compounds were tested in a wound healing assay but had no effect on migration driven by concentration gradient of the assay. The compounds also showed no activity in a tube formation assay using HUVECs, suggesting that the effects may be confined to their effect on invasion in aggressive cancer cells. We tested the two compounds in an anchorage-independent soft agar assay and found a concentration-dependent inhibition of colony formation, but the compounds did not completely inhibit colony formation, consistent with their lack of toxicity. Finally, we tested the effects of compounds on tumor cell colonization of bone in an ex vivo assay that involves co-culturing MDA-MB-231 cells for 7 days on mouse calvarial bone segments. Both compounds at 5 uM significantly inhibited bone colonization and blocked pathological responses to tumor, as monitored by the markers of bone destruction, RANK ligand and tartrate-resistant acid phosphatase. The effects of XHS32 and XHS34 were comparable to the drug zoledronic acid, which is clinically-approved for treatment of osteoporosis and bone metastases. Unlike zoledronic acid, which impairs metastasis by blocking bone resorption, XHS32 and XHS34 directly act on pathways associated with promoting cancer cell invasion to bone. The lack of toxicity of our compounds makes them promising candidates for the development of small molecules to prevent and reverse bone metastasis in breast cancer.

Synthesis and/or pharmacology of novel potential antitumor agents

Faculty
COVALENT INHIBITION OF FGFR OVERCOMES METASTATIC RESISTANCE TO HER2 TARGETED THERAPIES IN BREAST CANCER

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Directed therapies using kinase inhibitors (Lapatinib) and antibodies (Trastuzumab) to target human epidermal growth factor receptor 2 (Her2) have served as a testament to the potential of molecular-targeted therapies in this breast cancer subtype. However, Her2-targeted therapies are plagued by the pitfalls of intrinsic and acquired resistance, particularly in the metastatic setting. Along these lines, metastasis and acquisition of drug resistance in breast cancer cells has been linked to epithelial-mesenchymal transition (EMT). To identify the unique aspects of these EMT processes we treated Her2-transformed cells either with TGF-β1 or long-term lapatinib exposure. These treatment regimes resulted in a robust EMT phenotype as characterized by the Nanostring EMT profiling array. Interestingly, only TGF-β-induced EMT demonstrated the ability to revert back to an epithelial phenotype upon removal of stimulation, and only TGF-β treatment cells demonstrated increased metastatic progression. However, both EMT protocols resulted in resistance to Lapatinib and second generation covalent ErbB inhibitors. Importantly, these resistant cell populations potently upregulate fibroblast growth receptor (FGFR) and can be readily targeted using, FIIN2, our recently developed covalent inhibitor of FGFR. In addition, combined treatment with Lapatinib and FIIN2 effectively eradicated both epithelial and mesenchymal cell populations. Finally, in vivo application of covalent FGFR inhibitors effectively inhibited the growth of Her2 positive patient derived xenografts that were harvested from a patient that failed on Trastuzumab and were resistant to the antibody drug conjugate T-DM1. Overall our data demonstrate that the EMT processes that facilitate drug resistance and metastasis are unique, but that they both share a common switch in growth promoting signaling pathways and thus can be similarly targeted using covalent inhibitors of FGFR kinase activity.

Synthesis and/or pharmacology of novel potential antitumor agents

Graduate Student