The Role of Interstitial Fluid Flow on Breast Cancer Progression

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by

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DEDICATIONS

This work is dedicated to my mother - Satou Abakob - and to my father - Amadou Noumbissi - who are both now deceased. I would not be who I am physically nor mentally if it were not for their love, guidance and fervent dedication to my education.

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ABSTRACT

The Role of Interstitial Fluid Flow on Breast Cancer Progression
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The majority of deaths associated with breast cancer result from the ability of the cancer cells to invade the surrounding environment and spread to distant sites. The tumor microenvironment includes a variety of biophysical forces whose effects are known to influence cancer invasion and progression. Interstitial fluid flow (IFF) is one such force that is present in normal breast tissue and elevated in tumors. The focus of this dissertation was to study the effects of IFF coupled with overexpression of the proto-oncogene HER2 on breast cancer progression. The combined roles IFF and HER2 play on invasion had never been studied, especially in the context of how they may together influence the transition from ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC).

Using 3-dimensional cell culture-based assays, we demonstrated that IFF activates molecular mechanisms that are HER2-independent and leads to invasion of different stages of tumor development through the phosphoinositide-3-kinase (PI3K). This phenomenon was observed both in single cells and in breast cancer-like acini structures. We identified that in normal cells, IFF-induced PI3K activation was modulated by EGFR, a growth factor whose signaling is tightly controlled in normal tissue. In invasive cancer cells, IFF-induced PI3K activation was modulated by the metastasis-associated chemokine receptor CXCR4. Furthermore, we observed that

IFF-induced CXCR4 signaling only occurred in cells that have undergone EMT. Finally, using an approach to isolate IFF responsive cells, we observed that HER2 positive epithelial cells that invaded in response to IFF developed mesenchymal-like characteristics and displayed a lower sensitivity to HER2 drug treatment. Taken together, our findings suggest for the first time the role IFF plays on cellular transformation and drug resistance. In addition, they reveal IFF as a major contributor to breast cancer progression and highlight its implications on breast cancer therapy.

INTRODUCTION

The majority of deaths associated with cancer result from cancer cell invasion and metastasis. The causes of cancer invasion have been the focus of many research studies but much remains unknown. Although the expression of ErbB2 in pre-invasive cells is a risk factor for invasion and cancer progression, not all cells expressing this proto-oncogene develop invasive phenotypes. It has become increasingly apparent that cancer progression does not solely depend on the functions of cancer cells. There exist a crosstalk between cancer cells, other cells of the tumor microenvironment, and physical properties of the neoplastic tissue and each participate in the acquired capability of invasive growth and metastasis. Change in interstitial fluid flow (IFF) is one example of altered biomechanical characteristics of tumor tissue that has been linked to cancer invasion. IFF is elevated in solid tumors and can modulate stromal and tumor cell invasion. The focus of this dissertation was to study the effects of IFF on breast cancer progression. The combined role IFF and ErbB2 expression play on invasion had never been studied, especially in the context of how together they may influence the transition from ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC).

We initially hypothesized that IFF activates ErbB2 pathways leading to invasion of non-invasive ErbB2 expressing DCIS cells. However this hypothesis was quickly proven wrong and we demonstrated instead that although IFF is ErbB2-independent, it leads to invasion of different stages of tumor development through the PI3K molecular pathway and epithelial-to-mesenchymal transition (EMT).

This dissertation is organized into 6 main chapters. In the first chapter, I give a thorough review of the current scientific knowledge on breast cancer invasion, IFF and EMT. Then, I present my findings and observations in the next four chapters organized into four independent papers. In chapter II, I highlight and support the observation that increased invasion due to IFF in breast cancer is independent of ErbB2 expression. Relying primarily on our 3D invasion assay, I used both invasive and non-invasive breast cancer cell lines overexpressing ErbB2. Pharmacological inhibitors against various members of the ErbB2 pathway were also used to test the importance of these proteins on flow specific invasion.

In chapter III, I present evidence that IFF induces EMT in pre-invasive cells. The objective of this chapter was to identify the potential role of IFF in EMT and the hypothesis was that IFF is able to increase invasion of cells by inducing the loss of epithelial markers and increasing the expression of mesenchymal characteristics in cells. There were 2 separate approaches used in this chapter in order to prove the hypothesis. The first method compared changes in gene and protein expression of EMT markers between cells after short exposure to IFF (24 hours). The second method used an approach to isolate flow responsive cells after multiple exposures to IFF and compared their protein profiles to cells not exposed to IFF. Both methods demonstrated that epithelial cells invading in response to IFF had decreased expression of epithelial characteristics. In addition, these flow responsive cells displayed a lower sensitivity to HER2 drug treatments.

The focus of chapter IV is on elucidating the signaling pathways activated by IFF in HER2 positive cells. By measuring the changes in protein expression and

phosphorylation of various cell membrane receptors in both pre-invasive and invasive cells after IFF exposure, I demonstrate that IFF induces invasion of breast cancer cells via activation of phosphoinositide-3-kinase (PI3K). Using both post-EMT and pre-EMT cells, I then show evidence that IFF-induced invasion and PI3K activation is modulated by CXCR4 only in cells that have undergone EMT. Furthermore, I conclude that EMT alters the response of cells to IFF with data showing that once pre-EMT cells are induced to undergo EMT through growth factor treatment, their response to IFF switch from CXCR4-independent to CXCR4-dependent.

In chapter V, I focus on the role IFF plays in initiation of invasion in breast cancer multicellular structures or acini. Here I first developed a 3-dimensional breast tissue model mimicking normal, DCIS-like, and IDC-like spheroid structures under IFF. The hypothesis of this chapter is that IFF alone is able to induce invasion initiation of DCIS-like structures. The data presented in this chapter using this model, supports our hypothesis. It was observed that IFF decreased levels of basement membrane proteins (laminin V and collagen IV), increased PI3K phosphorylation, and increased protrusions in pre-invasive spheroids.

The final chapter of this dissertation (chapter VI) highlights the main findings of the project and presents the scientific impacts of the research. Identifying the mechanisms involved in this flow specific invasion has enhanced our current knowledge of the role IFF plays on the breast tumor microenvironment. It may also serve as a basis for therapeutic development for the treatment of DCIS patients.

CHAPTER 1: BACKGROUND

1.1 Introduction to Breast Cancer

Cancer is the second most common cause of deaths in the United States, after heart disease, and it accounts for 1 in every 4 deaths [1]. Breast cancer is the number one most diagnosed cancer and the second cancer killer in women in the United States [2, 3] (Fig. 1). The majority of the deaths associated with this cancer, result from the ability of the cancer cells to invade the surrounding environment and spread to distant sites in the body. The 5-year survival rate of breast cancer falls from 98% for localized tumors to 23% for metastatic disease [4].

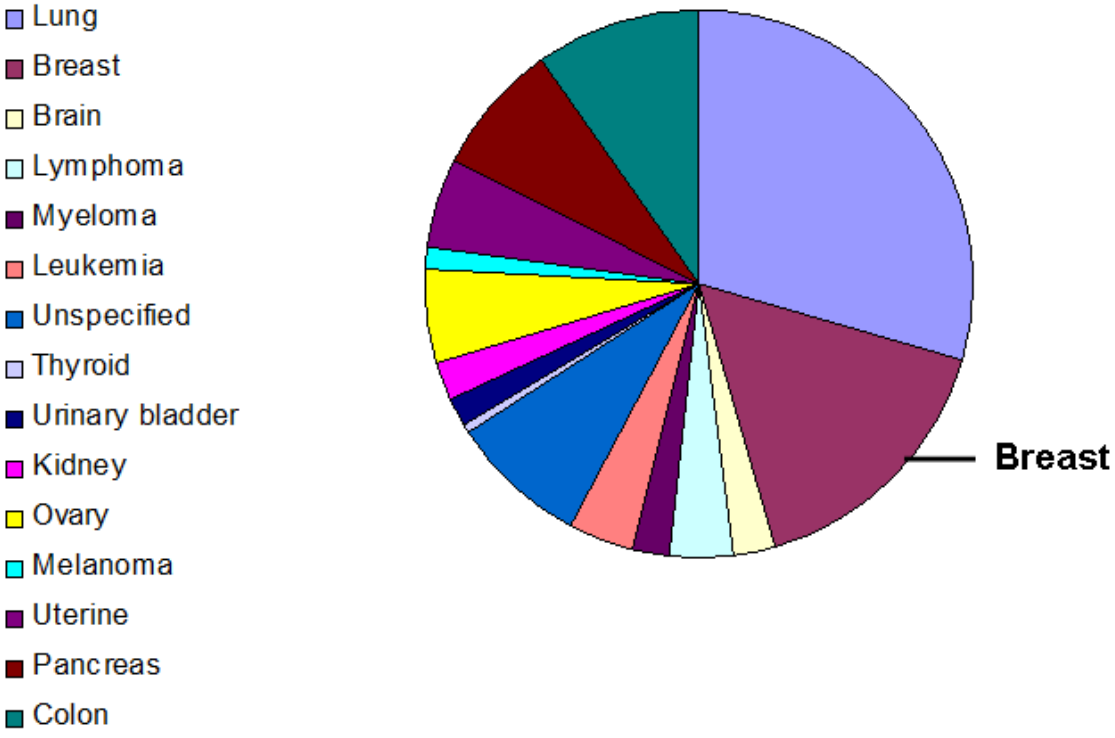

Figure 1: Cancer death rates among females in the United States. Modified from cancer facts and figures. American Cancer Society 2014.

Breast cancer is a multi-step diseases characterized by abnormal growth and spread of cells. Its progression involves transformation, tumor growth, invasion, and metastasis. In breast cancer in particular, constitutive activation of oncogenes like Ras and ERBB2, and downregulation of tumor suppressor genes like p53, are known to lead to transformation of cells and initiate tumor growth [5-7]. A recent notable review article identified a set of hallmarks or traits necessary to prolong the progression of cancer. These include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [8]. It is evident however that additional changes to the tumor microenvironment (the cells, matrix, and soluble molecules that surround the tumor) coupled with pre-existing genetic deregulation allow cells to progress through other cancer stages, as gene expression patterns are more similar between invasive and non-invasive tumor cells when compared to normal cells [9]. To successfully invade, the breast cancer cell must break away from the primary tumor, degrade proteins associated with the extracellular matrix (ECM) and the basement membrane, and migrate through surrounding tissue to blood and lymphatic vessels.

1.2 Stages of Breast Cancer

The normal mammary gland contains epithelial cells organized into hollow ducts and enclosed by a continuous basement membrane. These structures communicate with the stroma environment [10] present just outside each duct. Ductal carcinoma *in situ* (DCIS) is a pre-invasive stage of cancer characterized by the

proliferation of malignant cells within the lumen of mammary ducts. In this tissue, the basement membrane is still intact; however, an increase in stroma associated cells is observed, along with the over-expression of oncogenes that play significant roles in cancer progression such as ERBB2 and c-myc [11]. In invasive ductal carcinoma (IDC) tissue, the mammary ducts have lost their characteristic basement membrane, and this in part allows for the transformed cells to invade into the stroma and ultimately metastasize to other organs.

During cancer progression, the tumor microenvironment undergoes specific physiological changes that are categorized into distinct stages for clinical management. Stage 0 (also referred to as carcinoma *in situ*) is characterized by abnormal cell proliferation in the mammary duct (DCIS), lobules of the breast (LCIS), or nipple (Paget's) [12]. In stage I, the tumor size is less than 2 cm and cluster of cancer cells may or may not be present in the lymph node. In stage II, the tumor size is between 2 and 5 cm but has not spread to the lymph nodes or the tumor size is below 2 cm and additional tumors below 2 mm are detected in 1 to 3 axillary lymph nodes. In stage III, the tumor is any size and has spread to at least 9 axillary lymph nodes or lymph nodes near the collarbone and breastbone. In stage IV, cancer cells have spread to other parts of the body most notably the lung, brain, bone and liver [13].

1.3 Current DCIS management

DCIS is recognized as a late stage of cell deregulation and its histological patterns can vary from atypical hyperplasia to invasive breast cancer [14]. Increase

chromosomal imbalance, HER2 overexpression, p53 mutations, breakdown of the myoepithelial cell barrier, fibroblast proliferation, angiogenesis in the surrounding tissue [15-17] are among the numerous markers used to separate DCIS from normal tissue, atypical hyperplasia, and IDC. For example, loss of DNA heterozygosity is observed in 70% of high grade DCIS as opposed to 35-40% in atypical herplasia and 0% in normal tissue [18]. Data however suggests that DCIS represents a stage of breast cancer development where most of the molecular changes associated with IDC have already occurred although the tumor itself has not assumed the malignant phenotype [19, 20].

Because of the large variations in clinical behavior described above, DCIS is recognized as a very heterogeneous disease. This heterogeneity hampers the pre-identification of the 50% DCIS cases that never progress to IDC. Currently, there are four primary mode of treatment that are use to manage DCIS and prevent IDC. Up until the 1980s, mastectomy was the primary treatment [21]. However, due to the physical and psychological consequences associated with mastectomy, breast conserving surgery (also known as lumpectomy) has become the approach of choice in low-risk cases although mastectomy is still being used in 30-40% of cases [22]. Randomized clinical trials have identified that lumpectomy followed with radiation therapy can decrease the rates of local recurrence by 40-60% but this appears to be highly dependent on age, tumor size and molecular profiles [18, 23]. A fourth approach which combines lumpectomy with adjuvant therapy such as tamoxifen with or without radiation therapy has been developed for estrogen receptor positive DCIS [18].

Unfortunately, each of these treatment methods is accompanied by its share of side effects, emotional and psychological strain. One of the major hurdles in the management of DCIS is the issue of overtreatment especially in patients at low risk of developing IDC. The lack of proper understanding and identification of at risks markers has led the treatment of those DCIS patient that may not need treatment. For this reason, most patients diagnosed with DCIS are usually over-treated with harsh therapies even though the majority of these tumors never become lethal. The factors regulating the switch from localized to invasive breast cancer remain poorly understood and improving both disease survival and clinical management of patients will require understanding of the molecular mechanisms that regulate each of these steps and the identification of key factors at play.

1.4 Factors contributing to breast cancer invasion

HER2, a receptor tyrosine kinase, is over-expressed in more than 50% of DCIS cells and the risk of developing IDC from DCIS increases by 6-fold for cells overexpressing ERBB2 [24]. This increased invasion occurs via activation of members of the mitogen activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) pathways [25, 26] (Fig. 2). PI3K/Akt and P38-MAPK are potently activated by environmental stresses [27] and control the expression of matrix metalloproteinases (MMPs). Members of the MMP family degrade the basement membrane and stromal extracellular matrix, a precursor to invasion and metastasis. However, not all ERBB2 expressing DCIS develop into IDC and patients whose DCIS never progresses to IDC have an almost 100% chance of survival [18, 28].

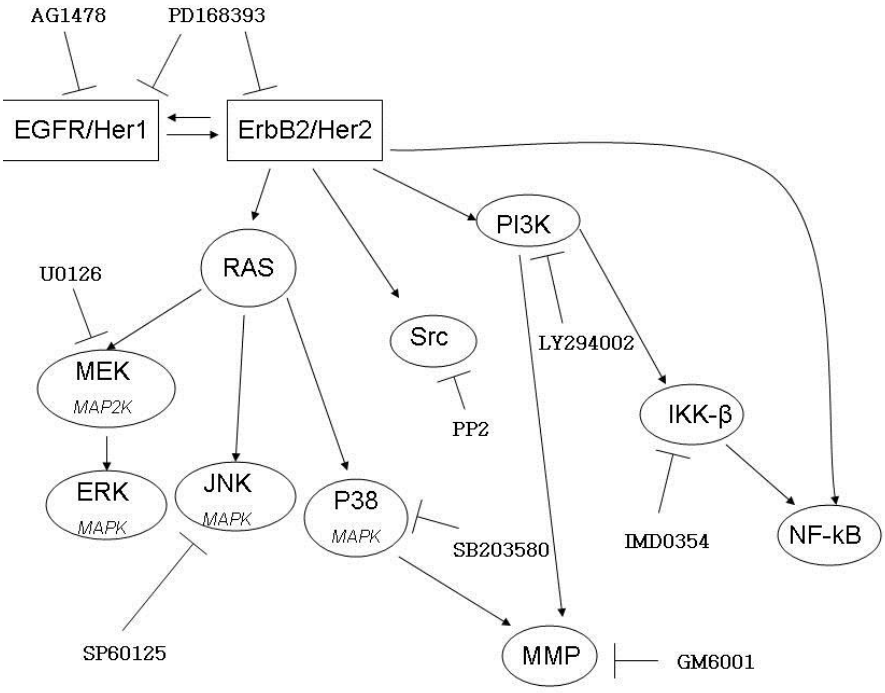

Figure 2: HER2 pathway along with known pharmacological inhibitors linked to their targets.

Like most tissue, breast tissue is comprised of parenchyma and stroma, two distinct compartments that interact through molecular and mechanical signaling. The growth and progression of breast cancer is known to depend on both the malignant potentials of the cancer cells and their response to stroma-associated signaling in the tumor microenvironment [29]. Macrophages, endothelial cells, and fibroblast are all non-malignant stromal cells that clearly have been linked to cancer promotion [30-35]. Stromal factors also contribute to initiation, growth, and spread of tumors. The implications of stroma cells in cancer have long been studied, and we know that normal fibroblast and mesenchymal cells provide signals to inhibit cancer cells

behavior while tumor associated fibroblasts enhance cancer progression [32]. These signals include differential expression of well known tumor suppressors and oncogenes such as ERBB2, p53 and PTEN in the fibroblast cells.

Both compartments of the cancer microenvironment (stroma and epithelial) are evolving together, and their constant interactions during this evolution effects the outcomes of the disease. The role of fibroblasts in cancer progression in particular has been the focus of numerous studies in the past two decades. Fibroblasts are the most abundant cells in animal connective tissue and are primarily responsible for synthesizing the extracellular matrix and collagen. They are found to act both as tumor suppressors and tumor promoters [32]. Normal fibroblasts inhibit differentiation of normal epithelial cells where as cancer associated fibroblasts (CAFs) have the ability to induce morphological transformation of epithelial cells [36, 37]. One characteristic of CAFs is their similarity to myofibroblasts which are differentiated fibroblasts found most abundantly in sites of inflammation and wound healing and enhance tumor phenotypes like proliferation, invasion and migration [38]. CAFs are responsible for the secretion of key chemokines and growth factors such as CXCL12, hepatocyte growth factor (HGF) and TGF-β whose receptors are expressed on the surface of cancer cells. In so doing, CAFs influence primary tumor growth, invasion and metastasis [39]. In addition, signal cross-talk between CAFs and tumor cells have been shown to modify the tumor microenvironment extra-cellular matrix and basement membrane [39, 40]. Changes in matrix mechanical properties were found to suffice in inducing fibroblasts transformation to myofibroblasts [41]. In addition, a study by Fleming *et al* observed that primary fibroblasts cells from

Caucasian American patients induced bigger tumors in mice than fibroblast from African American patients [42]. This study presented a fibroblast specific gene profile associated with race suggesting cancer discrepancies to be involved both in the parenchyma and stroma cells.

An important breakthrough in breast cancer research will be to uncover the factors, which along with genomic abnormalities, trigger DCIS to IDC transition. Identifying these factors will assist in tailoring patient-specific therapies given that current clinical management of DCIS has lead to over-treatment of patients who may never have developed IDC [43].

1.5 Epithelial to mesenchymal transition

For invasion to occur, significant cellular transformation needs to take place especially in *in vivo* structures. Epithelial-to-mesenchymal transition (EMT) is a process where epithelial cells loose their distinctive morphology and molecular characteristics to acquire a mesenchymal motile phenotype that is often associated with invasion. This phenomenon is indispensable during embryogenesis and tissue remodeling. In order for gastrulation and organogenesis to occur, progenitor cells through EMT are able to loosen their cell-cell contact, detach and undergo intrinsic cell motility toward specific regions of the developing embryo [44]. Although normally dormant in adult tissue, EMT is sometimes reactivated during wound healing and tissue regeneration.

EMT is observed to occur during tumor development and likely plays a role in tumor progression [45, 46]. In tumors, this process is characterized by the loss of

epithelial baso-apical polarity and cell-cell junctions, gain of spindle morphology, and activation of transcription factors that lead to increased invasion [47, 48]. EMT is almost always initiated with increased expression of transcription factors (SNAIL 1, SLUG, ZEB, TWIST) that modulate the expression of E-cadherin, a protein important for the characteristic cell-cell adhesion observed in epithelial cells and the main component of cadherin junctions [49]. Along with decreased expression of E-cadherin, EMT is coupled with increased expression of mesenchymal markers like vimentin, alpha smooth muscle actin, N-cadherin and fibronectin [48].

The induction of EMT during tumor development is a consequence of external cues in the tumor microenvironment [50-52]. Several signaling pathways (Notch, β-catenin), growth factors (EGF, TGF-β1, FGF) and hypoxia are known inducers of EMT. Along with its ability to induce migration and invasion in cells, EMT is demonstrated to generate cells with stem like properties which include the ability to self-renew and initiate tumor [53, 54]. and circulating stem cells have undergone EMT [55]. EMT has also been implicated in therapy resistance. In breast cancer, residual cells that survived chemotherapy were characterized by the expression of many EMT-associated genes [56] and these residual cells also share characteristics of tumor initiating cancer stem cells [57-59].

1.6 Interstitial fluid flow

Cells in normal tissue are subject to forces that result from their interactions with other cells and the ECM. Changes in these forces can influence cellular survival, motility, gene expression, and differentiation [60]. In addition, there are

biomechanical consequences of tumor growth that enhance cancer progression. As the tumor grows and expands, a host of mechanical forces and physical properties of the tumor microenvironment are altered.

Matrix stiffness [61], fluid pressure [62], matrix density [63], and interstitial fluid flow (IFF) [62] (Fig. 3) are all altered during cancer progression. IFF in particular is higher in tumor tissue when compared to normal due to elevated interstitial fluid pressure [64].

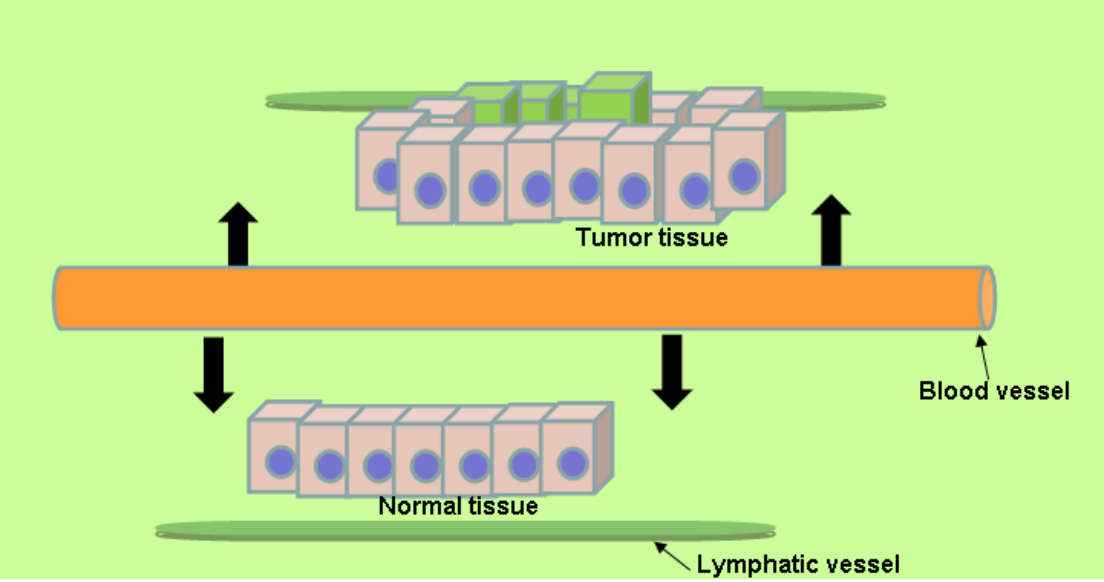

Figure 3: Interstitial fluid flow is defined as the movement of fluid within tissues. This fluid leakage originating from blood vessels is narmally drained in lymphatic vessels.

Levels of IFF have been measured to range from 0.1 to 1 μm s^{-1} in normal tissue and 0.5 to 55 μm s^{-1} in tumor tissue [65-68]. This elevated IFF is driven by steep fluid pressure gradients at the tumor margin [69-72]. Increased IFF - caused by angiogenesis and lymphangiogenesis, and increased vascular permeability - has been correlated with lymph node metastasis in human carcinoma, increased cellular

organization and migration of tumor, fibroblast, endothelial, immune and mesenchymal stem cells [67, 68, 73-83]. In addition, IFF alters extracellular gradients of growth factors and chemokines. It is further associated with transporting antigens and other soluble factors to lymph nodes [82, 84], and applying forces directly to cells (via fluid shear) and indirectly via the matrix [85].

To date, different studies have proposed mechanisms to explain the cell migration induced by elevated IFF, although most have focused on already-invasive cells. In fibroblasts, IFF induced myofibroblast differentiation and secretion of matrix metalloproteinases (MMPs), thus indirectly aiding tumor cell migration through matrix priming [41, 86]. Shieh et al showed that fluid flow enhances fibroblast migration via a mechanism dependent on transforming growth factor (TGF)-β1 and collagenase [26]. They proposed a model in which fluid flow causes stromal remodeling by fibroblast which facilitates tumor cell invasion. Shields et al demonstrated that fluid flow increased the metastatic potential of many cancer cell lines by creating a CCR7-dependent autologous chemotactic gradient around cells in 3D [75, 86, 87] (Fig. 4). Polacheck et al identified yet another mechanism where IFF was shown to affect cellular fluid drag and matrix adhesions via integrins and focal adhesions, and interestingly leading to migration against the direction of flow [77, 78]. In metastatic cells, IFF altered extracellular chemokine gradients to accelerate glioma cell migration via CXCR4 [75, 76, 78]. IFF was further demonstrated to modulate MMP activity through cell surface glycocalyx [79, 80, 88]. While we know some of its effects on the tumor microenvironment, the specific molecular pathways

triggered by interstitial fluid flow in tumor cells are still unclear, especially those that play a crucial role in initiating invasion in breast cancer cells.

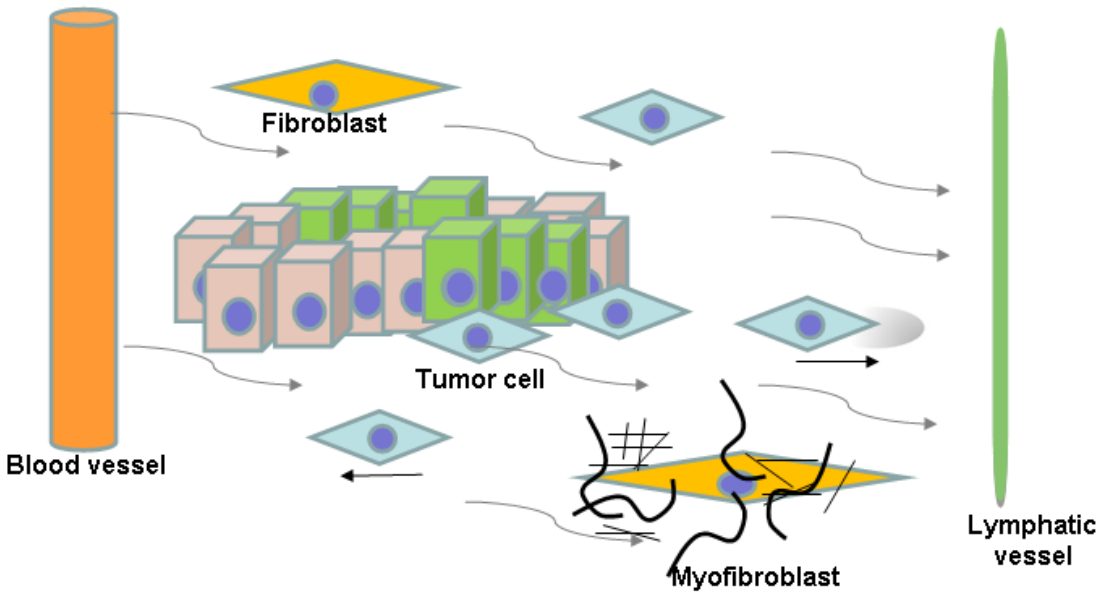

Figure 4: Consequences of interstitial fluid flow (IFF) in tumors. IFF has been shown lead the differentiation of fibroblasts into myofibroblasts, and to encourage tumor cells invasion by inducing fibroblast degradation of matrix and creating autologous chemotaxis.

1.7 Outstanding questions

Breast cancer invasion in general and the transition from DCIS to IDC in particular is a complex process regulated by a multitude of factors (EMT, tumor and stroma associated factors). While we know some of its effects on tumor and stromal cells, the specific molecular pathways triggered by elevated interstitial fluid flow in tumor cells are still unknown, especially those that may play a crucial role in initiating invasion in breast cancer tissue. Nevertheless, mechanical forces alone are likely not sufficient to cause invasion. Deregulation at the gene level and changes in tumor-stromal interactions may all play a part to increase invasion, all driven by

elevated interstitial fluid flow. The goal of this project is to elucidate the role these different components play in the transition from non-invasive breast cancer to invasive disease. Although much attention has been given to the tumor microenvironment and its potential application in cancer therapy, more work is needed to unveil the factors of the microenvironment that are key players in the transition from DCIS to IDC.

CHAPTER 2: Interstitial fluid flow-induced invasion is HER2 independent

2.1 Introduction

Cancer is a multi-step disease and its progression involves transformation, tumor growth, invasion, and metastasis. Breast cancer is the second-leading cause of cancer-associated deaths in women and its mortality is predominantly due to the metastasis of cancer cells. For metastasis to occur, cells have to develop an ability to invade the surrounding stroma, migrate to distant sites, and survive in foreign environments. Several factors have been identified as regulating cancer cell invasion and metastasis. Genetic mutations such as HER2 amplification and overexpression [89, 90] along with mechanical changes to the tumor microenvironment [91, 92] are factors that have each been correlated with breast cancer invasion but are rarely studied in combination.

ERBB2 is an oncogene that encodes for the receptor tyrosine kinase HER2, a protein known to increase the aggressiveness of breast cancer [93]. Poor breast cancer prognosis has been correlated with the overexpression of HER2 in 15-30% [94, 95] of all human breast cancers. In addition, HER2 is overexpressed in more than 50% of ductal carcinoma *in situ* (DCIS) cells – a pre-invasive stage of breast cancer. The risk of developing invasive ductal carcinoma (IDC) from DCIS is six times higher when cells overexpress HER2 [24]. The therapies currently used to treat DCIS patients tend to vary depending on the receptor profile of the cells. They usually include mastectomy, lumpectomy followed with radiation therapy and hormone therapies.

However, approximately 30% of patients who are diagnosed with DCIS will develop invasive breast cancer despite these harsh therapies [18, 93].

Interstitial fluid flow (IFF), which is defined as the movement of fluid through the tissue, is elevated in tumors compared to normal tissue [66, 67]. IFF was previously shown to induce tumor, fibroblast, and endothelial cells migration [75, 76, 78, 83]. While we know some of its effects on tumor and stromal cells, the specific molecular pathways triggered by elevated interstitial fluid flow in tumor cells are still poorly described, especially those that aid in initiating invasion of breast cancer cells in combination with altered gene expression profiles. Specifically, the combined role HER2 expression and interstitial fluid flow play on invasion has never been studied, particularly in the context of how they may influence progression at different stages of breast cancer.

We hypothesized here that interstitial fluid flow induces invasion of HER2 positive breast cancer cells by supporting the activation of the HER2 pathway and causing increased downstream signaling of important intracellular signal transducers such as phosphoinosite-3-kinase (PI3K). Identifying the combined roles of interstitial fluid flow and Her2 expression in breast cancer invasion will expand our understanding of why some cancers progress though invasion and metastasis and other do not.

2.2 Methods

Cell Culture

To model normal breast epithelium, DCIS, and IDC, we used a previously developed model of breast cancer based on MCF-10A human mammary epithelial cells engineered to overexpress HER2 [96, 97]: MCF-10A human mammary epithelial cells, MCF-10As retrovirally transduced with wild-type *ERBB2* (NeuN) and MCF10A overexpressing a constitutively active mutant of *ERBB2* (NeuT). When cultured in 3D conditions, these cells exhibit behavior of normal (MCF10A), pre-invasive *ERBB2* positive (NeuN) and invasive (NeuT) cancer phenotypes [96, 97]. These cells were maintained in DMEM/F12 supplemented with 5% donor horse serum (Atlanta Biologicals, Atlanta, GA), 20 ng/ml epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ), 10 µg/ml insulin (Sigma-Aldrich, Saint Louis, MO), 100 ng/ml cholera toxin (Enzo Life Sciences, Farmingdale, NY), 500 ng/ml hydrocortisone (Sigma Aldrich), and 1% penicillin/streptomycin (Mediatech, Herndon, VA). Cell lines naturally expressing HER2 were also used: BT474, MDA-MB-453 and SKBR3. BT-474 and MDA-MD-453 cells (ATCC, Manassas, VA) were grown in DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1% penicillin/streptomycin. SKBR3 cells were grown in McCoy's 5A (Mediatech) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained in a humidified environment at 37^{o}C and 5% CO_2.

EGF Chemoinvasion

50 µl of 50% growth-factor-reduced Matrigel solution was coated on transwell membranes and allowed to gel at 37^{o}C for 2 hours. Cells were resuspended in basal media 20,000 cells total was added to each culture inserts. Basal media

(control) or 1 ng/ml EGF media was added below the transwell membranes. After 24 hours, cells on the upper side of the culture insert were removed with cotton swabs. Cells on the underside of the cell culture insert were fixed, stained and counted as described below for the 3D-invasion assay. Chemoinvasion was measured by counting the number of cells that invaded through the matrix and migrated across the porous membrane. Experiment was repeated at least twice with 3 replicates each time.

3D Invasion Assay

Interstitial fluid flow was applied to cells as previously described [75, 86, 98]. Briefly, single cells were embedded in a matrix composed of 1.3 mg/ml rat tail tendon collagen type I (BD Biosciences, San Jose, CA) and 1 mg/ml Matrigel™ basement membrane matrix (BD Biosciences) at a final concentration of 5 x 10^5cells/ml inside culture inserts with 8 μm diameter pores (Millipore, Billerica, MA). For static (control) conditions, serum-free media levels inside the insert and outside in the well were kept approximately equal, resulting in minimal pressure difference across the gel and no interstitial flow. For IFF conditions, serum-free media was added under the insert (100 μl) and above the gel (650 μl). The pressure difference generated by these volumes is approximately 1.3 cm H_2O (or 1 mm Hg). After 24 hours of either physiological interstitial flow (approximate flow velocity ~0.1 μm/s) or static conditions, invasion was measured by counting the number of cells that invaded through the matrix and migrated across the porous membrane. Migrated cells on the underside of the culture insert membranes were fixed in 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Cells were

then stained with Alexa Fluor 488-conjugated phalloidin (1:50) (Life Technologies) and 4′,6-diamidino-2-phenylindole (DAPI) (1:500) (MP Biomedicals, Santa Ana, CA). Fluorescence was visualized on a widefield fluorescence microscope (Leica Microsystems). DAPI-stained nuclei on five randomly selected locations of each membrane were counted. Phalloidin stain was used to confirm positive DAPI stains corresponded to cells. Percent invasion was calculated with the following equation:

$$\%Invasion = \frac{100 * (AverageCellCount * MembraneSurfaceArea)}{(\mathrm{Im}ageArea * NumberofCellsSeeded)}$$

Experiments were repeated at least twice with a minimum sample size of n=3.

Western Blotting

Following the invasion assay, total and phospho-protein levels were determined by western blot. Cells were isolated using 2.5mg/ml collagenase D (Roche, Indianapolis, IN) for 30 minutes. The resulting solution was centrifuged, and the pellet was washed with PBS and resuspended in RIPA lysis buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM Tris-HCl at pH 8, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF and 1 mM Na_3VO_4). Lysates were then cleared via centrifugation at 16,000 x g for 20 minutes at 4°C. The antibodies used were: rabbit polyclonal anti-phospho PI3K and total PI3K (1:500, Cell Signaling, Danvers, MA), rabbit polyclonal anti-β-actin (1:3000, Cell Signaling), anti-HER2 (1:1000, Cell Signaling), rabbit polyclonal anti-phospho and total epidermal growth factor receptor (EGFR) (1:500, Abcam), goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:10000, Abcam).

Data and Statistical Analysis

Data are expressed as mean ± standard error of the mean. Differences among treatment groups were tested using Student's t-tests (for two groups) or two-way analysis of variance (ANOVA) (for three or more groups) as appropriate using GraphPad Prism. When ANOVA identified a significant difference, a Bonferroni post-test was employed for multiple comparisons. Differences were accepted as significant at $p < 0.05$.

2.3 Results

EGF induces invasion of MCF10A and NeuN

We relied primarily on MCF10A, NeuN, and NeuT as our breast cancer model. The main difference between these cells lies in their expression of HER2. MCF10A are nontumorigenic mammary epithelial cells. NeuN and NeuT are cell lines deriving from MCF10A that overexpress wild type HER2 and mutant HER2 respectively. All 3 cells lines express similar levels of the other HER family member EGFR (Fig. 5A). In addition, each cell line represents a specific stage of breast cancer progression primarily as a consequence of their HER2 expression (MCF10A: normal-like, NeuN: DCIS-like, NeuT: IDC-like), which is a known inducer of invasion in cells [89, 99, 100]. The invasion capability of the cell lines is demonstrated in Fig. 5B. Using an EGF-dependent chemotaxis assay, the cells were plated on top of a layer of reconstituted basement membrane matrix (Matrigel) and 1 ng/ml EGF was added to the underside of the cell culture insert as a chemoattractant. We observed that MCF10A cells only invaded in the presence of EGF. NeuN cells also responded

to the EGF gradient with a 10-fold increase of invasion; however NeuT cells were insensitive to EGF, exhibiting a high level of invasion in the absence of any external stimulus.

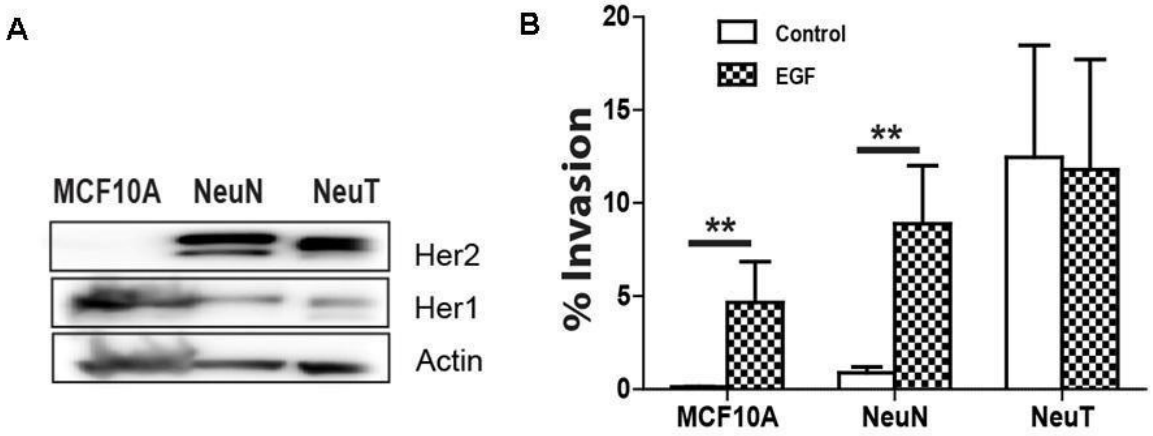

Figure 5: EGF induces invasion of MCF10A and NeuN: The cell lines used in this study model 3 separate staged of breast cancer, normal, HER2 positive pre-invasive and HER2 positive invasive cells. A) Representative western blot of epidermal growth factor family family members HER1 (commonly known as EGFR) and HER2 (Neu). All 3 cell lines express HER1, however detectable levels of HER2 were only observed in NeuN and NeuT. β actin was used as loading control. B) EGF induces invasion of the non-invasive cells MCF10A and NeuN but does not affect NeuT invasion. Chemotaxis assay: cells were seeded on a layer of matrigel in a cell culture insert. 1ng/ml EGF was added to the underside of the cell culture insert as a chemo-attractant. Invasion was measured after 24 hours by counting the number of cells that migrated and invaded through the matrigel and pores of the cell culture insert. Experiment was run in triplicates. All values are mean ± SEM. Student t test (: $p < 0.01$)**

Interstitial fluid flow induces invasion of both HER2 positive and negative cells.

Using our 3D IFF invasion assay, we measured the effects of IFF on invasion (See appendix figures). Invasion was significantly greater in flow conditions compared to static in parental MCF10A and MCF10A cells overexpressing HER2 (NeuN and NeuT) (Fig. 6). We further verified the IFF response of breast cancer cell lines with natural HER2 overexpression (SKBR3, MDA-MB-231 and BT-474) and again observed increased invasion under IFF (Fig. 6). The only HER2 negative cell

line in the panel of cells was MCF10A, and these cells exhibited low basal (static) invasion compared to NeuN and NeuT. This was expected given that these are non-tumorigenic epithelial cells and the previously described role of HER2 in breast cancer invasion [89]. Based on the observed effect of EGF on MCF10A invasion and EGF's previously observed role in invasion [101-103], we decided to look at the role of EGF and its receptor EGFR on IFF-induced invasion of MCF10A.

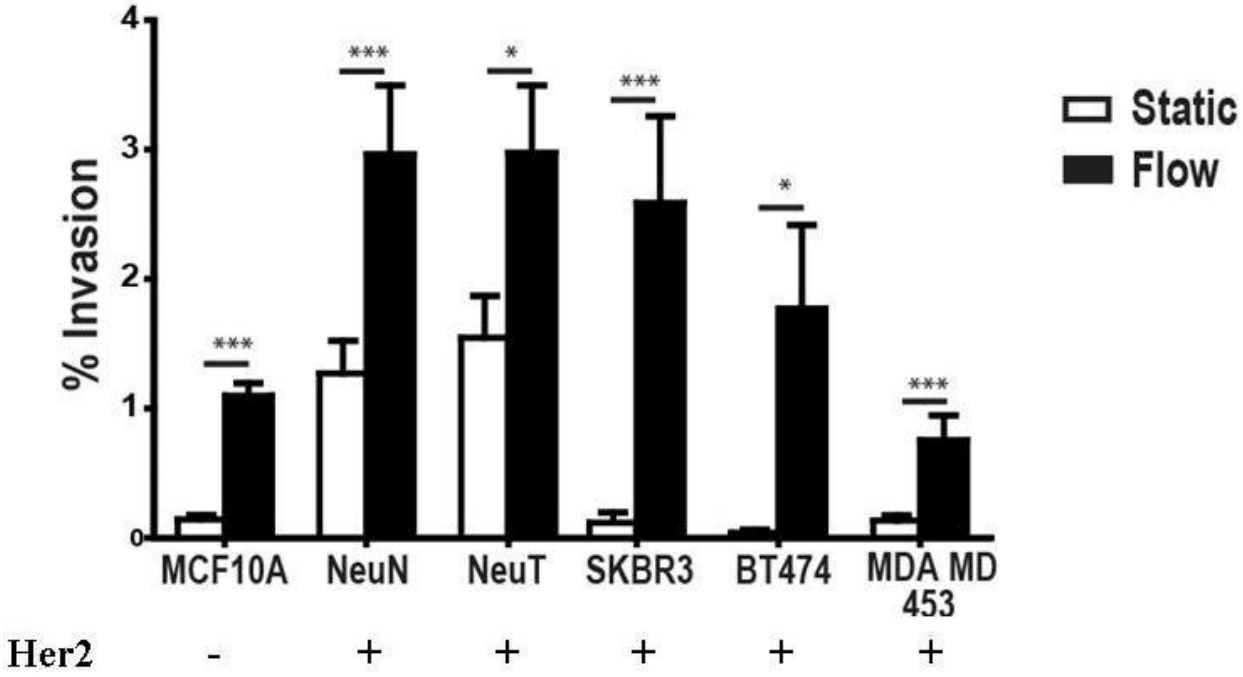

Figure 6: Interstitial fluid flow increases invasion of HER positive and negative cells. Percent invaded cells after 24 hours in 3-D IFF assay using our breast cancer model cells and additional HER2 overexpressing cell lines. All values are mean ± SEM. Student t test (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$), n>12.

EGFR activity is necessary for interstitial fluid flow induced invasion in MCF10A.

Parental MCF10A cells do not express detectable levels of HER2, however they do express EGFR (Fig. 5A). The observation that MCF10A invaded in the presence of EGF and/or IFF led us to block EGFR using the pharmacological

inhibitor AG1478 in our 3-D invasion assay. When blocking EGFR, we observed a significant decreased in IFF-induced invasion of the cells (Fig. 7A), suggesting that EGFR is required for IFF induced invasion in these cells. A key member of the molecular pathway activated by IFF in cells to induce invasion is PI3K (see chapter 4 for further discussion). The regulatory subunit of PI3K (p85) is almost always phosphorylated in the presence of IFF, however its modulator was not identified in MCF10A. IFF-induced PI3K phosphorylation was significantly decreased in these cells when treated with AG1478 (Fig. 7B), suggesting that EGFR activation is upstream of PI3K phosphorylation in IFF-induced invasion of MCF10A cells.

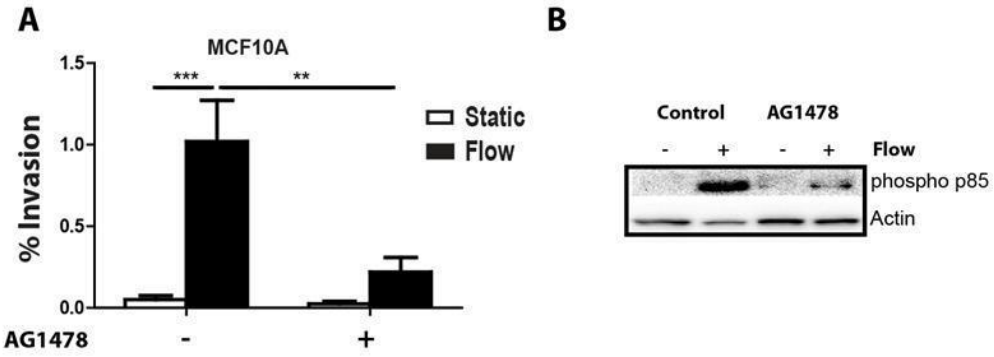

Figure 7: EGFR activity is necessary for flow induced invasion in MCF10A. A) Changes in flow-induced invasion in the presence or absence of EGFR (HER1) inhibitor (AG1478) in MCF10A. B) Representative western blot of phospho-p85 after 24 hours in 3D invasion assay with and without the AG1478; β actin was used as loading control. All values are mean ± SEM. Student t test and 2-way ANOVA (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$), n>6.

EGFR and HER2 are not necessary for IFF-induced invasion in NeuN and NeuT

In contrast, blocking EGFR or HER2 with AG1478 and/or lapatinib did not significantly alter IFF-induced invasion in NeuN and NeuT. In NeuN, both EGFR and HER2 are necessary for static invasion because in the presence of these inhibitors,

static invasion was ablated (Fig. 8). However, IFF-induced invasion was still pronounced in the presence of one or both inhibitors. EGFR and HER2 are known to induce random invasion in cells, which is initiated by the heterodimerization of both receptors. The fact that IFF is still able to enhance NeuN invasion in the presence of inhibitors to both HER2 and EGFR suggests that IFF-induced invasion occurs independently of these receptors in these cells. IFF induced invasion in NeuT is also independent of these receptors (Fig. 8), despite HER2 being constitutively phosphorylated in these cells (Fig. 5A).

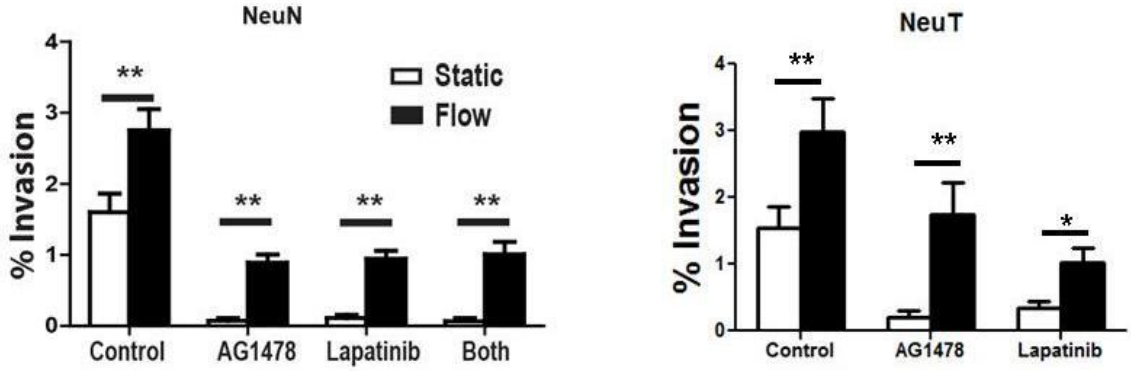

Figure 8: EGFR and HER2 activity are not necessary for flow induced invasion in NeuN and NeuT. A) Changes in invasion in response to HER1 and HER2 inhibitors in NeuN. NeuN response to AG1478, Lapatinib or both; neither inhibitors ablated IFF response. B) Changes in invasion in response to HER1 and HER2 inhibitors in NeuT. Neither inhibitors ablated IFF response. All values are mean ± SEM. Student t test (*: $p < 0.05$. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$); n>6.

Furthermore, IFF does not alter activation of HER2 as seen by the lack of change in phosphorylation of HER2 when comparing NeuN cells exposed to flow and control static conditions (Fig. 9A). In addition western blots of total HER2 protein in the presence of the HER2 inhibitor, lapatinib, revealed that although this inhibitor

decreased total levels of HER2, it did not alter the increased PI3K phosphorylation observed in IFF samples (Fig. 9B).

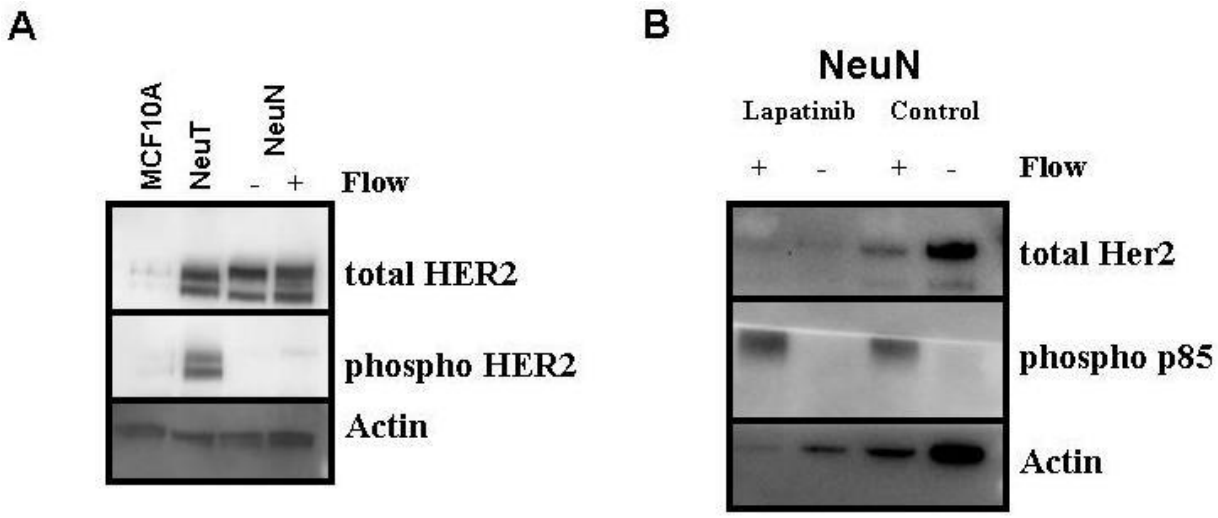

Figure 9: Inhibiting HER2 does not alter flow induced activation of PI3K. A) Representative western blot of phosphorylated and total HER2 in MCF10A, NeuN, and NeuT. β actin was used as loading control. HER2 is constitutively phosphorylated in NeuT and IFF does not activated HER2 phosphorylation in NeuN. B) IFF induces phosphorylation of PI3K regulatory subunit p85 and this is achieved independent of HER2. Representative western blot of total HER2, phosphor PI3K in the presence of HER2 inhibitor (Lapatinib). Inhibiting HER2 (upstream of PI3K) or mtor (downstream of PI3K) do not alter flow induced activation of PI3K, further supporting our claim that IFF induced invasion is independent of HER2 in NeuN and NeuT.

2.4 Discussion

In this study we characterized the role of EGFR and HER2 in regulating interstitial fluid flow induced invasion in MCF10A, NeuN and NeuT, three cells lines exhibiting distinct invasion patterns and modeling three separate stages of breast cancer. EGFR inhibition prevented both IFF induced invasion and phosphorylation of PI3K in MCF10A. In contrast, both EGFR and HER2 inhibition decreased invasion of static (control) NeuN and NeuT but did not alter IFF-induced invasion and PI3K activation.

In MCF10A, a non-tumorigenic cell line, we observed invasion was strongly dependent on EGFR, a growth factor receptor whose signaling is tightly controlled in normal tissue. In these cells, invasion was only observed when EGF was added to the system or in the presence of IFF. EGFR was identified as the activator of IFF-induced PI3K (Fig. 7). In addition, these cells lost their IFF-induced invasion when EGFR was inhibited. The fact that IFF was able to induce a low but significant increase in migration and invasion in these cells was shown in a previous study [75], however a mechanism was never identified. EGFR is the most studied membrane tyrosine kinase receptor in cancer. Mutations and over amplifications of this receptor have long been implicated in most cancer initiation and progression [103-105]. The most commonly demonstrated mode of IFF induced invasion is autologous chemotaxis, in which IFF creates a chemokine gradient around the cells allowing them to migrate in the direction of flow. However, when endogenous levels of EGF was added to MCF10A in the presence of IFF in order to ablate all peripheral cellular gradients, the cells invaded more in response to IFF suggesting that MCF10A do not respond to IFF through an EGF-dependent autologous chemotaxis. In contrast, we hypothesize that IFF may induce EGF production in cells or indirectly activate EGFR in an EGF-independent manner. Although this was not tested in our current study, additional experiments are underway. Nonetheless this strongly suggests the potential role IFF may play in EGFR-dependent cancers, an area that has never been examined, and further studies may be beneficial in improving therapeutic intervention of this type of cancer.

The combined role of members of the epidermal growth factor receptor family in breast cancer invasion, especially EGFR and HER2, has been explored in these and other cells [28, 89, 100, 106-109] but never in the context of IFF. HER2 is an established regulator of invasion in breast cancer cells. Its activity is usually regulated by heterodimerization with its other family members (EGFR, HER3 and HER4) and together, they transactivate key kinases necessary for the transcription of genes associated with cell motility [101, 108]. Although it has previously been shown that IFF plays a role in cancer invasion [41, 75, 78, 86], the molecular mechanisms underlying its effects on cells are still poorly understood. We initially hypothesized that IFF in combination with HER2 induced invasion. The data presented in this study strongly suggest that although HER2 is necessary for most invasion in HER2 positive cells, IFF induces a pathway independent of HER2 and these pathways will be presented in great details in the following chapters.

This study further reveals that IFF may play a role in HER2 drug resistance since HER2 positive breast cancer cells can still invade even when treated with anti-HER2 therapies, given the lack of HER2 involvement in IFF induced invasion. Regardless, HER2 overexpression still modulates significant gene expression changes in cells and these persistent changes may affect IFF response, even if HER2 activation at that moment does not.

CHAPTER 3: Interstitial fluid flow induces epithelial to mesenchymal transition in cells

3.1 Introduction

The tumor microenvironment includes a variety of biophysical forces whose effects are known to influence cancer progression. Interstitial fluid flow (IFF) is one such force that is present in normal breast tissue and elevated in tumors. We previously demonstrated that IFF induces invasion of normal, pre-invasive, and invasive cells via activation of phosphoinositide-3-kinase (PI3K). This phenomenon was observed both in single cells and in 3D acini structures. In normal cells, IFF-induced PI3K activation was modulated by EGFR, a growth factor whose signaling is tightly controlled in normal tissue. In invasive cells, CXCR4 controlled IFF-induced invasion. In order to understand why different pathways are activated by IFF to enhance invasion in these cells, we questioned the role of epithelial-to-mesenchymal transition (EMT). EMT is a process observed in cancer progression where cells lose their epithelial characteristics and adopt a mesenchymal phenotype, which notably includes an increased propensity to invade.

In this study, we employed two separate approaches to determine if interstitial flow affects the EMT process. In the first approach, we profiled mRNA and protein expression differences of cells after 24 hours of exposure to IFF. In the second approach, we developed a method to isolate the population of cells that consistently invaded after three serial exposures to IFF. We compared the levels of EMT markers in these cells to those of regular cells and those isolated from three consecutive

invasion assays under static conditions. We further profiled the morphological and cellular proliferation differences between these three groups.

3.2 Methods

Cell Culture

Non-invasive and non-tumorigenic cells MCF10A, pre-invasive NeuN, and invasive NeuT cells were used. Cells were maintained in DMEM/F12 supplemented with 5% donor horse serum (Atlanta Biologicals, Atlanta, GA), 20 ng/ml epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ), 10 µg/ml insulin (Sigma-Aldrich, Saint Louis, MO), 100 ng/ml cholera toxin (Enzo Life Sciences, Farmingdale, NY), 500 ng/ml hydrocortisone (Sigma Aldrich), and 1% penicillin/streptomycin (Mediatech, Herndon, VA).

3D Invasion Assay

To isolate interstitial fluid flow responsive cells and static control, cells were embedded in a matrix composed of 1.3 mg/ml rat tail tendon collagen type I (BD Biosciences, San Jose, CA) and 1 mg/ml Matrigel™ basement membrane matrix (BD Biosciences) at a final concentration of 5×10^5 cells/ml inside culture inserts with 8 µm diameter pores (Millipore, Billerica, MA). For static (control) conditions, serum-free media levels inside and outside the insert were kept approximately equal, resulting in a minimal hydrostatic pressure difference across the gel and no measurable interstitial flow. For IFF conditions, serum-free media was added under the insert (100 µl) and above the gel (650 µl). The hydrostatic pressure difference

generated was approximately 1.3 cm H_2O (or 1 mm Hg). After 24 hours of either physiological interstitial flow (approximate flow velocity ~0.1 μm/s) or static conditions, invasive cells were defined as the cells that invaded through the matrix, migrated across the porous membrane, and attached to the underside of the membrane. Gels and cells on the upper surface of the membrane were removed with cotton swabs. Migrated cells on the underside of the culture insert membranes were washed once in PBS then incubated in trypsin for 10-20 minutes. Detached cells were then resuspended in full media. The resulting solution was spun down for 5 minutes at 1,000 rpm and plated in a 24 well plate. Once the invaded cells were expanded to an appropriate cell concentration, the experiment was repeated two additional times to ensure the resulting population was primarily made of cells that are highly responsive to IFF. To check for increased IFF induced invasion, cells in similar 3D invasion assay conditions were run in parallel. The migrated cells in this condition were fixed in 4% paraformaldehyde for 30 minutes and stained with 4′,6-diamidino-2-phenylindole (DAPI) (2 ug/ml) (MP Biomedicals, Santa Ana, CA). Labeled cells were visualized on a widefield fluorescence microscope (Leica Microsystems). DAPI-stained nuclei on five randomly selected locations of each membrane were counted. Phalloidin stain was used to confirm that positive DAPI stain corresponded to cells. Percent invasion was calculated with the following equation:

$$\%Invasion = \frac{100 * (AverageCellCount * MembraneSurfaceArea)}{(imageArea * NumberofCellsSeeded)}$$

Western Blot

Following the 3D invasion assay, protein levels were determined by western blot. Cells were isolated from the matrix using 2.5 mg/ml collagenase D (Roche, Indianapolis, IN) for 30 minutes. The resulting solution was centrifuged, and the pellet was washed with PBS and resuspended in RIPA lysis buffer containing protease inhibitors (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM Tris-HCl at pH 8, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF and 1 mM Na_3VO_4). Cell grown on 2D cell culture flasks were trypsinized, washed twice in PBS and resuspended in RIPA lysis buffer containing proteases inhibitors (Roche). Lysates were cleared via centrifugation at 16,000 x g for 20 minutes at 4°C. Antibodies used were: rabbit polyclonal anti-β-actin (1:3000, Cell Signaling), rabbit monoclonal anti-vimentin (1:1000, Abcam), rabbit monoclonal anti-E-cadherin (1:1000, Abcam), rabbit polyclonal anti-collagen type IV (1:1000, Abcam), and goat anti-rabbit HRP (1:10000, Abcam).

Immunofluorescence microscopy

For visualization of vimentin expression differences, cells were plated onto 8 well chamber slides at 10,000 cells per well. After 16 hours, cells were fixed in 4% paraformaldehyde in PBS for 30 minutes and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. This was followed by 1 hour in blocking solution (1% BSA, 0.1% goat serum). Cells were stained with rabbit monoclonal anti-vimentin (1:100) (Abcam) for 16 hours, washed in PBS 3 times. DAPI (2 ug/ml), and then incubated with Alexa Fluor 555 conjugated anti-rabbit IgG for 16 hours, followed by 3 more

washes in PBS. Fluorescence was visualized on a widefield fluorescence microscope (Leica Microsystems).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed on cDNA reversed transcribed using QuantiTech Reverse Transcription kit and QuantiTech SYBR Green PCR kit (Qiagen). Primers for epithelial markers human desmoplakin and integrin subunit α6 (Quantitech, Qiagen) were used to quantify mRNA expression differences between static and flow samples.

Cell proliferation assay

We performed an MTT colorimetric proliferation assay to determine differences in cellular proliferation and response to HER2 associated drugs. Cells were plated in 96-well plates (5,000 cells/well) with full media. Twenty-four hours after cell plating (day 0), media was changed to serum free treatment media (lapatinib, PD168393, control) and full media for each condition. Following the appropriate treatment period (1, 2, 3, 4 days), MTT reagent was added to each well and incubated for 4 hours. DMSO was used to dissolve the crystals and optical density readings at 570 nm were obtained using a spectrophotometer.

Data and Statistical Analysis

Data are expressed as mean ± standard error of the mean. Differences among conditions were tested using Student's t-tests (for two groups) or two-way analysis of

variance (ANOVA) (for three or more groups) using GraphPad Prism. When ANOVA identified a significant difference, a Bonferroni post-test was employed for multiple comparisons. Differences were accepted as significant at $p < 0.05$.

3.3 Results

We previously identified that IFF increases invasion on non-invasive cells (see Chapter 2). In the current study, we hypothesized that one of the ways IFF increases invasion of cells is by inducing EMT in pre-invasive cells. During EMT, cells lose their epithelial characteristics, which include loss of junction proteins such as E-cadherin [49, 110]. We therefore compared gene expression changes in NeuN cells exposed to IFF for 24 hours and control. Using this approach, we observed that IFF significantly lowered the levels of desmoplakin, a junction protein and integrin alpha 6, a part of the α6β1 and α6β4 receptors that bind laminin V (a basement membrane protein) (Fig. 10A). In addition, we observed increased expression of the mesenchymal cells marker vimentin in lysates from IFF samples of both NeuN and MCF10A (Fig. 10B-C).

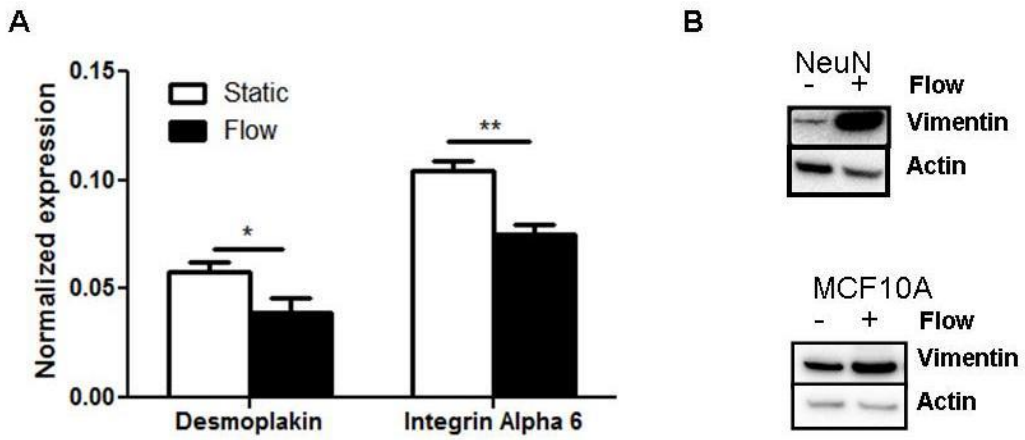

Figure 10: IFF induces the expression of EMT markers. A) RT PCR revealed IFF-dependent decreased mRNA expression of desmoplakin and integrin alpha 6, two genes highly expressed in epithelial cells. B) Western blot analysis shows vimentin levels are increased in both NeuN and MCF10A cells after exposure to IFF.

Collagen type IV is well studied as being one of the main protein components of the basement membrane [111, 112] where its expression is negatively correlated with breast cancer progression [113, 114]. It has however been recently identified as a positive EMT marker in mammary cells [115]. We questioned changes in the levels of this protein in MCF10A and NeuN and observed increase levels in the IFF conditions compared to control static conditions. Collagen IV levels increased in the presence of IFF in all cells tested (Fig. 11). In MCF10As, IFF-induced increase of collagen IV was ablated by the EGFR inhibitor AG1478. This inhibitor was previously demonstrated to reduce IFF-induced invasion (see chapter 2). Furthermore we observed that 3D spheroids of MCF10A and NeuN exposed to IFF also had an increased expression of collagen IV. This suggests that collagen IV plays similar roles in IFF signaling in both single cells and higher 3D complex structures. We previously identified that flow induced signaling occurs through EGFR in MCF10A

cells (Chapter 2). In line with this finding, we observed that the EGFR inhibitor AG1478 decreases collagen type IV levels in response to IFF (Fig. 11).

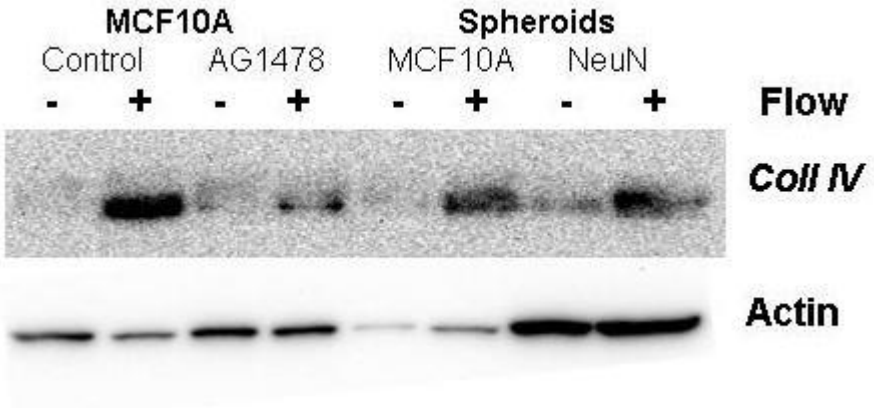

Figure 11: IFF increases protein levels of intracellular collagen type IV. Western blot analysis of MCF10A single cells in the presence or absence of EGFR inhibitor AG1478 as well as MCF10A and NeuN spheroids.

Although short exposure (24 hours) to IFF induced changes in expression of some EMT markers, the expression of key EMT-associated proteins like E-cadherin remained unchanged. For this reason we hypothesized that long term exposure to IFF is required for further EMT to occur. Following a new approach to isolate NeuT and NeuN population of cells that consistently invaded after three separate IFF invasion assay, we compared the expression of EMT markers in these cells (HF) to that of cells that consistently invaded under static conditions (HS). We tested changes in invasion after the isolation and observed that NeuT HF cells tended to become more invasive with more isolation rounds (Fig. 12) however this was not observed in NeuN HF.

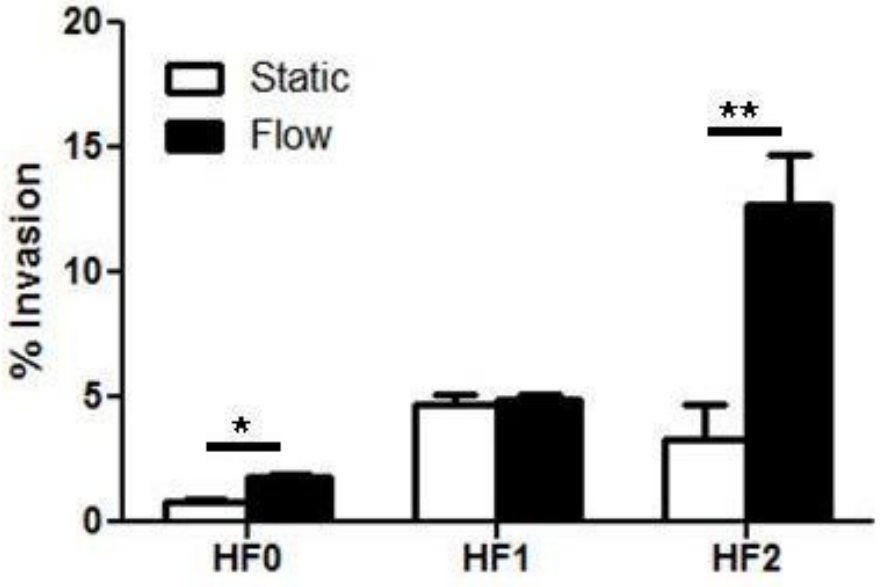

Figure 12: NeuT cells at each cell sorting stage became more and more invasive. HF0: invasion assay of regular NeuT, first round of cell sorting; HF1: second round of cells sorting; HF2: third round of cell sorting. Error bars represent SEM of 3 replicates; *p value < 0.05; **p value < 0.01.

NeuN HF cells exhibited lower levels of the epithelial marker e-cadherin (Fig. 13A). Levels of the mesenchymal marker vimentin were usually low in NeuN, localized in distinct area in the cells, not filamentous, and with no clear network. As cells undergo EMT, just as what is observed during treatment with the known EMT inducer transforming growth factor TGF-β1, the expression and localization of vimentin changes. In EMT cells vimentin levels increases and adopt a more uniform filamentous network throughout the cell (Fig. 13B). And this has been observed in other cells lines and systems [116-118]. We observed that compared to their static (HS) counterparts, NeuN HF cellular distribution of vimentin also became uniform like in NeuT (Fig. 13C). The cellular localization of vimentin also changed. In regular NeuN cells, vimentin intensity was lower and localized in distinct sections of the cell surrounding the nucleus. In HS cells, although vimentin intensity was higher, it

remained localized in distinct sections of the cell surrounding the nucleus. In contrast, vimentin intensity of HF cells was higher than regular cells and spread out uniformly throughout the cell.

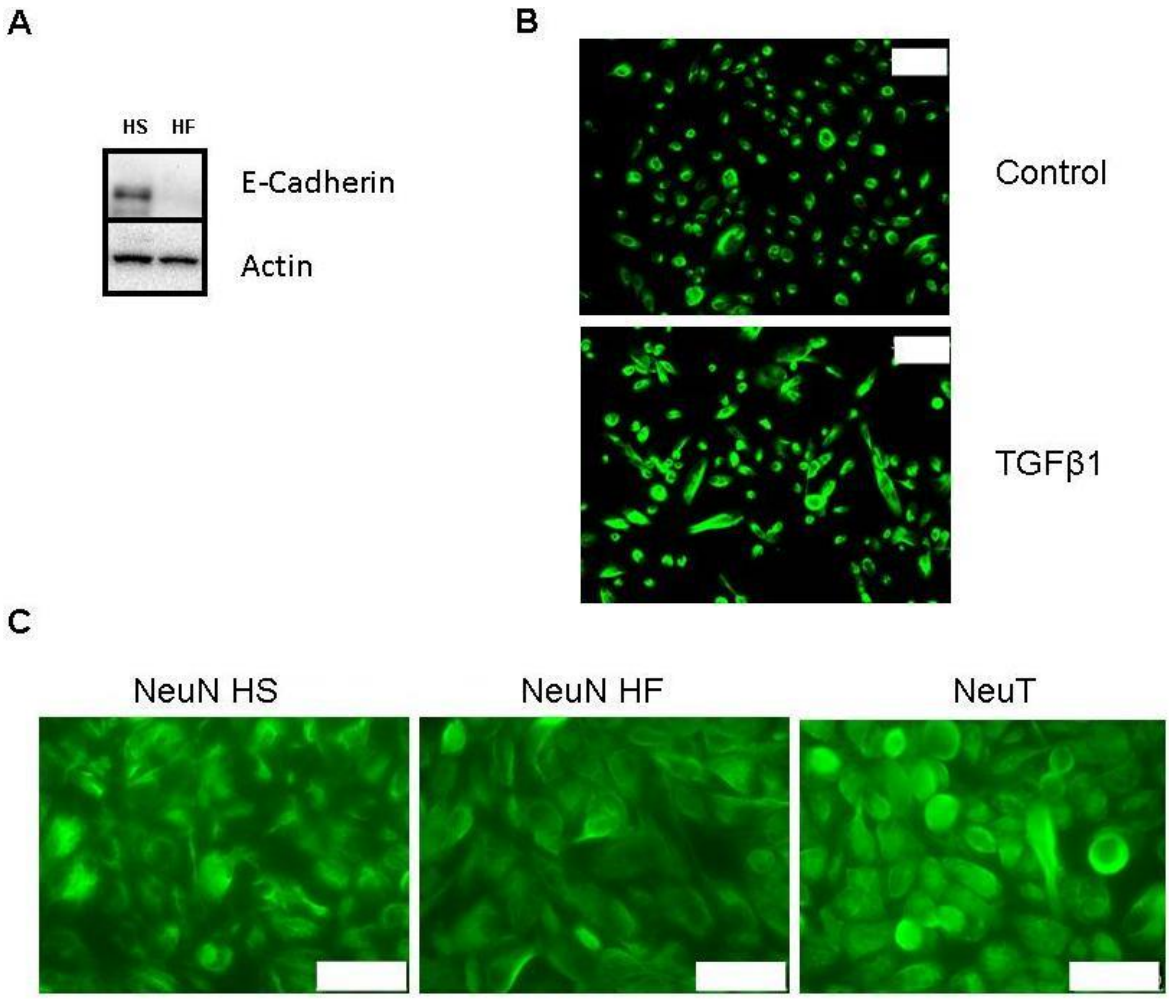

Figure 13: Flow responsive cells express higher levels of EMT markers. Static (HS) and flow (HF) responsive cells were isolated following a series of 3 cells sorting. A) NeuN HS cells express lower levels of E-cadherin compared to HF cells. White bar represents 100 µm B) NeuN EMT induction (TGF-β1) leads to increase vimentin levels along with uniform cellular distribution C) HF cells express similar vimentin distribution when compared to NeuT, a cell that has undergone EMT. White bar represents 50 µm.

A correlation between EMT and drug resistance has been established [119-121]. Mesenchymal-like cells are usually more tolerant of drug treatments compared

to their more epithelial-like counterparts [122, 123]. For this reason, we also aimed to identify the potential role IFF-induced EMT may play on resistance to HER2 therapy by comparing cell proliferation of HF, HS and Reg in the presence of known breast cancer drugs. While relying primarily on MTT assays, we first observed a significant decrease in proliferation of HF cells compared to regular and HS cells (Fig. 14A). Although both inhibitors significantly affected cellular viability of all three cell types, HF cells exhibited a lower sensitivity to both drugs by HF cells (Fig. 14B).

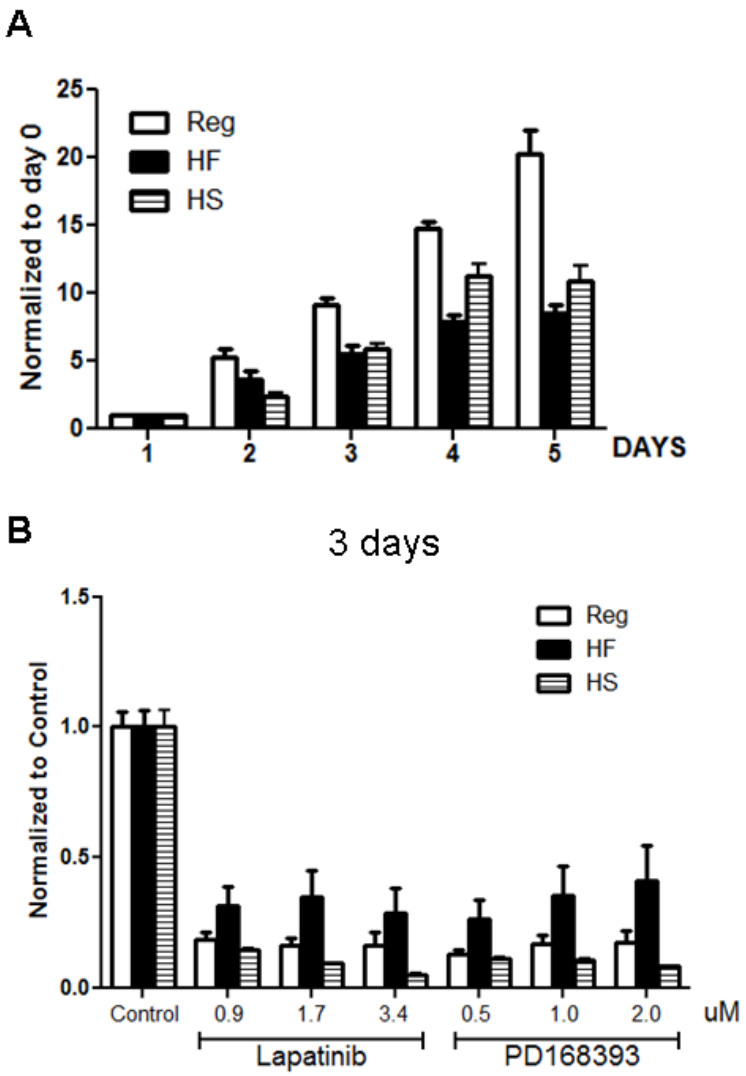

Figure 14: NeuN HF cells proliferate less and have a higher tolerance to drug treatment compared to their regular and HS counterparts. A) HF cells proliferate at a slower rate than HS and reg cells. Values where normalized to day 0 values. B) HF cells survived more in the presence of lapatinib and PD168393, 2 EGFR and HER2 inhibitors. Values where normalized to day control Error bars represent SEM of 2 separate experiments with 4 replicates each.

3.4 Discussion

In this study, we followed two separate approaches to study the role of EMT in IFF-induced invasion. In the first approach, we focused on changes to EMT markers due to short exposure to IFF. In the second approach, we aim to identify the

consequences of longer exposure to IFF. Both approaches revealed that IFF induces EMT in cells. In addition, the second approach revealed that IFF renders cells resistant to HER2 positive breast cancer therapies. Our data further suggest the classification of intracellular collagen type IV as an EMT marker.

We observed increased levels of vimentin and changes in vimentin filaments characterizations both after short and long IFF exposures. Vimentin filaments modulate cellular motility and invasiveness [124]. The fact that IFF enhances vimentin expression in cells further supports its role in cancer invasion. Loss of E-cadherin was only observed in cells after longer exposure to IFF. During EMT, the levels of E-cadherin are regulated both at the transcriptional level by transcription factors like Snail and Slug and at the post-translational level by focal adhesion kinases (FAK), SRC and receptor tyrosine kinases [49]. Using pharmacological inhibitors, we previously concluded that FAK, SRC and receptor tyrosine kinases such as EGFR and HER2 are not involved in IFF-induced invasion of NeuN since they did not affect IFF-induced invasion. In line with these findings, the post-translational regulation of E-cadherin was not expected. Because 24 hours may not be enough time to observe protein changes due to transcriptional regulation, this may explain why we only observed changes in E-cadherin levels when cells were repeatedly exposed to IFF (Fig. 13).

The role of collagen type IV in EMT is not clear and merits further investigation. We identified collagen IV to be up-regulated in correlations with EMT in cells exposed to IFF (Fig. 11) and this agrees with a recent findings where it was

identified as an EMT inducer [115]. Collagen IV has however been more correlated with basement membrane as it one of the main components after Laminin V.

Several studies have demonstrated a close connection between EMT and drug resistance [125, 126]. Mesenchymal stem cells express EMT markers, proliferate at a slower rate when compared to their epithelial counterparts, and are resistant to the majority of current cancer treatments [127-129]. Although tumor initiating capabilities may be a process independent of EMT [127], all three of those characteristics were observed in our IFF responsive cells. In HER2 positive cells, EMT is sometimes followed by a loss of HER2 overexpression [121] which may explain the spontaneous desensitization to HER2 targeted therapies. This phenomenon was observed in one of our HER2 positive cell lines which developed IFF-induced drug resistance (data not shown). Furthermore, the PI3K pathway typically regulates drug resistant cancer cell survival [130] specifically through PTEN, a PI3K inhibitor. We previously demonstrated that IFF induces strong PI3K phosphorylation (see Chapters 2, 4 and 5). It is possible that the combination of IFF-induced PI3K phosphorylation and IFF-dependent EMT induction together modulate cellular response to HER2 therapy specifically and cancer therapies in general.

Longer exposure to IFF is represented in this study as a series of three separate 24 hours IFF exposures. Although this is a longer time point than what is used in the majority of IFF studies, it is still very different when compared to *in vivo* system where IFF is always present. In addition, there may be a host of IFF consequences that are not captured in a 24 hours experiment. Future studies will focus on identifying the effects of continuous exposure to IFF in a 3D microenvironment.

This will better mimic patient tissue and allow us to identify those long term effects of IFF that are more relevant *in vivo.*

The fact that IFF alone is able to induce EMT by affecting the levels of key proteins like E-cadherin, collagen type IV and vimentin in cells along with altering drug response suggests its role as a major contributor to breast cancer progression and its involvement on patient therapy response.

CHAPTER 4: Epithelial-to-mesenchymal transition alters interstitial fluid flow-induced signaling in ERBB2-positive breast cancer cells

4.1 Introduction

Breast cancer is the most commonly reported form of cancer in women worldwide and is responsible for 13.7% of cancer related deaths in women [131]. The mortality rates associated with this and most other solid cancers is a consequence of cancer cell invasion into the surrounding stroma and metastasis to distant organs. Although many factors responsible for invasion and metastasis are still unknown, the influence of the breast tumor microenvironment, which consists of stromal cells, extracellular matrix, and soluble factors, on cancer progression is well established [10, 132].

While their importance in cancer progression has only been realized relatively recently, various biophysical factors are significantly altered in the tumor microenvironment. Changes in matrix density [63], stiffness [133], organization [61], interstitial fluid pressure [134], and flow [62] are all biophysical consequences of tumor growth that affect gene expression, proliferation, differentiation and invasion. Interstitial fluid flow (IFF), the movement of fluid through the tissue matrix, is elevated in tumors compared to normal tissue [91]. This elevated IFF is driven by steep fluid pressure gradients at the tumor margin [70]. Measured levels of IFF range from 0.1 to 1 μm s^{-1} in normal tissue, and 0.1 to 55 μm s^{-1} in tumor tissue [65-67]. Increased IFF is directly linked to lymph node metastasis in human cervical carcinoma, and increased cell motility and invasion *in vitro* in breast cancer, glioma, melanoma, and renal carcinoma cells [67, 75, 76, 79, 86]. Although increased IFF

appears to drive cancer cell invasion, the underlying mechanisms of interstitial fluid flow mechanotransduction are not fully understood.

To date, different studies have proposed mechanisms to explain the strong tumor and stromal cell migration induced by elevated IFF, although most have focused on highly invasive cells. In fibroblasts, IFF induced myofibroblast differentiation and secretion of matrix metalloproteinases (MMPs), thus indirectly aiding tumor cell migration through matrix remodeling [41, 86]. In metastatic cells, IFF altered extracellular chemokine gradients to accelerate tumor cell invasion via CXCR4 and CCR7-dependent chemotaxis [75, 76], and increased MMP activity via shear stress sensing through the glycocalyx [79]. More recently, IFF was shown to induce invasion against the direction of flow due to fluid drag forces and activation of integrins on the upstream side of the cell [77]. While we know some of its effects on invasive cells, the specific pathways triggered by IFF are still unclear, especially those that play a crucial role in the early stages of invasion.

At these early stages of invasion, the process of epithelial-to-mesenchymal transition (EMT) can play an important role. During EMT, epithelial cells lose their distinctive morphology and molecular characteristics and acquire a mesenchymal phenotype that is often associated with invasion. This phenomenon, although indispensable during embryogenesis and tissue remodeling, also occurs during tumor development and likely plays a role in tumor progression [45]. EMT is characterized by the loss of epithelial baso-apical polarity and cell-cell junctions, spindle-like cellular morphology, and activation of transcription factors that lead to increased invasion [47, 48]. The induction of EMT during tumor development is a consequence

of external cues in the tumor microenvironment like changes in levels of cytokines and growth factors [48, 51]. To date, the relationship between EMT and how cancer cells respond to IFF has not been investigated.

Thus, the overall goal of this study was to elucidate the molecular pathways activated by IFF in invasive and non-invasive breast cancer cells and determine whether EMT alters IFF signaling and response. Here we show for the first time that IFF causes an increase in tumor cell invasion via activation of phosphoinositide-3-kinase (PI3K) in ERBB2-positive breast cancer cells. Furthermore, we demonstrate that pre- and post-EMT cancer cells respond to IFF via different PI3K-dependent pathways.

4.2 Methods

Cell Culture

MCF10A and MCF10A-ERBB2 [135] (NeuN/NeuT) were maintained in DMEM/F12 supplemented with 5% donor horse serum (Atlanta Biologicals, Atlanta, GA), 20 ng/ml epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ), 10 µg/ml insulin (Sigma-Aldrich, Saint Louis, MO), 100 ng/ml cholera toxin (Enzo Life Sciences, Farmingdale, NY), 500 ng/ml hydrocortisone (Sigma Aldrich), and 1% penicillin/streptomycin (Mediatech, Herndon, VA). SKBR3, a breast cancer cell line that naturally expresses *ERBB2*, were grown in McCoy's 5A (Mediatech) supplemented with 10% FBS and 1% penicillin/streptomycin. BT474 and MDA-MB-453, other breast cancer cell lines that naturally express *ERBB2*, were grown in

DMEM (Mediatech) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained in a humidified environment at 37°C and 5% CO_2.

3D Invasion Assay

Interstitial fluid flow was applied to cells as previously described [75, 86, 98]. Briefly (Fig. 15), cells were embedded in a matrix composed of 1.3 mg/ml rat tail tendon collagen type I (BD Biosciences, San Jose, CA) and 1 mg/ml Matrigel™ basement membrane matrix (BD Biosciences) at a final concentration of 5 x 10^5 cells/ml inside culture inserts with 8 μm diameter pores (Millipore, Billerica, MA). For static (control) conditions, serum-free media levels inside and outside the insert were kept approximately equal, resulting in a minimal hydrostatic pressure difference across the gel and no measurable interstitial flow. For IFF conditions, serum-free media was added under the insert (100 μl) and above the gel (650 μl).

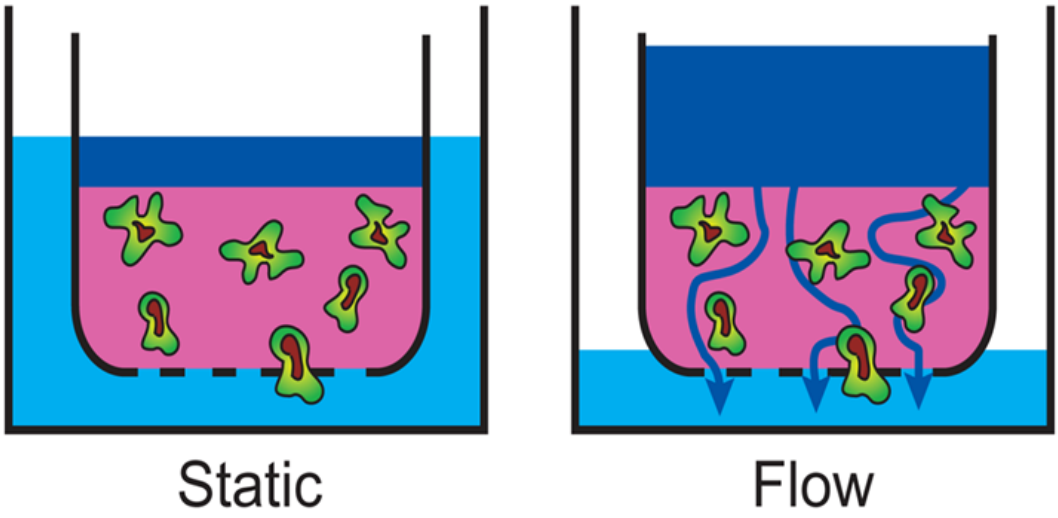

Figure 15: Schematic of the 3-D interstitial fluid flow invasion assay. Cells (green) are embedded in a collagen/Matrigel matrix (pink) media is added to simulate both control (static) and flow conditions.

The hydrostatic pressure difference generated was approximately 1 mm Hg. After 24 hours of either physiological interstitial flow (approximate flow velocity ~0.1 μm/s) or static conditions, invasion was measured by counting the number of cells that invaded through the matrix and migrated across the porous membrane. Migrated cells on the underside of the culture insert membranes were fixed in 4% paraformaldehyde in PBS for 30 minutes and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. Cells were then stained with Alexa Fluor 488-conjugated phalloidin (6 U/ml) (Life Technologies) and 4′,6-diamidino-2-phenylindole (DAPI) (2 μg/ml) (MP Biomedicals, Santa Ana, CA). Labeled cells were visualized on an epifluorescence microscope (Leica Microsystems). DAPI-stained nuclei at five randomly selected locations of each membrane were counted. F-actin staining was used to confirm that positive DAPI stain corresponded to cells. Percent invasion was calculated with the following equation:

$$\%Invasion = \frac{100 * \left(AverageCellCount * MembraneSurfaceArea\right)}{\left(imageArea * NumberofCellsSeeded\right)}$$

In some instances, percent invasion was normalized to the static control condition to allow for comparison between independent experiments. To determine the functional role of specific proteins, pharmacological inhibitors were used to block specific signaling proteins (see Table 1 for a list of inhibitors and corresponding concentrations presented in this study). When used, inhibitors were added to the collagen matrix and experimental medium at the appropriate concentrations. Experiments were repeated at least twice with a minimum sample size of $n = 3$ for each experiment (total minimum sample size $n = 6$).

Table 1: List of inhibitors, their targets, and concentrations used

Name	Protein target	Concentration (μM)
LY294002	PI3K	10, 50
PIK75	p110α	0.01
TGX221	p110β	0.1
AMD3100	CXCR4	12.6
WZ811	CXCR4	0.1
Pertussis toxin	$G\alpha_i$	0.1

TGF-β1 treatment

EMT was induced in NeuN cells via treatment with recombinant human TGF-β1 (Peprotech). An optimal concentration of TGF-β1 was first determined by treating NeuN cells at varying concentrations of TGF-β1 (3, 5, 10, 20 and 50 ng/ml) for 6 days. A concentration of 20 ng/ml was found to most effectively induce EMT (low E-cadherin expression, high vimentin expression, elongated morphology). NeuN cells were treated with this concentration in full media for 6 days, with a media change after 3 days.

Western Blot

Following the 3D invasion assay, total and phospho-protein levels were determined by western blot. Cells were isolated from the matrix using 2.5 mg/ml collagenase D (Roche, Indianapolis, IN) for 30 minutes. The resulting solution was centrifuged, and the pellet was washed with PBS and resuspended in RIPA lysis buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM Tris-HCl at pH 8, 0.1% SDS,

10% glycerol, 5 mM EDTA, 20 mM NaF and 1 mM Na_3VO_4). Lysates were cleared via centrifugation at 16,000 x g for 20 minutes at 4°C. Antibodies used were: rabbit polyclonal anti-phospho PI3K and total PI3K (1:500, Cell Signaling, Danvers, MA), rabbit polyclonal anti-β-actin (1:3,000, Cell Signaling), rabbit monoclonal anti-vimentin (1:1,000, Abcam), rabbit monoclonal anti-E-cadherin (1:1,000, Abcam), rabbit polyclonal anti-phospho and total CXCR4 (1:500, Abcam), rabbit polyclonal anti-p110α and β (1:1,000, Cell Signaling), and goat anti-rabbit HRP (1:10,000, Abcam).

Immunofluorescence

For visualization of EMT markers, cells were plated onto 8 well chamber slides at 10,000 cells per well. After 16 hours, cells were fixed in 4% paraformaldehyde in PBS for 30 minutes and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. This was followed by 1 hour in blocking solution (1% BSA, 0.1% goat serum). Cells were stained with rabbit monoclonal anti-vimentin (1:100) (Abcam) for 16 hours, then washed in PBS 3 times. Alexa Fluor 488-conjugated phalloidin (6 U/ml), DAPI (2 ug/ml), and Alexa Fluor 555 conjugated anti-rabbit IgG was incubated on the cells for 16 hours then washed in PBS 3 times. Fluorescence was visualized on an epifluorescence microscope (Leica Microsystems).

Microarray and Quantitative RT-PCR

Changes in gene expression in NeuN and NeuT cells were identified by microarray analysis. Total RNA was extracted from cells embedded in 3-D gels (6

biological replicates per sample) using RNeasy mini Kit (Qiagen, Germantown, MD). RNA was submitted to the Wistar Institute genomics core facility for amplification. Epicentre TargetAmp™ Nano Labeling Kit for Illumina® Expression BeadChip was used at 100 ng RNA and hybridized on an Illumina HT12v4 chip. Data were analyzed with PARTEK Genomics Suite and Database for Annotation, Visualization and Integrated Discovery (DAVID) online tools.

Quantitative RT-PCR (qRT-PCR) was performed on cDNA reversed transcribed using QuantiTech Reverse Transcription kit and QuantiTech SYBR Green PCR kit (Qiagen). Primers for human interleukin 6, desmoplakin, and integrin subunit α6 (Quantitech, Qiagen) were used to validate the microarray analysis and quantify mRNA expression differences.

Data and Statistical Analysis

Data are expressed as mean ± standard error of the mean. Differences among conditions were tested using Student's t-tests (for two groups) or two-way analysis of variance (ANOVA) (for three or more groups) using GraphPad Prism. When ANOVA identified a significant difference, a Bonferroni post-test was employed for multiple comparisons. Differences were accepted as significant at $p < 0.05$.

4.3 Results

Interstitial fluid flow induces invasion of breast cancer cells through PI3K activation

To model non-invasive and invasive cells, we used a previously developed model of breast cancer based on MCF10A human mammary epithelial cells

engineered to overexpress *ERBB2* [135]: MCF10A cells retrovirally transduced with either wild-type *ERBB2* (NeuN) or a constitutively active mutant of *ERBB2* (NeuT). When cultured in 3D conditions, NeuN cells exhibit behavior of pre-invasive *ERBB2* positive ductal carcinoma *in situ* (DCIS) [135], while NeuT cells behave like *ERBB2* positive invasive ductal carcinoma (IDC) [136]. Using a 3D invasion assay where single cells are embedded in stroma-like matrix, we measured invasion by counting the number of transmigrated cells (Fig. 15). We observed a 2-fold increase in invasion in response to 0.1 μm s^{-1} IFF in both NeuN and NeuT cells (Fig. 16A).

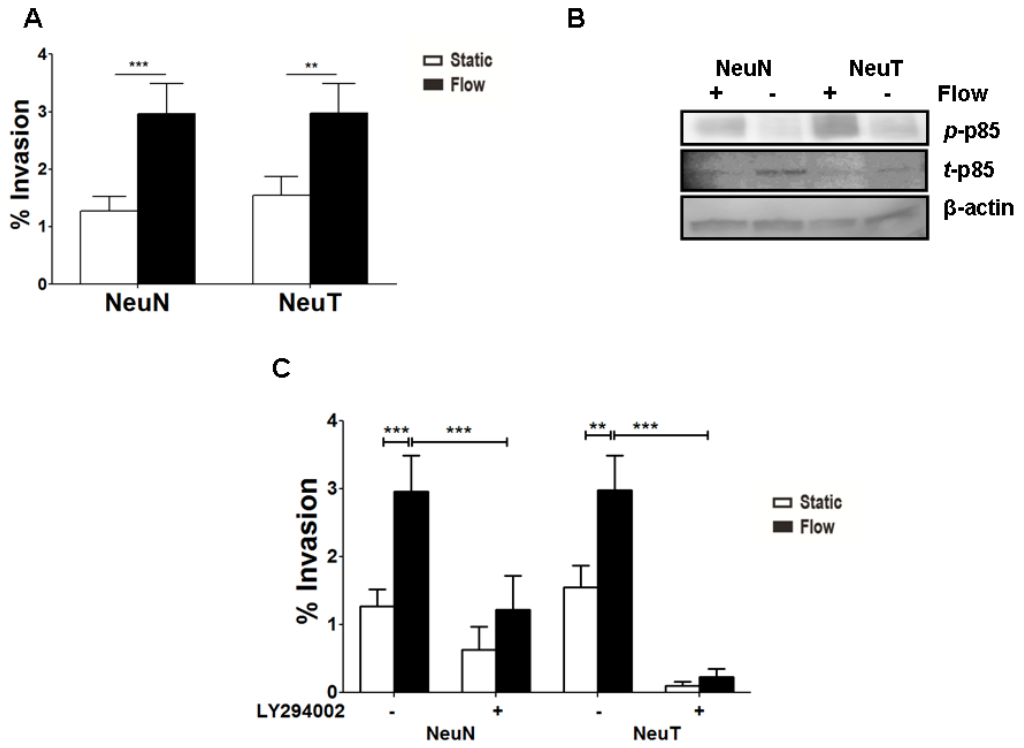

Figure 16: IFF-induced invasion occurs through p85 activation. A) IFF increases invasion of NeuN and NeuT. Percent invaded cells after 24 hours in 3-D IFF assay. All values are mean ± SEM. Student t test (*: p < 0.05; **: p < 0.01, ***: p < 0.001), n>12. B) IFF induces activation of PI3K in NeuN and NeuT. Representative western blot of phosphorylation of PI3K regulatory subunit (p85) after 24 hours of static or flow conditions in 3D flow invasion assay; β actin was used as loading control. C) PI3K activity is necessary for IFF-induced invasion in both cell lines. IFF-induced invasion is decreased in the presence of 10 μM LY294002, a pan PI3K inhibitor. All

values are mean ± SEM. Student t test and 2-way ANOVA (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$), n>6.

To determine the signaling pathways involved in the observed IFF-induced invasion, we examined pathways known to be important in invasion of HER2 positive breast cancer cells (HER2, MAPK, PI3K). We isolated cells from gels after the 3-D invasion assay and harvested protein from the resulting cells. Western blot revealed an increase in phosphorylated p85 (the regulatory subunit of class I PI3Ks) in both NeuN and NeuT cells treated with IFF (Fig. 16B). To confirm that our results were not specific to the engineered MCF10A cell lines, treatment with IFF of other breast cancer cells overexpressing ERBB2 including SKBR3, BT474, and MDA-MB-453 also induced increased invasion coupled with increased PI3K phosphorylation compared to static conditions (Fig. 17 A-C).

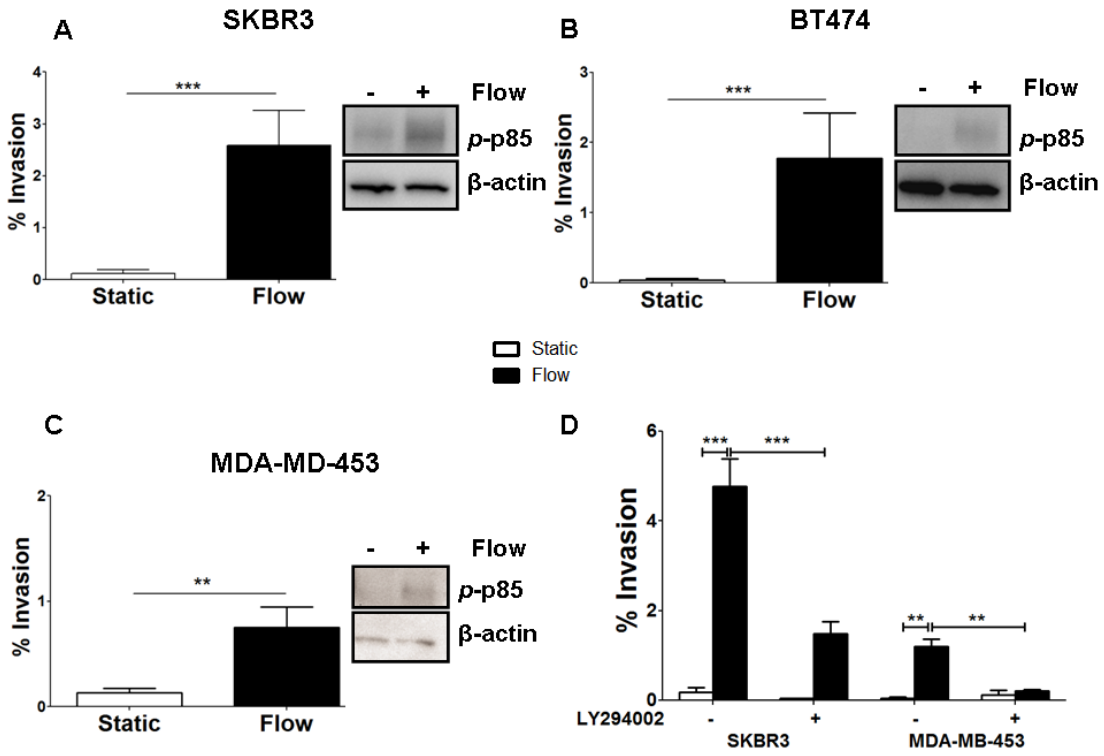

Figure 17: IFF induced invasion through PI3K is also observed in other HER2 positive cell lines. IFF induces invasion of SKBR3 (A), BT474 (B), MDA-MB-453 (C) coupled with PI3K activation. Percent invaded cells after 24 hours in 3-D IFF assay and representative western blot of phosphorylation of PI3K. D) SKBR3 and MDA-MB-453 show decreased IFF-induced invasion in the presence of 50 uM LY294002, a pan PI3K inhibitor. All values are mean ± SEM. Student t test and 2-way ANOVA (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$), 15>n>6.

To test whether PI3K activity was required for IFF-induced invasion we treated cells with the PI3K inhibitor LY294002 (a pan-PI3K inhibitor). Treatment with LY294002 decreased IFF-induced invasion in NeuN, NeuT (Fig. 16D) and breast cancer cells SKBR3 and MDA-MB-453 (Fig. 17D). These data suggest that IFF increases invasion in ERBB2 overexpressing breast cancer cells via activation and subsequent downstream signaling through PI3K.

p110β modulates response to interstitial fluid flow in NeuT but not NeuN cells

Class I PI3Ks are present in cells as heterodimers composed of a regulatory (typically p85 or p55) and catalytic subunit (p110) [137]. The p110 catalytic subunit is responsible for propagating downstream signaling through its kinase activity [138]. Using inhibitors that specifically target the β (TGX-221) or α (PIK-75) p110 catalytic subunit of PI3K, we observed that in NeuN cells, only the p110α inhibitor blocked IFF-mediated invasion (Fig. 18A). However in NeuT cells, inhibiting both p110α and p110β decreased IFF-induced invasion (Fig. 18B). NeuN and NeuT cells expressed similar protein levels of p110α and p110β (Fig. 18C), thus ruling out altered levels of these isoforms as the reason for this difference.

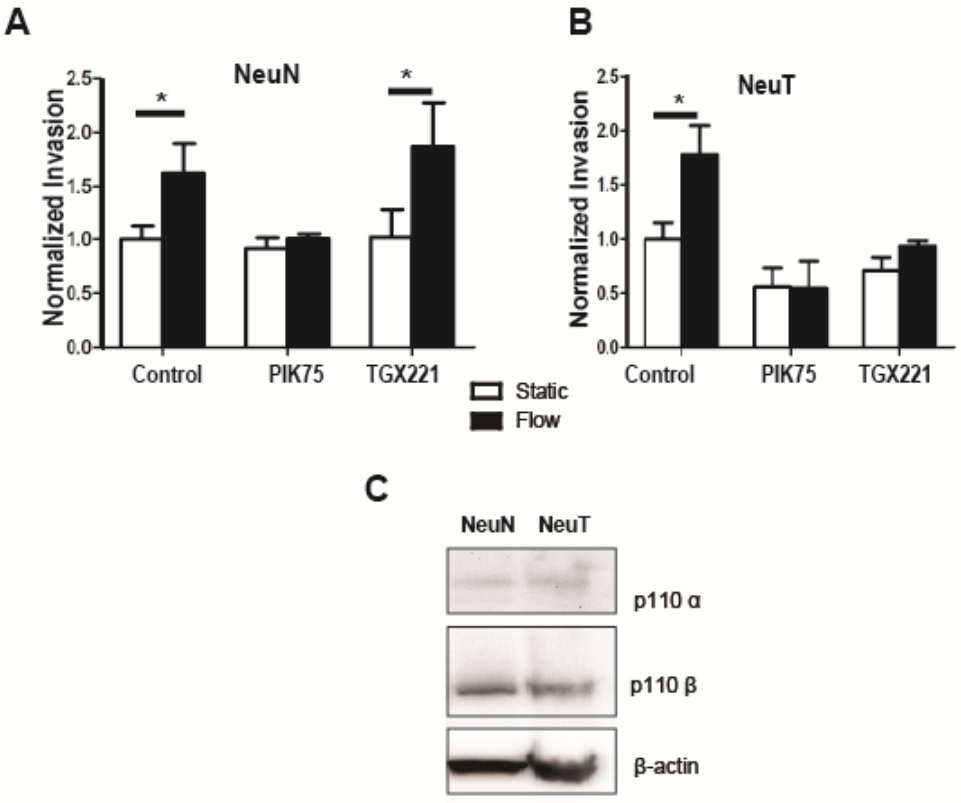

Figure 18: Different p110 isoforms are necessary for IFF induced invasion in NeuN and NeuT. A-B) Both p110α and p110β are necessary for flow response in NeuT only. Cellular response to P110 isoforms specific inhibitors PIK75 and TGX221 (p110α and p110β inhibitors respectively)

in NeuN (A) and NeuT (B). All values are mean ± SEM. Student t test (*: $p < 0.05$. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$), n=6. C) Both cells express similar levels of p110 isoforms. Representative western blot of p110α and β in NeuN and NeuT; β actin was used as loading control.

CXCR4 regulates PI3K activity in response to interstitial fluid flow in NeuT cells

Since previous studies have linked p110α to receptor tyrosine kinase signaling and p110β to G protein coupled receptors (GPCRs) [139-141], we investigated the role of chemokine receptors (a family of GPCRs) in the IFF-induced invasion of NeuT cells. CXCR4 is a known modulator of breast cancer invasion [142, 143] and IFF-induced glioma cell invasion was previously shown to be dependent on CXCR4 [76]. Treating NeuT cells with antagonists of the chemokine receptor CXCR4 (AMD3100 or WZ811) significantly reduced IFF-induced invasion (Fig. 19A). In contrast, AMD3100 and pertussis toxin ($G\alpha_i$ subunit inhibitor of all GPCRs [144, 145]) had no effect on IFF-mediated invasion of NeuN cells (Fig. 19B), suggesting that chemokine receptors, including CXCR4, are not involved in the molecular pathway activated by IFF in NeuN cells. CXCR4 inhibitors also had no effect on IFF-mediated invasion in the non-invasive HER2 positive cell line SKBR3 (data not shown).

To test whether CXCR4 signaling was associated with PI3K activation, we examined the effects of treating NeuT or NeuN cells with AMD3100 on p85 phosphorylation during IFF-mediated invasion. IFF-induced PI3K phosphorylation was decreased in NeuTs when the cells were treated with AMD3100 but phosphorylation was only weakly inhibited in NeuN (Fig. 19C). Interestingly, CXCR4 protein levels were similar in NeuN and NeuT cells (Fig. 20).

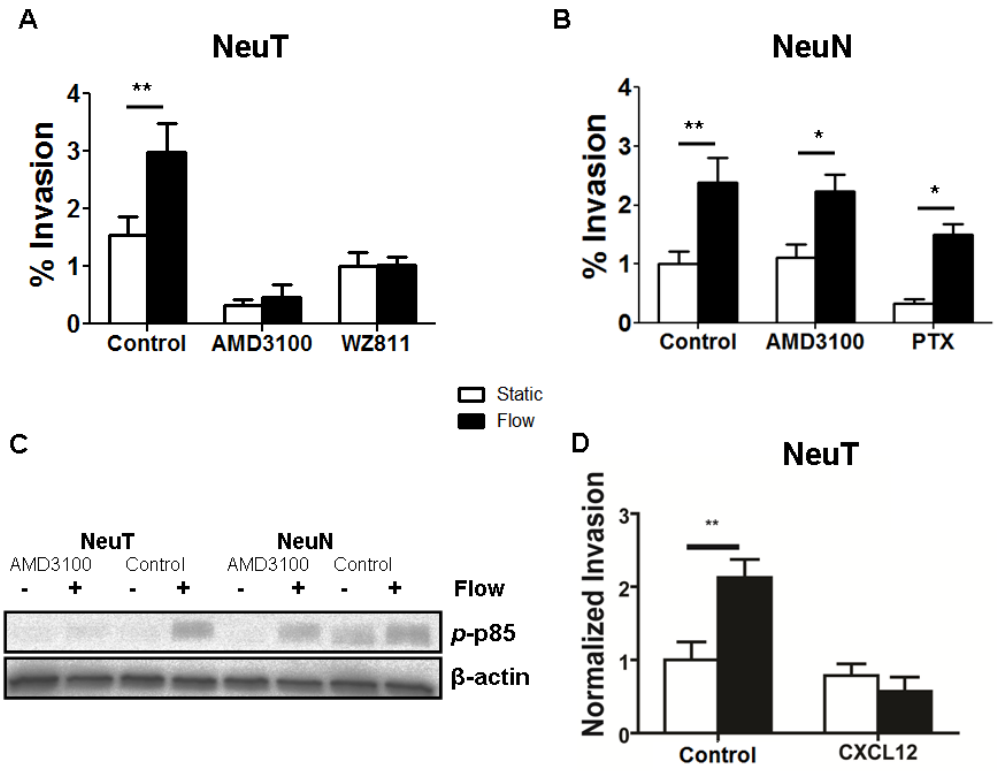

Figure 19: CXCR4 activity is required for IFF-induced invasion in NeuT but not NeuN. A) Changes in invasion in the presence or absence of CXCR4 inhibitors (AMD3100 and WZ811) in NeuT. B) AMD3100 and pertussis toxin are not necessary for IFF-induced invasion in NeuN. C) AMD3100 inhibits IFF-induced PI3K activation in NeuT but not in NeuN. Representative western blot of phospho-p85 after 24 hours in 3D invasion assay with and without the inhibitor in NeuN and NeuT; β actin was used as loading control. D) Changes to flow induced invasion when exogenous CXCL12 is added to surrounding matrix and media. NeuT respond to fluid flow only in the presence of a CXCL12 gradient. All values are mean ± SEM. Student t test (*: $p < 0.05$. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$); $n \geq 6$.

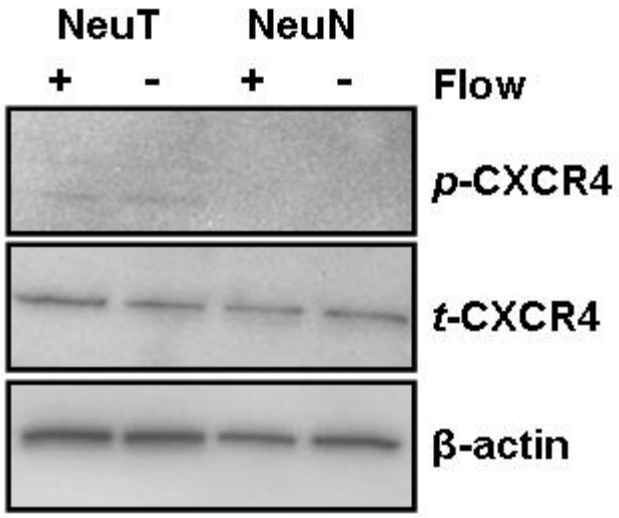

Figure 20: NeuN and NeuT express similar levels of CXCR4 and IFF does not alter CXCR4 total and phosphorylated protein levels. Representative western blot of total CXCR4, phosphorylated CXCR4 with loading control actin.

CXCR4 and its ligand CXCL12 have been previously implicated in IFF-mediated glioma invasion by autologous chemotaxis [76]. In autologous chemotaxis, a transcellular gradient is generated by the combination of autocrine chemokine secretion and interstitial flow [87]. To determine if a CXCL12 gradient was necessary for IFF-induced invasion of NeuT cells, exogenous CXCL12 was added to the surrounding media and matrix at a uniform concentration of 80 ng/ml (10nM). At this concentration, the cells' CXCR4 receptors should be approaching saturation (K_D ~14 nM) [146], effectively blocking gradient sensing. Under these conditions NeuT cells no longer responded to IFF (CXCL12 condition) compared to a 2-fold increase in invasion in the control flow condition (Fig. 19D). These data suggest that NeuT cells, unlike NeuN cells, require a gradient of CXCL12 for IFF-induced activation of PI3K and invasion, consistent with chemokine-dependent autologous chemotaxis.

TGF-β1-induced EMT alters the mechanism of interstitial flow-induced invasion in NeuN cells

Having identified separate IFF-induced pathways in NeuN and NeuT cells, our next goal was to determine some of the fundamental differences between these cells responsible for the observed differences in IFF-induced invasion. NeuN cells adopted more epithelial-like shapes on 2D cell culture plates and invaded in clumps of cells similar to their parental MCF10A counterparts (Fig. 21). However NeuT invaded as single cells and were characterized by a spindle-like morphology.

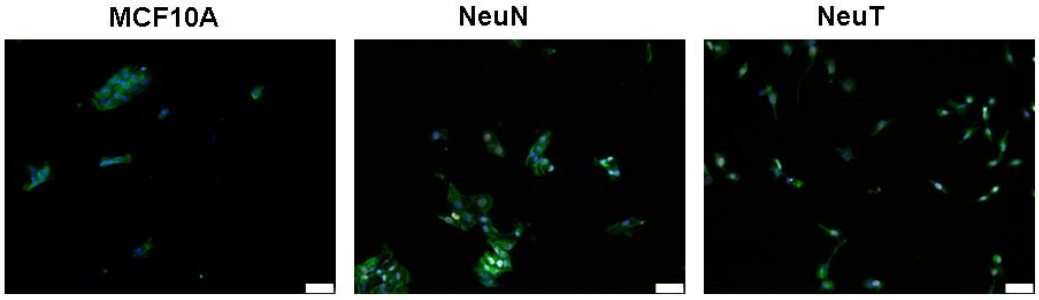

Figure 21: NeuN invade in clumps similar to the non-tumorigenic epithelial cell line MCF10A. NeuT however invade as single cells. Invaded cells were stained directly on membranes with DAPI (blue) and Phalloidin (green). White bars represent 50 µm.

Thus, we hypothesized that NeuT cells may have undergone EMT and this may explain the difference in IFF response. We probed for protein expression of epithelial and mesenchymal markers, and observed loss of E-cadherin and increased levels of vimentin in NeuT cells when compared to NeuN cells (Fig. 22 A, B). Both of these proteins are known markers of EMT [49].

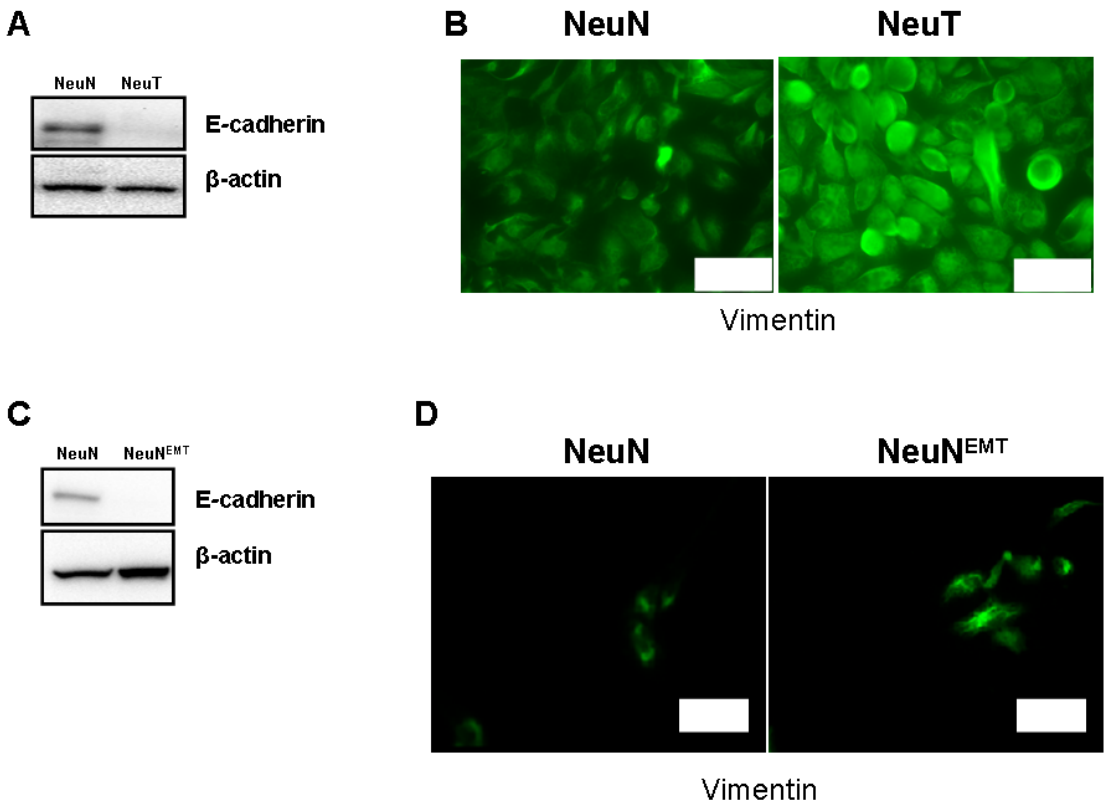

Figure 22: NeuT cells have undergone EMT similar to TGF-β1 dependent EMT induction in NeuN. A) Compared to NeuN, NeuT cells express lower levels of epithelial markers. Representative western blot of E-cadherin; β actin was used as loading control. B) Compared to NeuN, NeuT cells express higher levels of mesenchymal markers. Representative immuno-fluorescence of vimentin from cells plated on collagen I coated cover-slip. C) Compared to control, NeuNEMT cells express lower levels of epithelial markers. Representative western blot of E-cadherin; β actin was used as loading control. D) Compared to control, NeuNEMT cells express higher levels of vimentin. Representative immuno-fluorescence of vimentin from cells attached to the underside of membrane after IFF invasion assay. White bars represent 50 µm.

To examine gene differences between NeuN and NeuT cells further, we profiled gene expression using microarray analysis. Table 2 lists a number of known EMT-associated genes that were significantly different when comparing NeuT and NeuN mRNA expression ($\log_2$ transformed fold change is represented). The expressions of all these genes agree with published EMT-associated data [147-149].

Table 2: List of EMT genes differentially expressed between NeuT and NeuN

The genes below are published EMT genes whose expression profile agrees with our dataset. They were identified in our microarray data set with a significant log_2 transformed expression ratio (NeuT/NeuN) of at least ± 1.4

EMT UP-REGULATED GENES		
Gene Symbol (name)	**NeuT/NeuN**	**P value**
CDH2 (N-cadherin)	**+2.7**	**$6.8x10^{-8}$**
GNG11 (guanine nucleotide-binding protein)	**+2.9**	**$7.2x10^{-11}$**
IGFBP4 (insulin-like growth factor-binding protein 4)	**+1.9**	**$1.2x10^{-7}$**
STEAP1 (metalloreductase STEAP1)	**+3.4**	**$1.1x10^{-9}$**
VCAN (versican)	**+1.5**	**$1.6x10^{-8}$**
WNT5A (Protein Wnt-5a)	**+1.6**	**$2.0x10^{-8}$**
EMT DOWN-REGULATED GENES		
Gene Symbol (name)	**NeuT/NeuN**	**P value**
PPPDE2 (Desumoylating isopeptidase 1)	**-1.4**	**$4.0x10^{-7}$**
CDH1 (E-cadherin)	**-5.4**	**$8.4x10^{-9}$**

We validated the microarray result with quantitative RT-PCR of three of the identified genes (Fig. 23). These results supported the hypothesis that NeuT cells display a more mesenchymal-like phenotype when compared to NeuN cells.

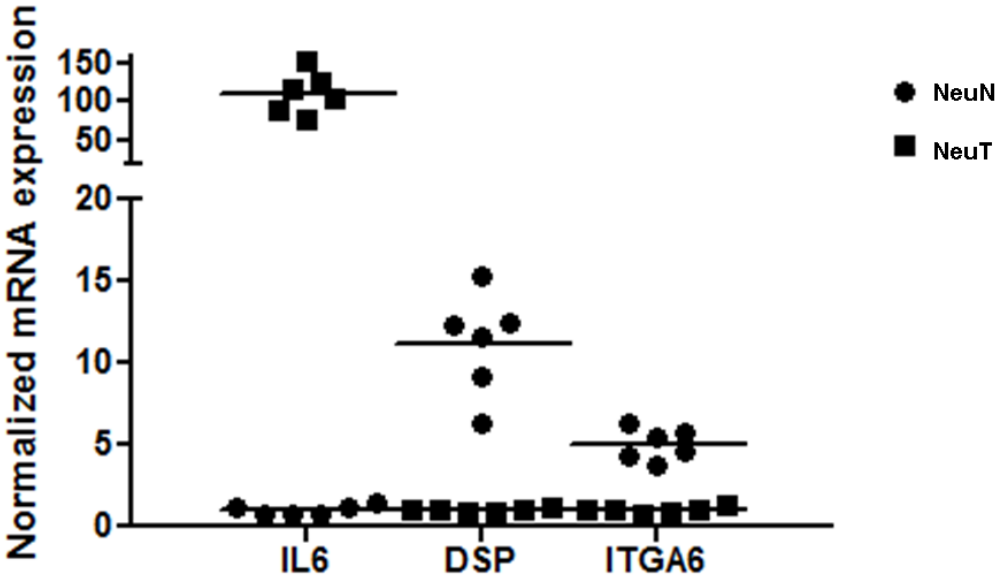

Figure 23: Validation of microarray data using quantitative RT-PCR. Interleukin 6 (IL6) expression is higher in NeuT. Desmoplakin (DSP) and integrin alpha 6 (ITGA6) expression is lower in NeuT.

To determine whether EMT contributes to the altered IFF response, EMT was induced in NeuN cells using the known EMT inducing growth factor TGF-β1 [48, 150]. The cells were treated for 6 days with 20 ng/ml TGF-β1 and EMT induction was confirmed using western blot and immunofluorescence. $NeuN^{EMT}$ cells show lower levels of E-cadherin and higher levels of vimentin when compared to their control NeuN counterparts (Fig. 22C, D). As expected, the NeuN cells induced to undergo EMT had higher levels of basal invasion (Fig. 24B). However, in line with our previous findings in NeuT cells, we observe that IFF-induced invasion in $NeuN^{EMT}$ cells was inhibited by both pertussis toxin and CXCR4 (Fig. 24A-B). In

addition, IFF-induced PI3K phosphorylation in the presence of AMD3100 was significantly reduced (Fig. 24C) in NeuN^{EMT} cells, similar to NeuT cells (Fig. 19C). Consistent with this result, treatment of NeuN^{EMT} cells with p110β inhibitor (TGX221) reduced IFF-mediated invasion, in contrast to their untreated NeuN counterparts (Fig. 24D), but similar to NeuT cells (Fig. 18B). Thus, these data support the hypothesis that ERBB2 expressing breast cancer cells that have undergone EMT invade in response to IFF through a CXCR4- and p110β-dependent mechanism.

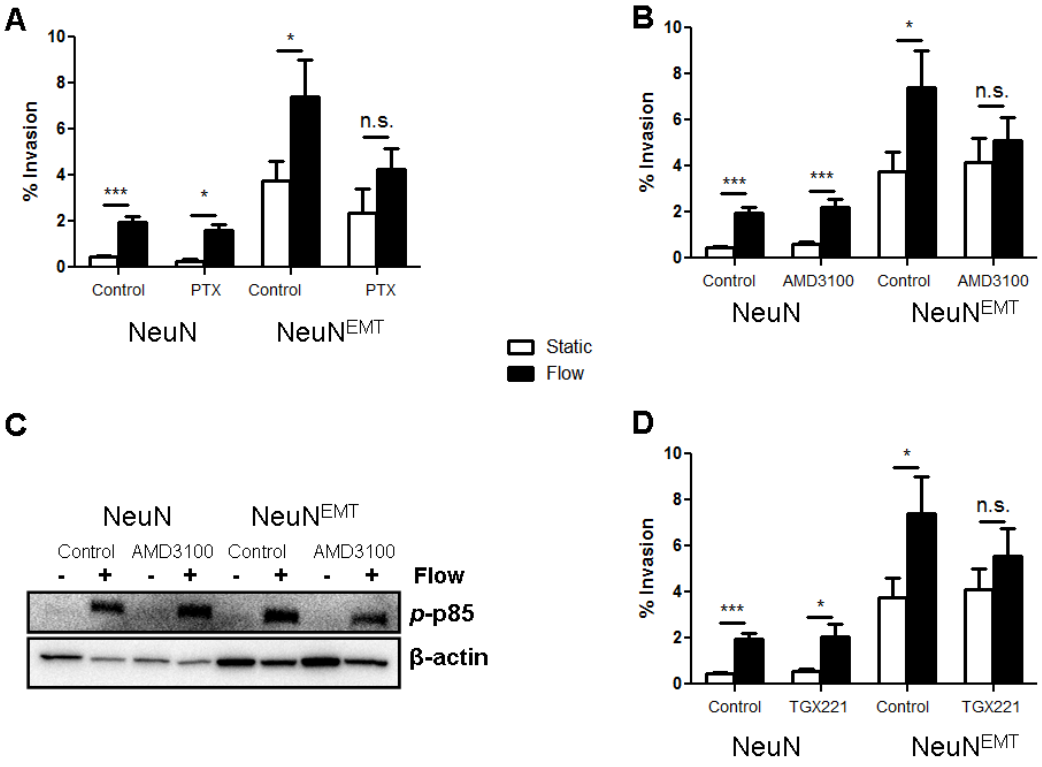

Figure 24: TGFβ1-induced EMT in NeuN leads to CXCR4- and p110β-dependent IFF-induced invasion. A) Changes in invasion in the presence or absence of pertussis toxin. Inhibiting GPCR activity in NeuN^{EMT} blocks IFF-induced invasion but not in control NeuN. B) Changes in invasion in the presence or absence of CXCR4 inhibitors, AMD3100. Inhibiting CXCR4 activity in NeuN^{EMT} blocks IFF-induced invasion but not in control NeuN. C) AMD3100 decreases IFF-induced PI3K activation in NeuN^{EMT} but not in control. Representative western blot of phospho-p85 after 24 hours in 3D invasion assay with and without the inhibitor; β actin was used as loading control. D) Changes in invasion in the presence or absence of p110β inhibitors, TGX221.

Inhibiting CXCR4 activity in NeuN^{EMT} blocks IFF-induced invasion but not in control NeuN. All values are mean ± SEM. Student t test (*: p < 0.05. *: p < 0.05; **: p < 0.01, ***: p < 0.001); n≥6.

4.4 Discussion

In the present study, we examined the role of key intracellular signaling pathways that mediate interstitial fluid flow-induced invasion in ERBB2-expressing breast cancer cells. In all breast cancer cells tested, we found that IFF induced activation of PI3K as measured by p85 phosphorylation. In addition, these breast cancer cells require PI3K activity for IFF-mediated invasion as inhibition of PI3K and the p110α catalytic subunit blocked IFF-mediated invasion. However, cells that have undergone EMT (NeuT and NeuN^{EMT} cells) require additional signals, including activation of chemokine receptors. Indeed, we showed that inhibitors of CXCR4 and p110β, a p110 isoform that is GPCR-regulated [139-141], specifically blocked IFF-mediated invasion in breast cancer cells that have undergone EMT. Our data suggest a model where, as cells undergo EMT, the signaling pathways activated by interstitial fluid flow to induce invasion change significantly (Fig. 25).

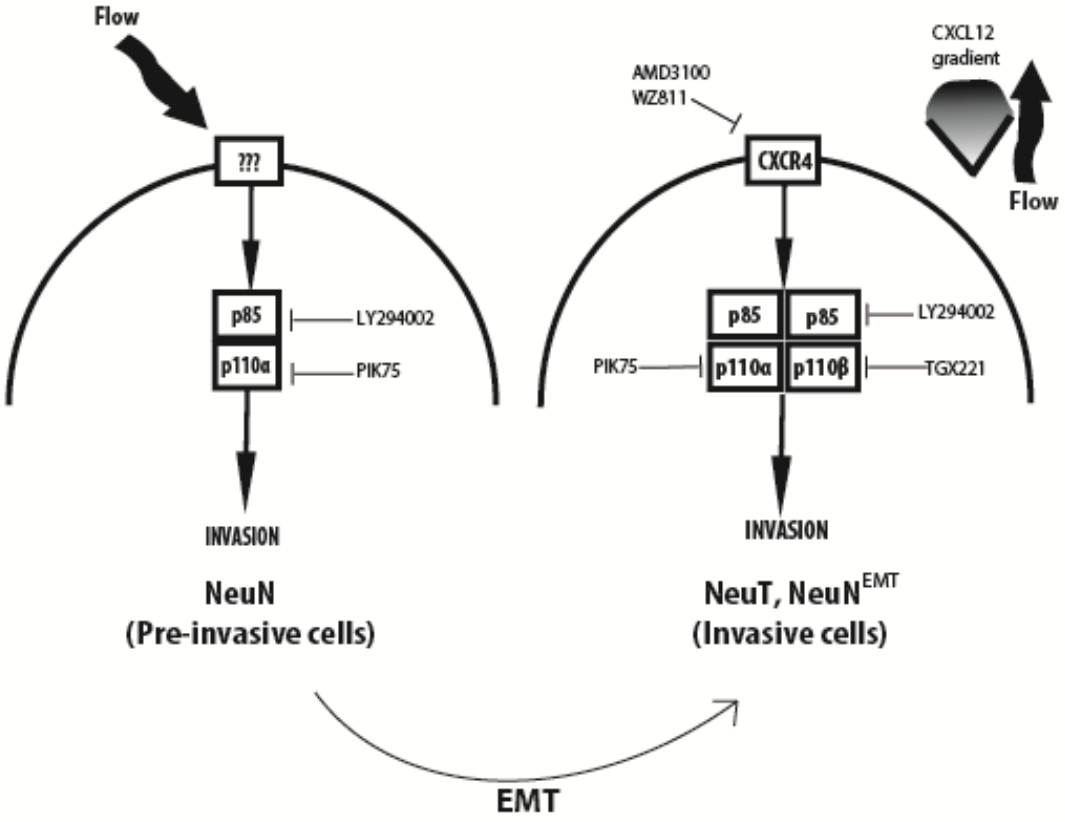

Figure 25: Proposed mechanism of interstitial fluid flow in ERBB2 positive breast cancer. Separate signaling pathways are activated in response to IFF in our cell model but they all converge at class I PI3K. In pre-invasive cells (NeuN) IFF activates PI3K through an unknown receptor leading to increased invasion via the p110α catalytic subunit. In invasive cells similar to those that have undergone EMT (NeuT, NeuNEMT), IFF activates PI3K through CXCR4 which leads to increased invasion via both p110 catalytic subunits. This occurs because the combination of autocrine CXCL12 secretion and IFF creates a chemokine gradient around the cells, driving chemoinvasion in the direction of IFF via autologous chemotaxis [75].

The PI3K pathway plays a central role in numerous cellular processes crucial for cancer progression. This pathway has been implicated in invasion, proliferation, transformation and cell survival [151, 152]. Mutations or alterations of this pathway are the most frequent in all human cancers, making it an important target of cancer therapeutics [151]. This work supports the importance of targeting the PI3K pathway in order to curtail breast cancer invasion, especially in those with prominent IFF.

PI3K is present in cells as a heterodimer complex made up of a regulatory and catalytic subunit. Cancer specific mutations have been observed in the four catalytic p110 isoforms (α, β, δ, γ) of the class I kinase [152]. IFF led to phosphorylation of p85 in all cells tested, suggesting that class I PI3K is central to signals activated by IFF. However, as cancer cells underwent EMT, we found changes in p110 subunit dependency. Only breast cancer cells expressing a mesenchymal phenotype (determined by E-cadherin and vimentin expression) required p110β activity. As mentioned above, p110α has been linked to downstream activity of receptor tyrosine kinases, while p110β has been linked to G protein coupled receptors [139-141]. This is consistent with our data that EMT cells required the GPCR and chemokine receptor CXCR4 for IFF-induced invasion. Our findings suggest that breast cancer cells undergoing EMT require both PI3K catalytic subunits p110α and p110β for IFF-mediated invasion and that this pathway may be targeted for blocking invasion of aggressive breast cancers. We show that by specifically blocking either p110α or p110β, we can decrease IFF-induced invasion in EMT-like cells. Our findings suggest that drugs targeting p110 isoforms may be used to block invasion of aggressive breast cancers and that may have less toxic compared to using pan-PI3K inhibitors.

We demonstrated that IFF-induced PI3K activation occurs through separate upstream receptors, leading to downstream signaling in a p110 isoform-specific fashion (Fig. 25). IFF-specific activation of PI3K in cells that have undergone EMT (NeuT and $NeuN^{EMT}$) occurs via CXCR4. CXCR4 is a known prognosis marker for breast cancer metastasis [142] and had been implicated in breast cancer invasion

[153]. The role CXCR4 plays in IFF-induced glioma invasion was previously described as a result of autologous chemotaxis through its ligand CXCL12 [76]. Here, as the cells secrete CXCL12, IFF creates a CXCL12 gradient around the cells in the direction of flow, leading to a higher chemokine concentration on the downstream side of the cells. We did not observe an increase in total or phosphorylated CXCR4 due to IFF. This suggests that IFF does not alter the levels of these proteins but may alter how the cells migrate by creating a small but biologically relevant transcellular gradient [87]. The fact that this phenomenon is only observed in cells that have undergone EMT suggests that in addition to inducing invasion in pre-invasive cells, IFF may play a role in sustaining invasion as cells move through the stroma individually. Invasive cells, like those which have undergone EMT, have been shown to be more sensitive to chemokine gradients [154, 155]. This may allow them to home to specific organs producing a chemoattractant [156, 157], but it may also facilitate IFF-induced invasion, as we have outlined here. Although basal invasion levels between NeuN and NeuT are similar, the mechanism by which these cells invade appears to be different. NeuN cells have a tendency to invade collectively while NeuT invade as single cells (Fig. 21). We further profiled mRNA and protein of these cells and observed that NeuT lacked key epithelial cell-associated proteins/genes (E-cadherin, integrin α6) and expressed higher levels of mesenchymal markers (N-cadherin, vimentin) suggesting that EMT alters the mode of invasion of cells, and this leads to changes in the response of cells to IFF. To test this idea specifically, we showed that induction of EMT in NeuN converted the IFF invasion response from chemokine-independent to chemokine receptor-dependent.

Although our data suggest potential relevance of our findings to treatment of HER2 positive DCIS, the study focuses on invasion of single cells embedded in a matrix. DCIS cells *in vivo* are organized in 3-dimensional complex structures that are highly dependent on cell-to-cell interactions and basement membrane restrictions [18]. Their invasion outside the stroma is also dependent on stroma-associated cells, which are well known to have both positive and negative influences on cancer progression [10]. In the future, investigating the role of IFF on 3-dimensional DCIS structures in a more clinically relevant matrix will lead to better understanding of the role of IFF on breast cancer invasion. In addition, questioning the role of known mechanotransducers by targeting proteins such as integrins and glycocalyx, which have both been implicated in IFF-induced response [77, 79], may help identify the upstream mediator of PI3K phosphorylation in NeuN cells. Further studies should also focus on identifying the implications of EMT in altering IFF response in other cancers (e.g. melanoma, glioma and renal cell carcinoma).

Taken together, our results demonstrate for the first time that interstitial fluid flow increases invasion of cells via separate mechanisms depending on the stage of the cancer cells. This is the first study to examine the effect of IFF on separate stages of breast cancer, especially in the context of how it may influence the early steps of tumor progression. We showed that IFF increases the invasion of different types of human mammary breast cancer cells through activation of PI3K. We further demonstrated that EMT alters how cells respond to IFF, engaging a CXCR4/CXCL12-dependent autologous chemotaxis mechanism that signals through p110β. Understanding these cellular responses to interstitial fluid flow will increase

our understanding of how biophysical forces interact with molecular factors to drive breast cancer progression and may help identify novel therapeutic regimens.

CHAPTER 5: Interstitial fluid flow induces invasion of non-invasive breast cancer spheroids

5.1 Introduction

Ductal carcinoma *in situ* (DCIS) is a pre-invasive stage of breast cancer where the cells, although abnormal in their proliferation and gene expression profile, are non-invasive. They remain within the confined structures of the mammary duct enclosed by the basement membrane. In response to still unknown triggers, these cells develop the ability to invade beyond the basement membrane and infiltrated the surrounding stroma. The resulting cancer stage is known as invasive ductal carcinoma (IDC). Unfortunately, factors regulating the invasive and metastatic behavior of cancer cells are complex and much remains unknown [8].

Breast cancer-associated mortality is primarily the result of IDC, where cells are able detach from the primary tumor and metastasize to distant tissues and organs such as the lymph nodes, bone marrow, liver, lungs, and brain [158, 159]. The 5-year survival rate of breast cancer falls from 98% for localized tumors to 23% for metastatic disease [4]. These collective findings underscore the importance of identifying both the molecular and biophysical factors that put patients at risk of invasion and metastasis, which will allow for the development of better diagnostics and treatments. Improving both disease survival and clinical management of patients will require understanding of the molecular mechanisms that regulate this transition and the identification of key factors at play.

In breast cancer, it is well established that genetic dysregulations, coupled with changes in the tumor microenvironment, play key roles in the transition from

ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC) [10, 32, 33, 60, 160]. *ERBB2* is an oncogene that encodes for the receptor tyrosine kinase HER2, a protein known to increase the aggressiveness of breast cancer [93]. Poor breast cancer prognosis including metastasis has been correlated with the overexpression of HER2 in 15-30% [94, 95] of all human breast cancers. Additionally, not only is HER2 overexpressed in more than 50% of DCIS cells, but the risk of developing IDC from DCIS is six times higher when cells overexpress HER2 [24]. Approximately 30% of patients who are diagnosed with DCIS will develop invasive breast cancer despite therapy [18, 93]. This increased invasion occurs via activation of members of the mitogen activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) pathways [25, 26]. For this reason, many patients diagnosed with localized tumors are over-treated with harsh therapies even though the majority of these tumors never become lethal. The most common treatment for DCIS is lumpectomy followed by radiation therapy. Additional treatments include mastectomy and hormonal therapy and these are usually followed by numerous physical and emotional side effects.

We previously showed in chapters 2, 3, and 4 that interstitial fluid flow (IFF), the movement of fluids within the interstitial space, induces the invasion of normal and pre-invasive cells in 3D through a PI3K-dependent pathway. In our previous experiments along with experiments performed in other groups [75, 76, 86], the invasion of single cells embedded in a stroma-like matrix was measured. These single cell experiments help elucidate the intricate signaling mechanism employed by IFF in clinical systems where the cells are invading as individual entities such as in metastasis. They however are not relevant in identifying the role of IFF in the

individual cellular detachment from the primary tumor that is required in the initial steps of invasion. The effect IFF has on the early steps of tumor invasion of DCIS-like acini or spheroids has never been studied. In the current study, we aimed to identify the role IFF plays in initiating invasion by otherwise non-invasive 3-D normal and DCIS-like spheroid structures.

5.2 Methods

Cell Culture

To model normal breast epithelium, DCIS and IDC, we used a previously developed model of breast cancer based on MCF-10A human mammary epithelial cells engineered to overexpress HER2 [96, 97]: MCF-10A human mammary epithelial cells, MCF-10As retrovirally transduced with wild-type *ERBB2* (NeuN) and MCF10A overexpressing a constitutively active mutant of *ERBB2* (NeuT). These cells were maintained in DMEM/F12 supplemented with 5% donor horse serum (Atlanta Biologicals, Atlanta, GA), 20 ng/ml epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ), 10 µg/ml insulin (Sigma-Aldrich, Saint Louis, MO), 100 ng/ml cholera toxin (Enzo Life Sciences, Farmingdale, NY), 500 ng/ml hydrocortisone (Sigma Aldrich), and 1% penicillin/streptomycin (Mediatech, Herndon, VA). Cell lines naturally expressing HER2 were also used: BT474, MDA-MB-453 and SKBR3. BT-474 and MDA-MD-453 cells (ATCC, Manassas, VA) were grown in DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1% penicillin/streptomycin. SKBR3 cells were grown in McCoy's 5A (Mediatech) supplemented with 10% FBS and 1%

penicillin/streptomycin. All cells were maintained in a humidified environment at 37^{o}C and 5% CO_2.

3D Acini Invasion Assay

MCF10A, NeuN and NeuT cells were grown in acini following a protocol adapted from [161] and then subjected to interstitial fluid flow. Briefly, 24-well cell culture plates were coated with 100 µl growth-factor-reduced Matrigel. Cells were resuspended in 3D medium (DMEM/F12, 2% donor horse serum, 10 ug/ml insulin, 100 ng/ml cholera toxin, 500 ng/ml hydrocortisone, 1% penicillin-streptomycin, 5 ng/ml EGF) containing 2% growth-factor-reduced Matrigel and plated at a concentration of 5,000 cells/ml. After 12 days, acini were dislodged by gentle pipetting and seeded in 1.3mg/ml collagen gels inside 8 µm diameter pore cell culture inserts. Spheroids were then exposed to flow and static conditions for 48 hours. For static (control) conditions, serum-free media levels inside and outside the insert were kept approximately equal, resulting in a minimal hydrostatic pressure difference across the gel and no measurable interstitial flow. For IFF conditions, serum-free media was added under the insert (100 µl) and above the gel (650 µl). Every 4-6 hours, media was replenished above the gels of the flow transwells using flow-through media.

Immunofluorescence Microscopy

Gels were fixed and stained with Alexa Fluor 488-conjugated phalloidin (1:50), DAPI (1:500), and either mouse monoclonal anti-laminin V (1:100) or rabbit

monoclonal anti-collagen IV antibody (1:100) (Abcam, Cambridge, MA) to visualize the basement membrane.

Fluorescent images were analyzed using MATLAB and its Image Processing Toolbox. Pictures were reduced into binary images using the Otsu algorithm and the background pixels were removed in order to de-noise the image. In order to separate individual spheroids and define their outline, the area of each staining was calculated using the *regionprops* MATLAB function. Collagen IV fluorescent intensity was normalized to total F-actin intensity to obtain loss of collagen IV levels. The total number of protrusions per spheroid was counted manually. Two additional counters (each blinded) were used to double check counter bias.

Western Blotting

Following the invasion assay, total and phospho-protein levels were determined by western blot. Spheroids were isolated using 2.5 mg/ml collagenase D (Roche, Indianapolis, IN) for 30 minutes. The resulting solution was centrifuged, and the pellet was washed with PBS and resuspended in RIPA lysis buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM Tris-HCl at pH 8, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF and 1 mM $Na_3VO_{4)}$. Lysates were then cleared via centrifugation at 16,000 x g for 20 minutes at 4°C. The antibodies used were: rabbit polyclonal anti-phospho PI3K and total PI3K (1:500, Cell Signaling, Danvers, MA), rabbit polyclonal anti-β-actin (1:3000, Cell Signaling), goat anti-rabbit HRP(1:10000, Abcam).

Data and Statistical Analysis

Data are expressed as mean ± standard error of the mean. Differences among treatment groups were tested using Student's t-test (for two groups) or two-way analysis of variance (ANOVA) (for three or more groups) as appropriate using GraphPad Prism. When ANOVA identified a significant difference, a Bonferroni post-test was employed for multiple comparisons. Differences were accepted as significant at $p < 0.05$.

5.3 Results

Interstitial fluid flow induces loss of basement membrane of DCIS-like acini

In this study, we used three types of cells that each represented a distinct stage of breast cancer progression. It was previously shown that when cultured in 3D condition, MC10A, NeuN and NeuT cells exhibited behavior expected of normal, pre-invasive-HER2 positive and invasive-HER2 positive cancer phenotypes [96, 97]. We cultured MCF10A, NeuN, and NeuT in a three-dimensional reconstituted basement membrane matrix. After 12 days, these cells formed separate types of acini structures (Fig. 26). MCF10A cells developed 3D spheroid structures that were surrounded by an intact basement membrane (positive collagen IV staining), no protrusions, and hollow centers (lumen). NeuN acini had lost the distinctive lumen but had intact basement membranes. NeuT 3D structures appeared more disorganized and less compact, and exhibited a discontinuous or non-existent basement membrane.

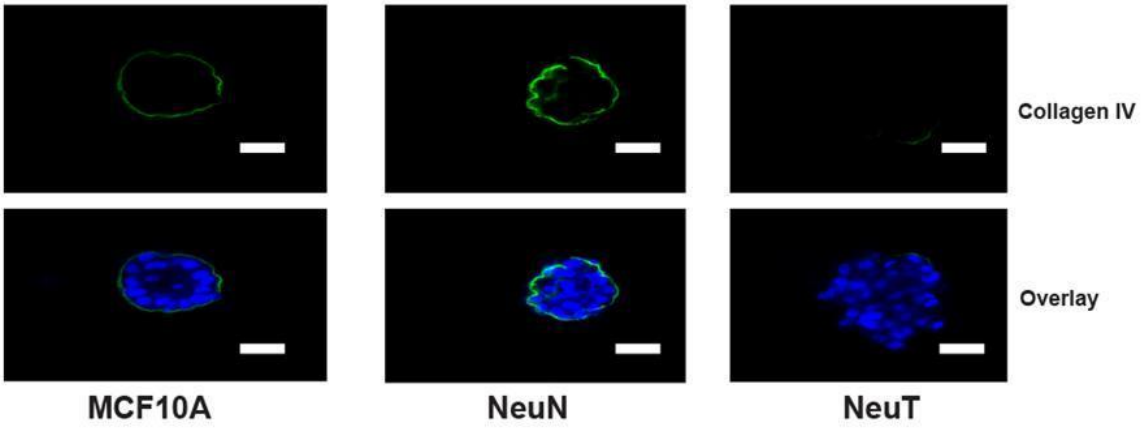

Figure 26: 3-D structures observed after 12 days on Matrigel. Basement membrane is represented as Collagen IV staining in green (top) and DAPI stained nuclei in blue. MCF10A and NeuN have intact basement membranes.

Once formed, these 3D multicellular structures were dislodged and embedded in collagen type I. IFF was applied for 48 hours and the pressure gradient was maintained by replenishing the media on the inside of the cell culture insert using flowed-through media. NeuN spheroids were used to determine the effects of IFF on HER2 DCIS-like acini. We observed a decrease in periacinar laminin V – a basement membrane associated protein - in response to interstitial flow (Fig. 27). In addition, a few of the acini that were subjected to IFF possessed invading cells in the vicinity of the spheroid (see arrow in Fig. 27) suggesting these cell originated from the main structure but had invaded.

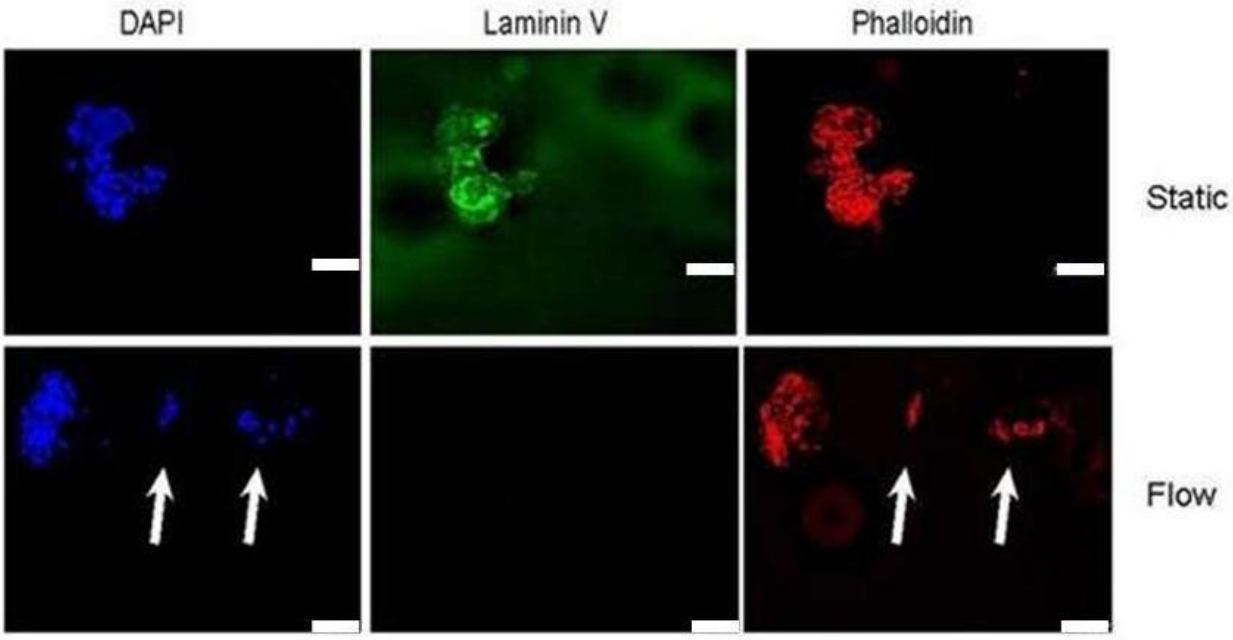

Figure 27: NeuN 12-day old acini plus 2 days under flow or static conditions; decreased laminin V staining in flow conditions and increase invasion.

The main two proteins components of the basement membrane are laminin V and collagen type IV. We decided to further confirm loss of basement membrane due to IFF using another protein marker and stained the spheroids with collagen IV. In line with the loss of laminin V staining previously observed, we saw a loss of basement membrane associated collagen IV staining in the IFF samples (Fig. 28A). Analysis of these NeuN acini showed a greater than 50% decreased in basement membrane associated collagen IV staining under IFF (Fig. 28B) ($p<0.05$). Interestingly, MCF10A acini also displayed an IFF-induced decrease in collagen IV staining; however in this case, the difference was not statistically significant. Figure 28B is the percentage ratio of collagen IV staining and total cell surface staining (as measured by F-actin staining).

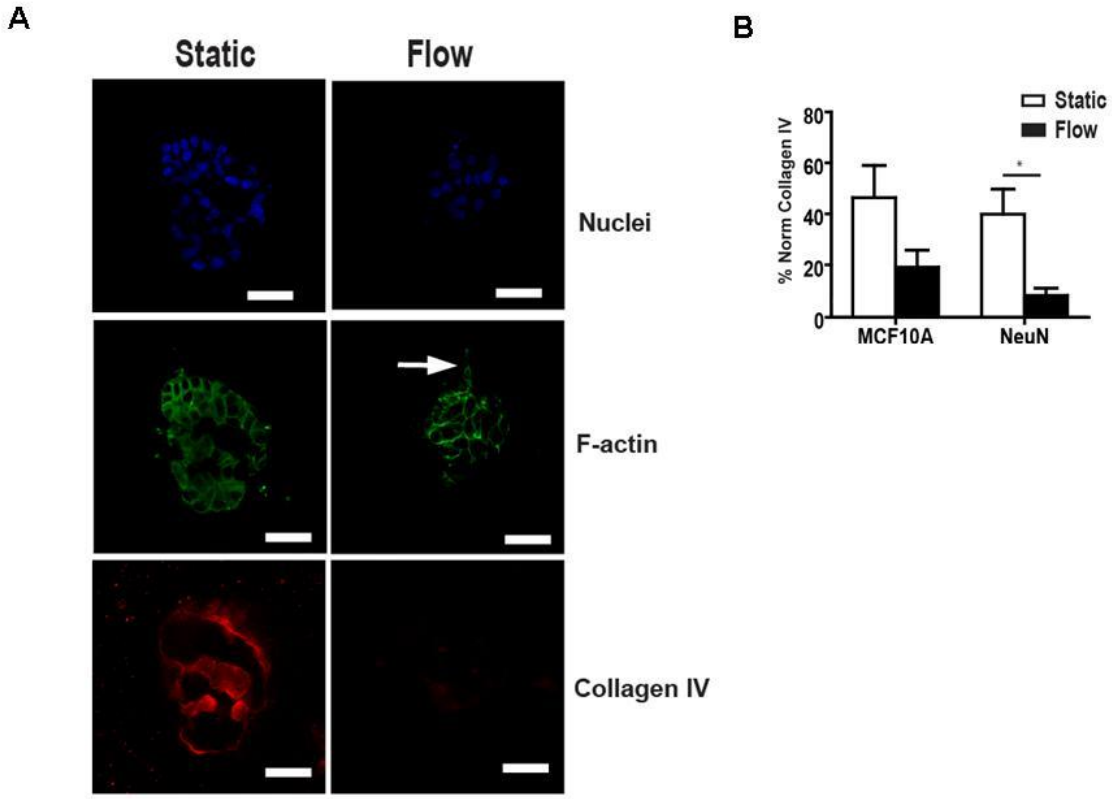

Figure 28: Interstitial fluid flow induces loss of basement membrane associated collagen type IV. A) NeuN 12-day old acini plus 2 days under flow or static conditions; decreased collagen IV staining in flow conditions and increase in invasive protrusions (arrow). B) Image analysis using MATLAB of collagen IV fluorescent intensity differences. Data is represented as percentage Collagen IV (red) stain when compared to Phalloidin (green) from NeuN flow: 9; NeuN static: 16; MCF10A flow: 16; MCF10A static: 16. All values are mean ± SEM. *: $p < 0.05$.

Interstitial fluid flow increases spheroids protrusions

Basement membrane degradation is a hallmark for initiation of 3D invasion along with increase spheroid protrusions. To quantify spheroids protrusions, we counted the number of outward cell protrusions per acini (as shown by the arrows in Fig. 29A). We manually counted the number of protrusion in each acinus and observed that IFF increased the number of cellular protrusions from MCF10A and NeuN spheroids (Fig. 29B-C). We defined spheroid protrusions as extensions

outward of the main structure and quantified them using two separate approaches. First we classified spheroids displaying at least one protrusion as invasive spheroids and compared that number to the total number of spheroids to obtain a percentage invasive spheroids value (Fig 29B). This allowed us to observe that IFF increased the number of MCF10A invasive spheroids from 0 to 17%. IFF increased the number of NeuN invasive spheroids from 20% to 71%. The vast majority of NeuT spheroids (89%) were already invasive regardless of IFF. Secondly, we manually counted the number of protrusion in each spheroid to identify the role of IFF in increasing the total number of protrusions per spheroids. Here we observed that although both MCF10A and NeuN flow samples had higher averages of protrusions, these were not statistically significant when compared to static spheroids (Fig. 29C).

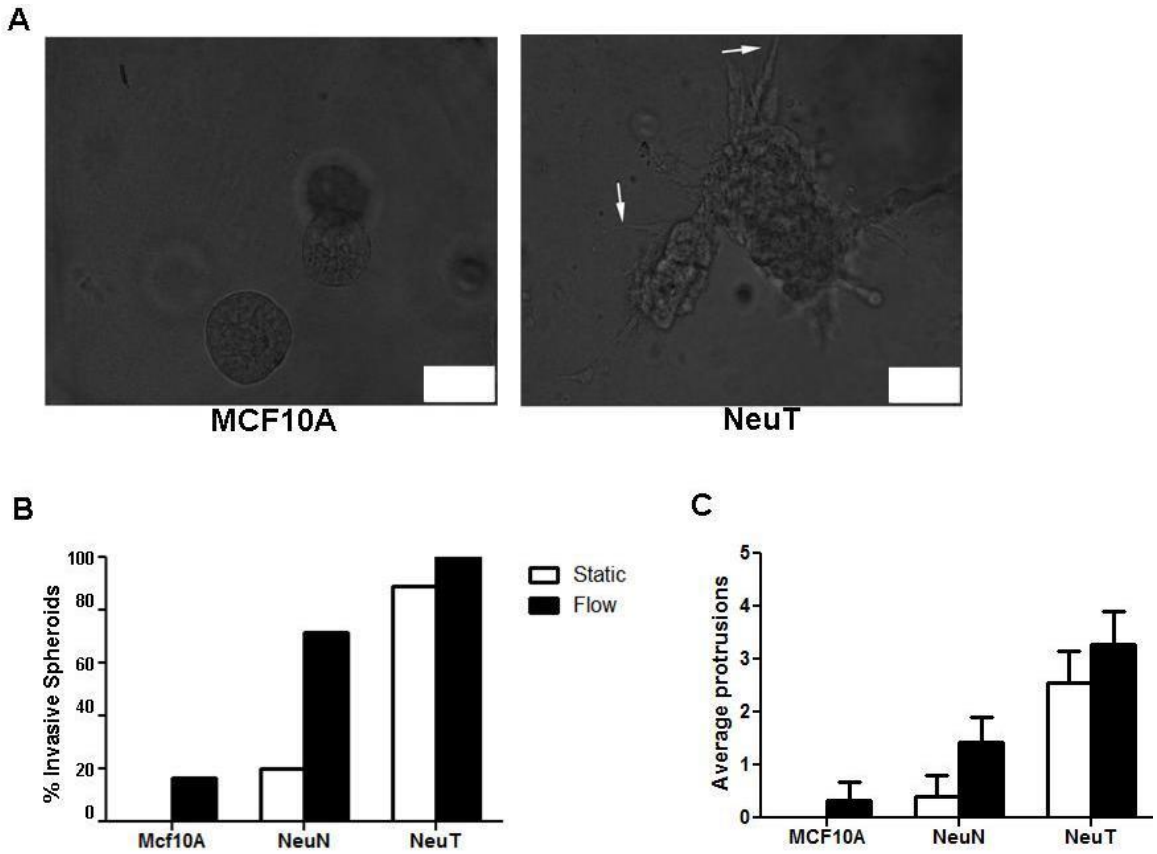

Figure 29: Interstitial fluid flow increases number protrusions of non-invasive breast cancer spheroids. A) Representative image of acini without protrusions (MCF10A) and acini with more than 6 protrusions (NeuT). Arrows points to protrusions. B) Percentage of acini with at least 1 protrusion. Protrusions were identified as extension from main structures. C) Average number of protrusions per spheroids. MCF10A static: 15; MCF10A flow: 25; NeuN static: 7; NeuN flow: 7; NeuT static: 10; NeuT flow: 8 in both A and B.

Interstitial fluid flow leads to PI3K phosphorylation of 3D spheroids

We previously observed that IFF increases invasion of single cells through PI3K phosphorylation (see Chapter 4). This led us to question the role of PI3K in IFF-dependent invasion of spheroids. In order to measure the level of this kinase in our samples, we isolated spheroids from gels after the 3-D invasion assay and harvested proteins. Western blot revealed an increase of phosphorylated p85 (the regulatory subunit of class I PI3K complex) in response to IFF in MCF10A and NeuN but not in NeuTs (Fig. 30).

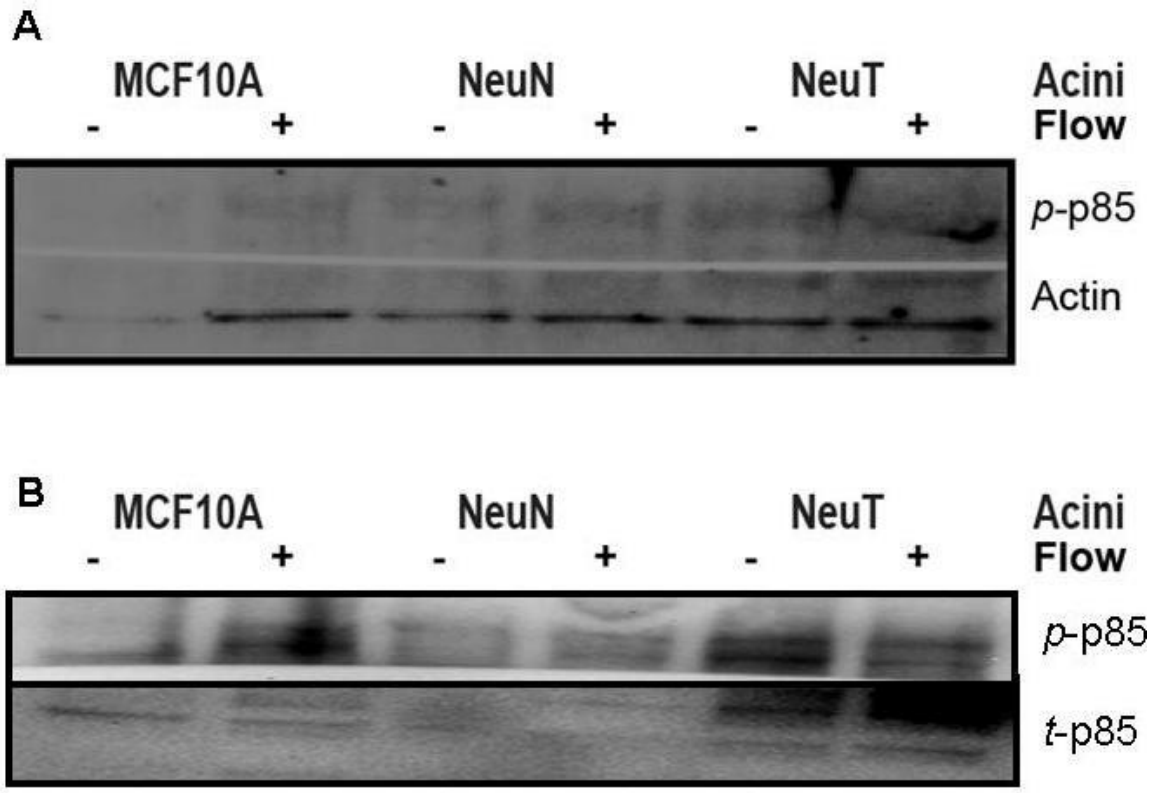

Figure 30: Interstitial fluid flow induces phosphorylation of PI3K in spheroids. Representative western blot of phosphorylation of PI3K regulatory subunit (p85) after 48 hours of static or flow conditions in 3-D acini structures embedded in a collagen matrix; A) β actin was used as loading control B) Total PI3K was used as loading control.

5.4 Discussion

Numerous studies have identified key components of the tumor microenvironments that alter tumor progression [30, 32, 34-36, 38-40, 60, 159, 162, 163]. Aside from the tumor and epithelial cells themselves, the tumor microenvironment includes all other components of the mammary gland (stroma-associated cells, matrix proteins, soluble factors and biophysical forces) and these have each been implicated in the suppression or promotion of tumor initiation, growth and development. Here we present the role of yet another microenvironment factor in the initial stages of cancer invasion and metastasis.

While we were aware of the ability of IFF to increase invasion of individual single cells, we aimed to investigate its effects on complex 3D multicellular acini that mimic tumor *in vivo* structures. The role IFF plays on 3D multicellular structures had never been studied. Hence, we showed for the first time that IFF modulated invasion of acini through increase in protrusion-positive spheroids, number of protrusions per spheroids, basement membrane associated protein degradation, and PI3K phosphorylation.

Early signs of tumor invasion and metastasis include basement membrane degradation, cellular protrusions and changes in cell and extracellular matrix interaction [8, 20]. The degradation or decreased expression of basement membrane associated proteins such as laminin V and collagen IV is a necessary step *in vivo* to allow for cell migration out of the mammary ducts into the surrounding stroma [113], and onwards into lymphatic and/or blood vessels. Because recreating the architecture of *in vivo* structures allow for more relevant physiological models in the study of cancer [164], we firstly developed a 3-D cell culture model that mimics interstitial fluid flow dynamics in normal, DCIS-like and IDC-like tumors. Secondly, we used this model to visualize the effects of IFF on the early steps of invasion which include basement membrane degradation and cellular protrusions.

In *in vitro* studies, cellular protrusions have been used as a positive marker for invasion [165-167] in addition to basement membrane degradation. The type of extensions and mode of cellular invasion away from the main tumor can be used to develop better cancer therapies. Cancer cells usually adopt distinct migration and invasion patterns. These are categorized as mesenchymal, amoeboid or collective cell

migration [49]. Each of these invasion patterns rely on a set of signaling cascades and these can be used as molecular targets in therapy development for advance disease in patients.

Most studies on IFF have been based on single cell migration and invasion within a matrix [76, 78, 86]. This approach is effective at identifying molecular signaling pathways activated by IFF and their consequences on cellular motility. However, their relevance to *in vivo* systems can only be inferred. Single cell migration out of a compact multicellular structure of cells requires loss of cell-to-cell contact that is tightly regulated by e-cadherins and integrins, and this phenomenon is not displayed in individual cells embedded in a matrix. Our newly developed approach allowed us to recapitulate pathological tissue architecture and allowed us to determine the effects of IFF on the invasion of these 3D structures.

In cancer, IFF originates from both leakage of normal blood vessels (outside the tumor) and tumor core (inside the tumor) leakage. Our current model did not consider the elevated IFF that is the result of additional angiogenic blood vessels present within the tumor especially in late stage DCIS and IDC. Although the flow velocities used in this study were closer to normal tissue IFF velocities, it was intriguing to demonstrate that such low IFF velocities were significant enough to initiate spheroids invasion. Future work will compare the effects of higher IFF velocities on both HER2 positive and negative acini.

Using a 3-D breast cancer model, we demonstrate for the first time that IFF induces the invasion of HER2 positive DCIS-like spheroids through a PI3K-dependent mechanism. We measured increased spheroid protrusions and loss of

basement membrane associated proteins (laminin V and collagen IV) in the presence on IFF. Further experiments are underway to elucidate the complete pathways activated by fluid flow in these structures. This study will allow us to identify therapeutic targets that may aid in DCIS management and treatment.

CHAPTER 6: Conclusions and future work

6.1 Major Findings and Significance

Introduction

The current study provides a foundation for interstitial fluid flow (IFF) signaling in HER2 positive breast cancer cells. Given that the combined role of IFF and HER2 expression in breast cancer invasion and progression had not been previously studied, this work identified key signaling proteins necessary in this pathway. The main findings of this study are that (a) physiological levels of IFF induce invasion of both single breast cancer cells and 3D spheroids via activation of PI3K; (b) although HER2 is necessary for most invasion in HER2 positive cells, and changes induced by HER2 overexpression probably plays an indirect role in IFF response, IFF induces invasion through a pathway independent of direct HER2 activity; (c) IFF mediated invasion requires CXCR4 signaling and a CXCL12 gradient in HER2 positive cells that have undergone EMT; and (d) IFF induces protein expression in cells consistent with EMT and renders cells more resistant to HER2-targeting therapies.

Previous IFF-induced mechanisms

Prior to the work presented here, IFF had been hypothesized to increase invasion of cells via two separate mechanisms. In the first mechanism, increased IFF-induced invasion is regulated by autocrine signaling in which IFF alters the levels of a specific invasion-inducing chemokine around the cells leading to migration in the direction of fluid flow. This mechanism is termed autologous chemotaxis and was

shown in three separate cellular systems and through two separate chemokines (CCR7 and CXCR4) [75, 76, 78]. Interestingly, all cells previously shown to invade through this mechanism are invasive and metastatic cells. We present evidence supporting CXCR4-dependent autologous chemotaxis in breast cancer cells. However, we demonstrate for the first time that this mechanism is specific to invasive cells like those that have undergone EMT, and non-invasive cells do not respond to IFF through chemokine signaling.

CXCR4/CXCL12 axis was previously identified as necessary for this IFF-induced autologous chemotaxis response in glioma cells [76]. Our results show that autocrine CXCL12 secretion and autologous CXCR4/CXCL12 chemotaxis is important only in IDC cells and not in pre-invasive cells. Because the levels of CXCR4 were not altered between our invasive and non-invasive cells, this suggests that during breast cancer progression, once the cells have acquired the invasive phenotype though EMT, they become more sensitive to chemokine gradients and develop a chemokine-dependent directional migration. The switch from chemokine dependent to chemokine-independent probably helps in facilitating metastasis and organ homing. It further emphasizes the importance of CXCR4 signaling in breast cancer invasion similar to what was observed in previous studies [143].

The second mechanism of IFF-induced invasion that was previously demonstrated is shown to be a consequence of IFF-induced changes to cellular interactions with the ECM via MMPs and adhesion proteins (integrins, focal adhesion kinases (FAKs) and CD44) [77, 79, 86]. Our finding do not suggest the importance of MMPs in the IFF-induced invasion of our breast cancer cell model since inhibition of

MMP activity using the pan-MMP inhibitor GM6001 did not ablate IFF-induced invasion. Because changes to MMP expression and activity are hallmark of cancer progression and the fact that these enzymes are highly secreted by metastatic cells to facilitate migration though matrix degradation, we cannot exclude their potential importance in the IFF-induced pathway. What we observed may be specific to the components of the matrix used in our model (e.g. the presence of matrigel as opposed to collagen-only which was used in studies identifying the important role of MMP in IFF-induced invasion), and the invasive potential of the cells (e.g. MMPs may be necessary for NeuT migration and not NeuN). This same principle can be applied to the role of FAK in IFF-induced pathway because we found that inhibiting this kinase did not disrupt IFF-induced invasion. We did not however question the role of integrins and CD44 molecules in our system, and this can serve as the basis of future studies (see future studies section below).

IFF and DCIS management

The identified specific mechanisms of interstitial fluid flow in initiating invasion can serve as a basis for therapeutic development in the treatment of DCIS patients. To date, most patients diagnosed with DCIS are treated with harsh therapies including lumpectomy, mastectomy, radiation therapy to prevent invasive breast cancer (IDC) even though more than 50% of DCIS will never progress to IDC [168, 169]. Our findings suggest that IFF velocities may serve as a potential marker for the classification of which DCIS patients are more at risk of developing IDC. This agrees with findings from a previous study showing a positive correlation between

interstitial fluid flow, pressure, and lymph node metastasis in several types of human cancers [67]. Additional studies still remain to be done nevertheless elevated levels of IFF may be correlated with higher rates of breast cancer invasion and metastasis especially *in vivo*.

IFF and PI3K

The PI3K pathway is known as one of the most important pathways in cancer and is implicated in cell growth, invasion, proliferation and differentiation [152, 170, 171]. For this reason, numerous cancer therapies targeting this pathway have been developed [172, 173]. PI3K is usually activated by receptor tyrosine kinases such as HER family members who bind the PI3K complex through either specific p85 binding sites or recruit chaperone proteins [174]. In many cancers however, constitutive activation of this pathway has been observed [175, 176]. The current project identifies IFF as a potential indirect contributor to PI3K activation in cancers. It also supports the importance of targeting this pathway in order to curtail breast cancer invasion in both DCIS and IDC, especially in those with abnormally elevated IFF. We show that by specifically blocking either p110α or p110β, we can decrease IFF-induced invasion in IDC cells. This means that in IDC patients requiring a specific p110 isoform for normal function or response to other unrelated therapies (depression, diabetes [176, 177]), the second isoform may be targeted. Inhibiting both isoforms using pan-PI3K inhibitors like most used currently in the clinic for breast cancer invasion can inadvertently affect the patients quality of life or health. Therefore, targeted p110 isoforms therapy will be more beneficial [178].

Compared to EGFR in MCF10A and CXCR4 in NeuT, we did not identify the specific upstream mediator of IFF-induced invasion in NeuN. Integrins are known activators of PI3K and their expressions are altered during cancer progression as the cells loose expression of specific basement membrane associated integrins ($\alpha_6\beta_4$) and gain expression of others such as $\alpha_5\beta_5$ [179]. Because integrins are known cellular transducers of mechanical signals, and we show through microarray analysis that the expression of both integrin $\alpha_6\beta_4$ and $\alpha_6\beta_6$ is down-regulated in NeuT compared to NeuN, this suggests these two integrins as potential upstream activators of IFF-induced PI3K in NeuN. Platelet-derived growth factors receptors (PDGFRs) are another potential IFF-induced PI3K activators in NeuN since these growth factors along with their receptors have been associated with shear stress induced invasion and PI3K activation [180-182].

The fact that all three identified pathways converge at PI3K and p110α suggest that this kinase is important for cellular invasion throughout cancer progression however its specific activator changes as the tumor progresses and the cells transform from epithelial characteristics to mesenchymal and their functions change.

IFF and HER2

The knowledge gained from this study has the potential to impact HER2 breast cancer management. A major hurdle in HER2 cancer therapy has been the *de novo* and acquired development of drug resistance observed in the clinic after months to years of therapy success [183]. Our findings demonstrate that HER2 positive cells

can still invade and proliferate in the presence of HER2 inhibitors when influenced by IFF. This reveals IFF as a contributor to HER2 drug resistance and tolerance. It also suggests that HER2 drug resistance may be prevented by developing new treatment approaches that modulate IFF levels in the tissue. In addition, combination therapies targeting both members of the here-presented IFF-activated molecular pathways (p110, CXCR4) along with current HER2 drug targets (Herceptin, Lapatinib) may be more effective at reducing the rates of invasion in HER2 positive breast cancer patients compared to HER2 therapies alone.

EMT is sometimes followed by a loss of HER2 overexpression in HER2 positive cells [121]. This may explain the spontaneous desensitization to HER2 targeted therapies observed in some patients. The fact that our data demonstrates that IFF alters the expression of EMT markers further suggests its impact on HER2 therapy resistance, cancer progression, and cancer cell differentiation.

Single cells versus spheroids

Most studies on IFF have been based on single cell migration and invasion [76, 78, 86]. Although this approach can effectively identify molecular signaling pathways activated by IFF and their consequences on cellular motility (see chapters 2, 3, and 4), single cells display invasion dynamics that are not relevant to in pre-invasive *in vivo* systems. Single cell migration out of a compact multicellular structure (mammary duct) requires alterations to both cell-to-cell and cell-to-ECM interactions that are not prominent in systems of individual cells embedded in a matrix. In addition, the basement membrane presents a physical barrier that cells need

to breakdown in order for invasion to occur. For this reason, results using our developed 3D spheroid model that recapitulate pathological tissue architecture are more relevant in studying the transition from DCIS to IDC. Using this approach, we demonstrate for the first time the impact of IFF on DCIS-like spheroids and this occurring also in a PI3K-dependent fashion. From these findings, we can postulate that IFF plays separate autonomous roles during cancer progression. First, it facilitates initiation of invasion out of the primary tumor site and second, it aid in directing single cell migration during metastasis.

Conclusion

In conclusion, this study provides evidence supporting the mechanism of autologous chemotaxis in IFF response, identifies additional IFF-induced mechanisms that are chemokine independent, and further demonstrates a hitherto undefined role of IFF on cell fate during breast cancer progression. In addition, our findings helped isolate the role each mechanical (IFF) and molecular factors (HER2) studied plays in initiating invasion, and has helped change the paradigm of how mechanical forces can interact with molecular factors to control tumor progression.

6.2 Future Work

The findings described here can be used to investigate several research topics. Our initial findings identified PI3K as a major component of all three IFF molecular pathways in all cell lines tested. Future work will focus on identifying both the upstream and downstream activators of PI3K that can modulate IFF-induced invasion

in HER2 positive non-invasive cancer cells and other HER2 negative DCIS-like cells. Our current hypothesis is that these may be integrins and/or PDGR as both are known inducers of PI3K and mechano-transducers. Targeting these receptors to questions their importance in IFF-induced signaling will require the use of pharmacological inhibitors, neutralizing antibodies or silencing RNAs. Further experiments to identify the complete pathway activated by IFF in each of these cells will also be beneficial.

Since IFF-induced invasion in DCIS cells occurred specifically through p110α, future breast cancer therapy development could focus on targeting this isoform in HER2 positive DCIS in order to decrease their risks of transitioning to IDC. This in turn may aid in decreasing the overall number of deaths associated with breast cancer.

One limitation of our study was the difference in pressure gradients between our model and most *in vivo* systems. The hydrostatic pressure difference generated by our 3D system was approximately 1 mm Hg; however levels *in vivo* are much larger despite the less permeable environment. This suggests that higher pressure gradients *in vivo* are required to generate a similar amount of IFF, and this may impact how cells respond *in vivo* compared to *in vitro* [67, 184]. Future studies should therefore question the role of members of the identified IFF molecular pathway such as PI3K and CXCR4 in a model system that includes IFF velocities and pressures closer to those levels observed *in vivo.* The pressure and velocity levels can be increased by incorporating a manual pump in the system. The pump can therefore be set to several physiological levels higher than what was used in this model and pathological levels (high and low measured levels in tumors). Changes to invasion and response to

pharmacological inhibitors targeting CXCR4 and PI3K can then be compared between these conditions.

In addition, animal studies will help bridge the gap of knowledge that currently exists between the mechanisms of IFF in the lab and in patients. Building upon the findings of the present study, we may be able to identify the role of normal and elevated levels of IFF in more complex but relevant systems (mice, human). Using MRI imaging for example to measure IFF velocities in the mammary fat pad of mice at multiple stages of breast cancer, IFF velocities could then be compared to the rates of invasion and number of metastasis, sites of metastasis and rate of survival. Identifying a correlation between flow velocities and invasion/metastasis in mouse models will aid in translating the findings of the current research to impacting human breast cancer. Through an improved understanding of IFF induced cell invasion, this research will have significant impact in helping determine how and why breast cancer cells invade, and what regulates the transition from DCIS to IDC.

Interstitial fluid flow is just one of the many biophysical stimuli *in vivo* that alter cellular invasion. It remains however still largely ignored in cancer research and therapy development. Understanding the consequences of interstitial fluid flow and other biomechanical forces in the tumor microenvironment on cellular behavior will revolutionize our understanding of the importance of these biophysical forces in driving breast cancer progression. This will not only lead to better clinical management of breast cancer patients in particular but other cancer patients in general and indirectly decrease the mortality rates of cancer.

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APPENDIX

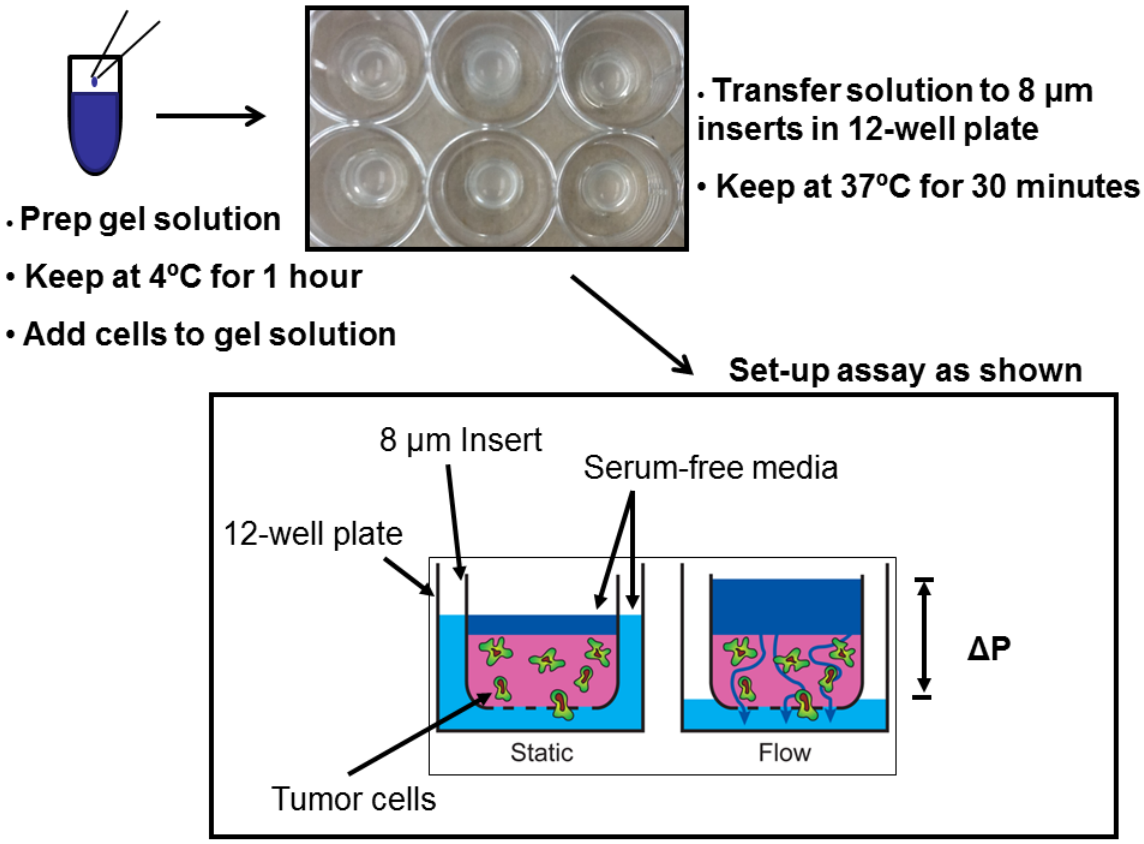

Figure 31: Schematic of the 3-D interstitial fluid flow invasion assay. First prepare gel solution using appropriate concentrations and volumes. Then add cells to gel solution and transfer to cell culture inserts. Finally add appropriate volume of media to each condition and incubate. Interstitial fluid flow is driven by a fluid pressure head.

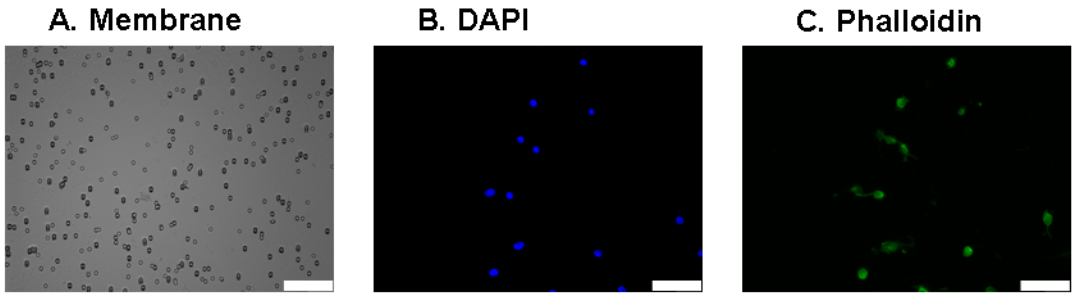

Figure 32: Representative invaded cells after an experiment (mutated MCF10A mammary epithelial cell line). The cells on the membranes were fixed and stained with DAPI and Alexa Fluor 488-conjugated phalloidin to facilitate counting of invaded cells; A) Picture of the membrane under bright field; B) DAPI stained nuclei (in blue); C) Alexa Fluor 488-phalloidin stained F-actin (in green). Scale bar represents 50µm.

Table 3: List of all inhibitors and neutralizing agents used in the projects along with their protein target

	Inhibitor/Neutralizing agent	Protein target
1	Lapatinib	**Her2**
2	Triciribine	**Akt**
3	IMD0354	**NFKB**
4	PD168393	**HER ½**
5	AG1478	**HER 1**
6	SP60125	**JNK II**
7	SB203580	**p38**
8	LY294002	**PI3K**
9	U0126	**MEK**
10	PP2	**SRC**
11	GM6001	**MMP**
12	AMD3100	**CXCR4**
13	WZ811	**CXCR4**

14	AS605240	**P110 gamma**
15	CAL 101	**P110 delta**
16	PIK75	**P110 alpha**
17	TGX221	**P110 beta**
18	PF573228	**FAK**
19	BEZ235	**MTOR**
20	Pertussis toxin	**GPCR**
21	SiRNA construct	**CXCL12**
22	Neutralizing antibody	**CXCL12**
23	Neutralizing antibody	**CXCR4**
24	Neutralizing antibody	**CCR7**

CURRICULUM VITAE

Skills

Soft: critical thinking, result orientation, initiative, thoroughness, self-development orientation
Laboratory: ELISA, flow cytometry, mammalian cell culture, primary cell isolation, 2D and 3D cell culture, HPLC, LC-MS/MS, mass spectrometry, protein extraction and purification, RNA and DNA isolation from blood, cells and tissue, real-time PCR, microarray data analysis, tissue digestion, western blotting, immuno-histochemistry, immuno-fluorescence, fluorescence microscopy, confocal microscopy, agarose gel electrophoresis, SDS-PAGE, 2D protein gels, IC50 determination, transfection, micro-cantilever related assays
Computer: Proficient in MATLAB, GraphPad Prism, Illustrator, Photoshop, Microsoft Office Suite
Languages: Fluent in French

Laboratory and Research Experience

Drexel University: College of Biomedical Engineering and Science
Jan 2011 - Present
Research Assistant/Pre-doctoral student

- Design and develop 3 dimensional breast cancer structures for the study of mechanical forces on cancer progression using 3D matrix
- Utilize on a daily basis most molecular biology techniques mentioned above to elucidate signaling pathways
- Conduct grant writing, manuscript preparations and conference presentations
- Oversee master level and undergraduate research projects on mechanobiology based projects
- Manage maintenance of laboratory equipment and orders
- Supervise and train 10 staff on laboratory techniques
- Guest lectured graduate level class of mechanobiology in cancer

Thomas Jefferson University: Kimmel Cancer Center
March 2006 - Dec 2010

Cancer Biology Research Assistant

- Prepared, ran and analyzed samples on HPLC, LCQ, Maldi Mass Spectrometer, and micro-cantilever machinery for biomarkers discovery
- Analyzed high throughput multidimensional proteomic and genomic databases for pathway analysis using R, Matlab
- Collaborated with a team of surgeons, oncologists, engineers and molecular biologists for profiling of breast cancer tissue

- Developed protocol for primary stem cell isolation and transfection
- Collected and organized information for publication and assisted in preparation of manuscripts and presentations
- Managed human specimen collection and processing (blood and tissue) and established primary cell culture lines

Temple University Hospital: Pulmonary Department
June - Sept 2004, 2005
Laboratory Technician

- Prepared, ran, and daily analyzed gel electrophoresis (agarose and polyacrilamide) for genetic biomarkers
- Set up and analyzed Radial-Immuno-Diffusion and Iso-Electric focusing gel for biomarkers
- Trained and supervised summer lab staff on laboratory techniques
- Participated and collaborated in clinical research involving Asthma and COPD drugs

Education

Drexel University, School of Biomedical Engineering, Science and Health Systems
GPA: 3.8/4.0
Ph. D. in Biomedical Science
Expected July 2014
Drexel University, School of Biomedical Engineering, Science and Health Systems
GPA: 3.7/4.0
M. S. in Biomedical Engineering
June 2012
Temple University, College of Science and Technology
B. S. in Biology, Minor in Psychology
Dec 2005

Publications (maiden name: Minkeu)

2014 - A. Tchafa, C. Ho, A. Kohli, M.J. Reginato, A.C. Shieh "Interstitial fluid flow induces invasion of non-invasive breast cancer spheroids" In preparation
2014 - A. Tchafa, M.J. Reginato, A.C. Shieh "Interstitial fluid flow induces epithelial-to-mesenchymal transition in cells" In preparation
2014 – A. Tchafa, M.J. Reginato, A.C. Shieh "Interstitial fluid flow-induced invasion is HER2 independent" In preparation
2104 – A. Tchafa, M. Ta, M.J. Reginato, A.C. Shieh "Epithelial-to-mesenchymal transition alters signaling response to interstitial fluid flow in HER2-positive cancer cells" In submission
2013 - A. Tchafa, R. Meng, R. Wassell, J.N. Quong, A.A. Quong "Increased cellular invasion and proliferation via the estrogen receptor after 17-β-estradiol treatment in

breast cancer cells using Stable Isotopic Labeling with Amino acids in Cell culture (SILAC)" Advances in Breast Cancer Research, Vol.2, No. 2, April

2012 - A. M. Tchafa, A. D. Shah, M.T. Duong, S. Wang, A.C. Shieh "Three-dimensional cell culture model for measuring the effects of interstitial fluid flow on tumor cell invasion" – Journal of Visualized Experiments, July

2012 - M. Gormley, **A. Tchafa**, R. Meng, Z. Zhong, A.A. Quong "Proteomic profiling of infiltrating ductal carcinoma reveals increased cellular interactions with tissue microenvironment" Journal of Proteome Research, February

2008 - I. Mercier, M. C. Casimiro, C. Wang, A. L. Rosenberg, J. Quong, **A. Minkeu**, K. G. Allen, C. Danilo, F. Sotgia, G. Bonuccelli, J-F. Jasmin, H. Xu, E. Bosco, B. Aronow, A. Witkiewicz, E. S. Knudsen, M. P. Lisanti "Human breast cancer-associated fibroblasts (CAFs) show caveolin-1 down-regulation and RB tumor suppressor functional inactivation: Implications for the response to hormonal therapy" Cancer Biology and Therapy, Vol7, Issue 8, August

Presentations (maiden name: Minkeu)

2013 - A. M. Tchafa, M. J. Reginato, A. C. Shieh, "Interstitial Flow Stimulates Invasion of ErbB2-overexpressing Breast Cancer Cells via PI3K Activation" BMES 2013 annual meeting, Seattle WA, September 25-28 – Oral presentation

2012 - A. M. Tchafa, M. J. Reginato, A. C. Shieh, "Combined role of interstitial fluid flow and ErbB2 expression on breast cancer progression", CSH Asia/ICMS joint conference on tumor microenvironment, Suzhou China, November 13-17 - Poster

2012 - A. M. Tchafa, A. C. Shieh, "Interstitial fluid flow increases invasion of ductal carcinoma in situ-like cells through PI3K-dependent mechanisms" NEBEC, Philadelphia PA, March 16-18 - Poster

2011 - A. M. Tchafa, A. C. Shieh, "Interstitial fluid flow and ErbB2 expression combine to initiate breast cancer cell invasion" AACR tumor microenvironment conference, Orlando FL, November 3-6 - Poster

2011 - R. Meng, **A. Minkeu**, A. A. Quong, "Proteomic profiling reveals increased immunological response and extracellular matrix-receptor interactions in invasive ductal carcinoma" AACR 102nd annual meeting, Orlando FL, April 2-6 - Poster

2009 - A. Minkeu, R. Meng, A. L. Rosenberg, A. A. Quong, "Secretome profiling of matched tumor and normal primary human mammary epithelial cells" AACR 100th annual meeting, Denver CO, USA, April 18-22 - Poster

2008 - A. Minkeu, J. N. Quong, A. L. Rosenberg, K. L. Brill, A. A. Quong, "Global protein expression differences between matched pairs DCIS/Normal and Invasive/Normal Primary HMEC and other physiological differences" San Antonio Breast Cancer Symposium, San Antonio TX, USA, December 10-14 – Poster (award winner)

2008 - A. Minkeu, R. Meng, R. Wassell, J.N. Quong, A.A. Quong, "Proteome Analysis of the Effects of Estradiol and Tamoxifen Stimulations on MCF7 Breast Cancer Cells using SILAC" Greater Philadelphia Bioinformatics Alliance fall retreat, Philadelphia PA, USA November 6 – Poster (1st Prize poster winner)

2008 - Z. Zhong, **A. Minkeu**, J. Quong, A. Quong "Decreased BRCA1 protein expression alert the response of MCF7 cells to Tamoxifen" AACR International Conference: Molecular Diagnostics in Cancer Therapeutic Development, Philadelphia PA, USA, September 22-25 - Poster

2007 - A. Minkeu, J. Quong, A. Quong, "Micro-Cantilever Based System for the Measurement of Protein Levels in Human Serum Samples" Biotech 2007, Philadelphia PA, USA, October 8-9 – Poster

2007 - J. N. Quong, A. L. Rosenberg, K. L. Brill, S. Farooqi, W. Yeow, **A. Minkeu**, A. A. Quong "Correlation of protein and gene expression for the stratification of breast cancer patients" Breast Cancer Symposium, San Franscico CA, USA, September 7-8 – Poster

Awards

- Outstanding Graduate Award - Research – Interdisciplinary Frontiers
  2014
  School of Biomedical Engineering, Science and Health Systems, Drexel University
- Doctoral Research Excellence Award, Highly Commended
  2014
  Graduate Student Association, Drexel University
- Travel Award
  2013
  Biomedical Engineering Society Annual Meeting, Seattle WA
- International Travel Award
  2012
  Cold Spring Harbor Asia/ICMS Joint Conference on Tumor Microenvironment, Suzhou, China
- AACR Minority Scholar Award
  2008
  American Association for Cancer Research
- First Prize Poster Winner
  2008
  Greater Philadelphia Bioinformatics Alliance Fall Retreat

Professional Organizations

- Biomedical Engineering Society (BMES)
  2012 to present
- American Association of Cancer Research (AACR)
  2008 to present
- American Association for the Advancement of Science (AAAS)
  sponsored membership 2010 to 2012

Volunteer Experience and Activities

- Volunteer with iPraxis, a University of Pennsylvania non-profit group promoting science education in underserved Philadelphia public schools.
  2013 - Present
- University of Pennsylvania Museum of Archeology and Anthropology, Philadelphia, PA
  International Classroom speaker to elementary, middle and high schools and special events
  2001 - Present
- Community Service: Haiti, New Orleans (Hurricane Katrina relief), South Dakota Lower Brule reservation, Dominican Republic
  Summer 2002, 2006, 2007, 2008, 2010