IDENTIFICATION OF A COMMENSAL RELATIONSHIP BETWEEN EPIGENETICALLY DISTINCT SUBPOPULATIONS DURING BREAST CANCER INVASION

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# IDENTIFICATION OF A COMMENSAL RELATIONSHIP BETWEEN EPIGENETICALLY DISTINCT SUBPOPULATIONS DURING BREAST CANCER INVASION

by

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Breast cancer is responsible for over 40,000 deaths each year in the United States. The majority of these deaths are not attributable to the primary breast tumor, but to metastases in vital organs. Tumor cell invasion is an early step in the metastatic cascade which can occur collectively by multiple cells cooperatively invading into the surrounding stroma. Primary patient breast tumors and patient-derived breast cancer cells can collectively invade yet how cells collectively invade is still largely unknown. It is well known that tumors contain heterogenous populations of cells yet traditional metastasis models focus

on the ability of a rare population of neoplastic cells to autonomously invade past the basement membrane surrounding the tumor, intravasate into blood vessels and disseminate throughout the body to colonize foreign tissues. We hypothesized that there is a stable subpopulation of tumor cells that is capable of initiating the invasion of another population. Using organotypic culture models, which provide a three dimensional environment that models stromal conditions, and real-time imaging, a technique in which cell behavior can be imaged in real time at a single cell resolution, we determined that breast cancer cell lines can contain populations of cells with differential invasive potential. Furthermore, we concluded that one population of invasvie cells is sufficient to induce the invasion of other noninvasvie cells. This suggests a new mechanism for breast cancer metastasis, in which subpopulations of cells can cooperate with each other as opposed to competing against each other, to invade and potentially metastasize. Future studies will focus on determining the requirements for the leader cells to induce invasion and the follower cells to migrate behind the leader cells, with the eventual goal of targeting specific tumor populations for diagnostic and therapeutic treatment.

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PUBLICATIONS

Dang, T.T, Prechtl, A.M & Pearson, G.W. Breast cancer subtype-specific interactions with the microenvironment dictate mechanisms of invasion. *Cancer Res* 71, 6857-6866.

Prechtl, A.M, Westcott, J. M, Dang, T.T & Pearson, G.W. Non cell-autonomous control of invasion by a subpopulation of tumor cells. *In preparation.*

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Arp2/3 – Actin-related protein 2/3

BRCA1 – Breast cancer 1

Cdc42 – Cell division cycle 42

DAB2 – Disabled-2

DCIS – Ductal carcinoma *in situ*

DOCK10 – dedicator of cytokinesis 10

ECM – Extracellular matrix

EGF – Epidermal growth factor

EMT – Epithelial to mesenchymal transition

EpCAM – Epithelial cell adhesion molecule

ER – Estrogen receptor

FACS – Fluorescent activated cell sorting

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

FOX – Forkhead box

FOXD1 – Forkhead box protein D1

GAP – GTPase-activating protein

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GDI – Guanine nucleotide dissociation inhibitor

GDP – Guanine diphosphate

GEF – Guanine nucleotide exchange factor

GTP – Guanine triphosphate

GTPase – Guanosine triphosphatase

H2B – Histone 2B

H&E – Hematoxylin and eosin

HER2 – Human Epidermal Growth Factor Receptor 2

IBC – Invasive breast cancer

INV– Invasive subpopulation

IR – Infrared

IGF – Insulin-like growth factor

ITGA11 – Integrin alpha-11

LCIS – Lobular carcinoma *in situ*

LCM – Laser capture microdissection

LPA – Lysophosphatidic acid

LPAR1 – Lysophosphatidic acid receptor-1

LPP3 – lipid phosphate phosphatase 3

MET – Mesenchymal to epithelial transition

MMP – Matrix metalloproteinases

NON – Noninvasive subpopulation

NSCLC – Non-small cell lung cancer

N-WASP – Neural Wiskott-Aldrich syndrome protein

PBS – Phosphate buffered saline

PDGFR α – Platelet derived growth factor receptor α

PPAP2B – Phosphatidic acid phosphatase type 2B

PR – Progesterone receptor

PVDF – Polyvinylidene Fluoride

QPCR – Quantitative polymerase chain reaction

RIPA – Radioimmunoprecipitation Assay

S.D. – Standard deviation

SDS-PAGE- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

S.E.M. – Standard error mean

shRNA – short hairpin ribonucleic acid

siRNA – small interfering ribonucleic acid

Twist1 – Twist-related protein 1

WASP –Wiskott-Aldrich syndrome protein

WAVE – WASP-family verprolin-homologous protein

# CHAPTER ONE
# Breast Cancer Background

## INTRODUCTION

### Overview

Breast cancer was responsible for approximately 40,000 deaths in 2011, making it the second leading cause of cancer-related death of women in the United States (Society, 2011). This deadly disease is characterized by the uncontrolled proliferation of mammary epithelial cells, which grow to form neoplastic lesions within the breast. If detected in the early stages of the disease, breast cancer survival rate is high, but as the disease progresses mortality rates quickly increase (Michaelson et al., 2003; Nothacker et al., 2009).

Breast cancer starts as a local disease, but over time can progress to a more malignant state. An early step towards malignancy is the invasion of tumor cells into the surrounding extracullular matrix (ECM), which can lead to the metastatic spread of cancer and eventually death (Steeg, 2003). Relatively little is known about the early steps of invasion and the progression to metastasis. My studies focus on investigating how neoplastic cells cooperate during invasion away from the primary tumor and defining molecular pathways associated with tumor cell invasion.

### Transition from normal to DCIS

The normal mammary epithelium is composed of lobules and ducts (Figure 1-1). Luminal epithelial cells that line the lobules within the breast secrete milk, which is

carried out of the breast by luminal epithelial cells that line the ducts (Hu et al., 2008). Luminal epithelial cells are anchored to myoepithelial cells which secrete a protein rich basement membrane (Bissell et al., 2002). Myoepithelial cells have been shown to play a protective role, preventing tumor cells from progressing to a more advanced stage, so it is not surprising that as tumors advance, they progressively lose their surrounding myoepithelial layer of cells (Adriance et al., 2005; Barsky and Karlin, 2005). The basement membrane separates the epithelial cells from the surrounding stroma, which is composed of structural proteins and other cell types such as fibroblasts, adipocytes, and immune cells (Gusterson et al., 1982; Tlsty and Coussens, 2006).

When cells enter into the hollow luminal space within a duct, they normally undergo apoptosis in response to the loss of polarity and attachment to the basement membrane (Debnath et al., 2002; Mailleux et al., 2008). When cells bypass the apoptotic signaling pathway in response to loss of basement membrane attachment, and continue to survive and proliferate in the luminal space, it leads to the formation of a solid mass within the mammary gland known as a carcinoma *in situ* (Figure 1-1). Most carcinomas *in situ* of the breast occur when abnormal luminal cells lining the ductal epithelium form noninvasive lesions known as ductal carcinoma *in situ* (DCIS), which are thought to be a precursor to developing a more malignant form of breast cancer known as invasive breast cancer (IBC) (Figure 1-1) (Burstein et al., 2004a; Hu et al., 2008). Lobular carcinoma *in situ* (LCIS) are noninvasive lesions derived from lobular epithelial cells. LCIS are less common than DCIS, however the occurrence of LCIS in one breast increases the chances of developing IBC in either breast (Akashi-Tanaka et al., 2000; Chuba et al., 2005).

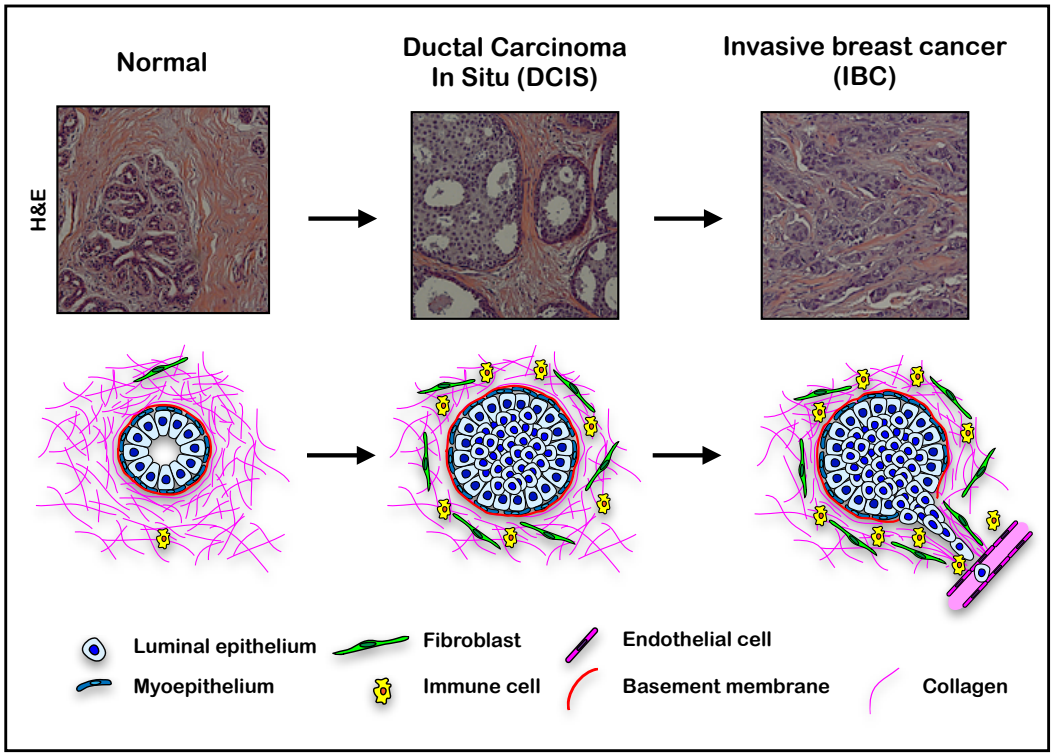



**Figure 1-1. The loss of epithelial organization and changes to the microenvironment can lead to tumor progression.** Breast cancer gradually progresses from a benign, noninvasive lesion to a malignant, invasive lesion. The top panel shows hematoxylin and eosin (H&E) staining of normal, benign (DCIS) and invasive (IBC) human breast tissue. Below each panel is a depiction of a cross-section of the mammary epithelial gland at each stage. Normally, luminal epithelial cells (light blue) are surrounded by myoepithelial cells (dark blue), which secrete a basement membrane (red), which serves to segregate the epithelial cells from the surrounding microenvironment which is composed of structural elements such as laminins and collagens as well as other cell types such as fibroblasts and immune cells. Breast cancer occurs when tumor cells accumulate within the ducts and lobules forming DCIS lesions. Over time benign lesions will likely progress to invade and metastasize throughout the body.

**Breast cancer invasion and metastasis**

Invasion is a hallmark of cancer and an early step in the metastatic cascade which requires cancer cells to invade past the basement membrane into the surrounding stroma, intravasate into the blood stream, travel to distant sites in the body, extravasate into a secondary tissue, and grow in a foreign microenvironment (Figure 1-2) (Hanahan and Weinberg, 2000; Pinder and Ellis, 2003; Steeg, 2003). However, what causes invasion of cells away from the primary tumor mass is still unknown. Blocking invasion would preclude cells from initiating the metastatic cascade, thus preventing metastasis from occurring. Understanding this early step in the metastatic cascade could lead to better diagnostic tools and treatments for breast cancer patients.

Tumor cell invasion is associated with multiple factors, involving both intrinsic variations in the molecular pathways of the cell, and external components of the tumor microenvironment. Several studies have attempted to identify genes associated with invasion in tumor progression (McSherry et al., 2007). Studies using gene expression profiling to compare progressive stages of breast cancer have been performed in an attempt to identify DCIS and IBC-specific signatures (Ma et al., 2003; Schuetz et al., 2006). However, distinct signatures for various pathological stages of breast cancer were not found, perhaps due to intratumor heterogeneity that exists within tumors (McSherry et al., 2007). One gene expression study that addresses the heterogeneity within tumors was performed by Wang et al. They used an *in vivo* invasion assay to isolate invasive rat breast tumor cells to compare against the bulk tumor and were able to identify a potential invasive cell gene signature (Wang et al., 2004). These gene expression studies have

been successful in identifying a few genes that may be important in invasion progression and diagnosis in breast cancer, but little overlap in genes required for invasion from previous expression studies has been observed. Additional studies are needed to identify and determine genes associated with the *in situ* to invasive transition, which may be targetable for breast cancer treatment and diagnosis.

Treatment for early stage breast cancer currently involves surgical resection and/or radiotherapy of the detectable tumor. To avoid relapse of undetectable tumor cells in distant tissues, chemotherapy, radiation, or endocrine therapy, is given to over 80% of breast cancer patients after their local treatment of surgical resection or radiotherapy (Weigelt et al., 2005). Most patients do not require adjuvant therapy, as the local treatment is generally sufficient to remove the disease from the patient. However, because we currently do not have accurate prognostic markers to predict who will relapse and require additional treatment, many patients are over-treated and must endure the resulting toxic side effects (Weigelt et al., 2005). Thus, prognostic markers targeting invasion could be identified to accurately distinguish patients that are most at risk for developing life-threatening metastases.

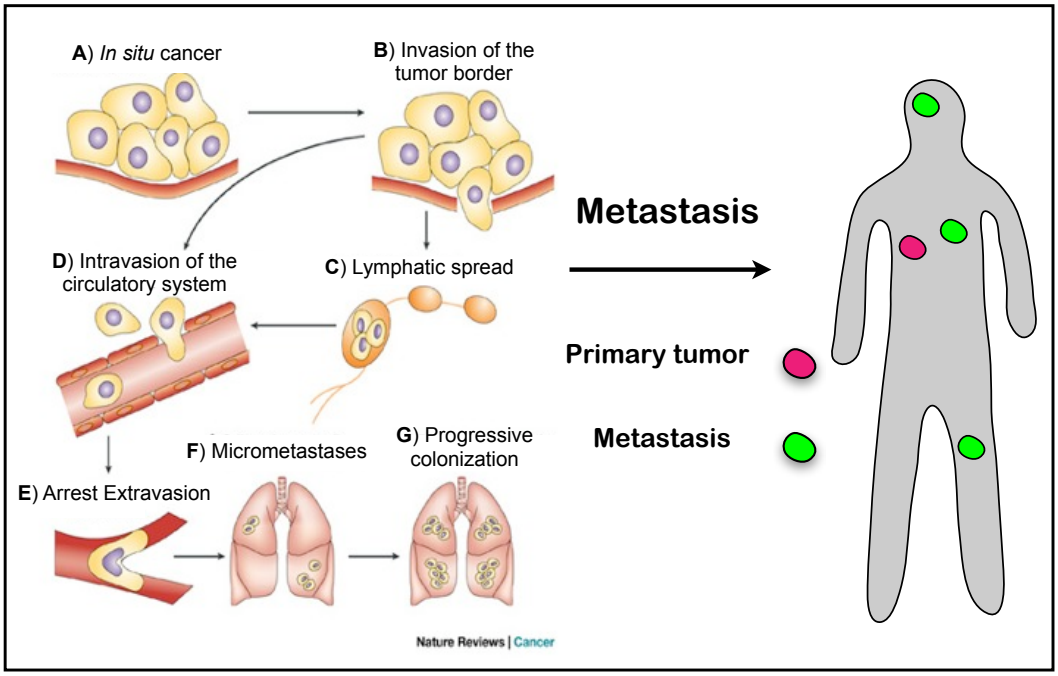



**Figure 1-2. Invasion is a critical step in the metastatic cascade.** A schematic of the metastatic cascade. (**A**) Cells bypass normal growth restrictions to proliferate uncontrollably then can (**B**) invade past the basement membrane into the surrounding stroma where they have access to the (**C**) lymphatic system and (**D**) vasculature. They can then spread to distant sites in the body and (**E**) extravasate and (**F**) survive in critical organs, where they can lie dormant for years. (**G**) Cells can then grow and form macrometastases, which can result in the death of the patient. Figure modified from (Steeg, 2003).

**Mechanisms of cell motility**

Motility is necessary for cells to invade and migrate away from the primary tumor (Vignjevic and Montagnac, 2008). Cells move by extending plasma membrane protrusions known as lamellipodia, sheet-like projections, and filopodia, finger-like projections, at the leading edge of the cell (Ridley, 2011). Once the plasma membrane protrusion has extended, the leading edge establishes adhesions with the surrounding substratum (Friedl and Wolf, 2003b). Homotypic adhesion proteins known as cadherins and cell-matrix adhesion proteins called integrins both are important in promoting adhesion in epithelial cells and can relay regulatory signals to the cell (Aplin et al., 1998; Hanahan and Weinberg, 2000). Actomyosin-based contractile forces, possibly mediated through focal-adhesion linked stress fibers, translocate the cell body forward, and the anterior portion of the cell is retracted forward as adhesions disassemble at the trailing edge of the cell (Mattila and Lappalainen, 2008). This basic mechanism of migration is essential during embryonic development and is also utilized in invasion during tumorigenesis (Hall, 2005; Ridley, 2011; Thiery et al., 2009).

**Small Rho GTPases-regulators of invasion**

Members of the Rho guanosine triphosphatase (GTPase) family are key regulators of the actin cytoskeleton, controlling cell migration and invasion (Yilmaz and Christofori, 2010). Rho GTPases cycle between active guanine triphosphate (GTP) -bound and inactive guanine diphosphate (GDP) -bound states. Activation by GTP and GDP is tightly regulated by guanine nucleotide exchange factors (GEF), GTPase activating proteins (GAP), and guanine nucleotide dissociation inhibitors (GDI) (Hall,

2005; Jaffe and Hall, 2005). GEFs activate Rho GTPases by stimulating the dissociation of GDP, allowing subsequent GTP binding (Figure 1-3) (Erickson and Cerione, 2004). Conversely, GAPs inactivate Rho GTPases by enhancing GTP hydrolysis resulting in an inactive Rho-GDP bound state (Hakoshima et al., 2003). GDIs sequester RhoGTPases in an inactive GDP bound state and prevent activation by GEFs (Bishop and Hall, 2000).

Small Rho GTPases regulate many crucial biological functions including cell migration, proliferation, and gene transcription by activating downstream effector proteins (Van Aelst and D'Souza-Schorey, 1997). Rho, Rac, and Cdc42 are three of the most well studied small Rho GTPases that play a major role in regulating cytoskeletal dynamics. RhoA is involved in the formation of stress fibers and focal adhesion complexes while Rac is associated with the formation of broad, sheet-like, actin-rich projections that generally form at the leading edge of a cell, known as lamellipodia (Hall, 1998; Ridley and Hall, 1992; Ridley et al., 1992). Rac stimulates the formation of lamellipodia through the activation of WASP-family verprolin-homologous protein (WAVE), which in turn activates Actin-related protein 2/3 (Arp2/3). Arp2/3 stimulates actin nucleation and branching, thus leading to lamellipodia formation (Figure 1-3) (Jaffe and Hall, 2005).

Cell division cycle 42 (Cdc42) is involved in the formation of filopodia, which are actin-rich, finger-like membrane projections (Nobes and Hall, 1995). Cdc42 directly binds and activates neural Wiskott-Aldrich syndrome protein (N-WASP), or the hemopoietic-specific WASP, which activates Arp2/3, thus causing actin nucleation and filopodia formation (Figure 1-3) (Jaffe and Hall, 2005; Rohatgi et al., 1999). Filopodia have roles in would healing through the promotion of cell-cell adhesion, they serve as

chemokine gradient sensors to provide guidance cues on neuronal growth cones, and they have an important role in cell migration (Mattila and Lappalainen, 2008). Filopodia probe the microenvironment around a cell containing adhesion molecules and receptors that promote adhesion of the leading edge, thus promoting cell migration and motility (Mattila and Lappalainen, 2008).

The presence of filopodia in cancer cells is a critical for invasion in cancer (Vignjevic et al., 2007). These cellular projections aid in the migration of a cell away from the primary tumor thus allowing for the invasion of a cell into the surrounding ECM. This acquisition of an invasive phenotype is often correlated with a transition from an immobile, epithelial state to a de-differentiated, mesenchymal state known as epithelial to mesenchymal transition (EMT).

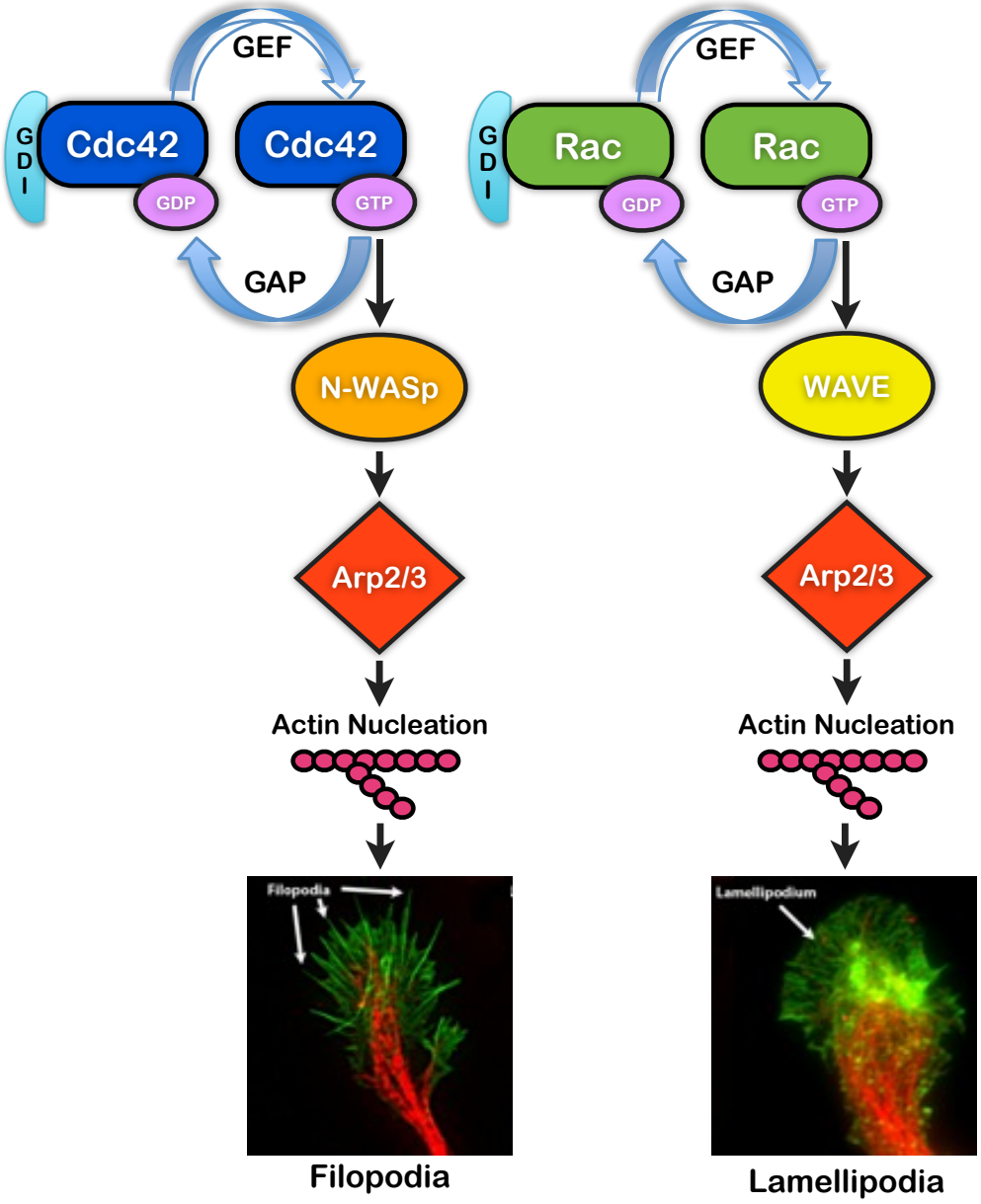



**Figure 1-3. Cdc42 and Rac regulate filopodial and lamellipodial dynamics.** GEFs activate small Rho GTPases, Cdc42 and Rac, by stimulating the dissociation of GDP, allowing subsequent GTP binding. Conversely, GAPs inactivate small Rho GTPases by increasing GTP hydrolysis to GDP. GDIs sequester inactive GDP-bound small Rho GTPases in the cytosol, preventing activation by GEFs. GTP-bound Cdc42 activates N-WASP, thereby activating ARP2/3, which promotes actin

nucleation and filopodia formation. GTP-bound Rac activates WAVE that activates ARP2/3 and thus actin polymerization and lamellipodia formation; F-actin (green), microtubules (red). Images from http://en.wikipedia.org/wiki/Growth_cone.

**EMT and MMPs are classical mechanisms of invasion**

One method cancer cells acquire migratory and invasive abilities is associated with cells undergoing an epithelial to mesenchymal transition (EMT) (Tomaskovic-Crook et al., 2009). EMT involves a change in cellular morphology and signaling pathways which cause an epithelial cell to display mesenchymal characteristics including a loss of cell adhesion proteins and cell polarity (Thompson et al., 2005). Cells that have undergone EMT can be identified by decreased levels of epithelial markers such as E-cadherin, a protein that mediates cell-cell contacts, and increases in mesenchymal markers Vimentin, smooth muscle actin, and N-cadherin (Foroni et al., 2011; Kalluri and Weinberg, 2009). Increased expression of transcriptional repressors of E-cadherin such as Snail and SIP-1 are also indicative of EMT and have been shown to be upregulated in invasive patient tumor samples (Nakagawa and Takeichi, 1995; Peinado et al., 2007; Peinado et al., 2004). Interestingly metastatic nodules often display highly epithelial as opposed to mesenchymal characteristics (Chao et al., 2010). This finding has been rationalized by the concept of EMT to MET, mesenchymal to epithelial transition, which also occurs in development (Davies, 1996; Foroni et al., 2011). It is thought that tumor cells undergo EMT to invade away from the primary tumor, survive in the vasculature, and seed foreign tissues. Once at the metastatic site, the cell undergoes MET which may facilitate growth and establishment of the metastatic lesion. Thus, tumors can acquire the necessary ability for invasion away from the primary site via EMT, and revert back via MET to the

epithelial state required for growth at the secondary site.

EMT has been associated with single cell invasion, which is characterized by individual cells invading past the basement membrane and dissolving their contacts with surrounding cells (Figure 1-4) (Thiery, 2002). Invasion can also occur collectively or can be facilitated by cells within the microenvironment such as fibroblasts (Figure 1-4) (Gaggioli et al., 2007; Rorth, 2009). Collective cell invasion is characterized by multiple cells invading as groups, strands, or clusters while maintaining their cell-cell junctions and is observed in both breast cancer patient tumors and transgenic mice (Conklin et al., 2011; Provenzano et al., 2006).

Invasion can be influenced by the activity of proteases such as matrix metalloproteinases (MMPs), which degrade components of the ECM thus facilitating invasion (Stetler-Stevenson, 1990; Talvensaari-Mattila et al., 1998). There are 21 known MMPs, some are more closely associated with cancer aggressiveness than others. MMP-2 and MMP-9 for example correlate with poor prognosis in breast cancer (Machesky and Tang, 2009; Talvensaari-Mattila et al., 1998). However, MMPs are not required for all types of invasion, for example macrophages utilize an MMP-independent mode of motility known as amoeboid movement, which can induce an MMP-independent mode of invasion in breast cancer cells (Guiet et al., 2011).

MMPs are generally produced by stromal cells in the microenvironment and not by their epithelial counterparts, but as cells acquire mesenchymal characteristics thorough EMT they gain the ability to secrete MMPs. Gene expression studies show that MMPs are upregulated in patient tumors and cell lines that display EMT-positive profiles. The EMT profile is distinct among heterogeneous breast cancer intrinsic subtypes.

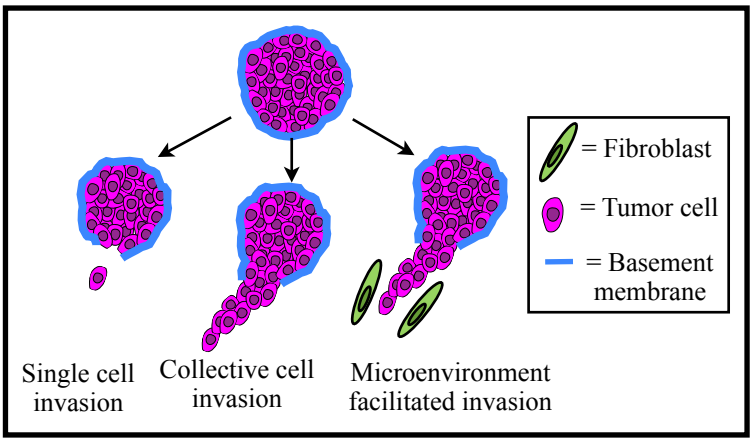



**Figure 1-4. Modes of tumor cell invasion.** EMT can induce single cell invasion, characterized by individual cell invasion and dissociation away from the primary mass. Invasion can also occur collectively when multiple cells form invasive projections, invading past the basement membrane and into the surrounding stroma. Components of the microenvironment, such as fibroblasts, can also contribute to invasion.

### Intertumor heterogeneity- Breast cancer intrinsic subtypes

Breast cancer can be classified into five different subtypes based on global gene expression analysis, but how these subtypes relate to invasion is not well studied. The breast cancer subtypes include Luminal A, Luminal B, Her2-positive, Claudin-low, and Basal-like (Prat and Perou, 2011). The intrinsic subtypes have been found to occur in both breast cancer cell lines and mouse models (Herschkowitz et al., 2012; Kapp et al., 2006; Prat et al., 2010). HER2 positive, Basal-like and Claudin-low tumors are hormone receptor negative subtypes which tend to have poorer patient outcomes in comparison to the hormone receptor positive Luminal A and B subtypes, with Luminal A tumors exhibiting the best prognosis (Fan et al., 2006; Sorlie et al., 2003).

The intrinsic subtypes observed in breast cancer suggest that tumors likely contain distinct genetic alterations that contribute to their responsiveness to treatment

(Rouzier et al., 2005; Slamon et al., 2001). The luminal tumors are hormone receptor positive, expressing estrogen receptors (ER) and progesterone receptors (PR). Luminal A tumors represent about 30% of breast cancers while luminal B tumors represent around 14% of breast cancers. Both are characterized by high expression of luminal markers, the presence of estrogen and progesterone receptors, and the lack of HER2 expression. However, luminal B tumors are distinguished by a higher rate of proliferation, poorer prognosis, and the relative expression of biomarkers compared to luminal A tumors (Cheang et al., 2009).

Basal tumors represent about 20% of breast cancers, are triple negative which means they lack ER, PR and HER2 receptors, and are associated with germline mutations in the tumor suppressor gene, breast cancer 1 (*BRCA1*) (Sorlie et al., 2003; van 't Veer et al., 2002; Zhang and Powell, 2005). HER2 positive tumors represent about 8% of all breast cancers and are characterized by the presence of the proto-oncogene HER2, a transmembrane tyrosine kinase that when overexpressed can lead to cancer, and lack of estrogen and progesterone receptors (Sergina and Moasser, 2007). HER2 amplification within tumors is an example of how stratification of breast cancer into subtypes can be utilized to target cancer for specific therapeutics. Trastuzumab is a humanized monoclonal antibody that inhibits proliferation and survival of neoplastic cells specifically in HER2 dependent tumors, and is now used as part of the first line treatment against these tumors (Baselga et al., 2006; Hudis, 2007).

Claudin-low tumors represent about 10% of all breast cancers and are triple negative. These tumors display low expression of luminal markers, high expression of EMT markers, and tend to be highly invasive thus imparting a poor prognosis on the

patient (Herschkowitz et al., 2007; Prat et al., 2010). Claudin-low tumors are enriched for features associated with mammary stem cells and cancer stem cells, which may also contribute to the poor prognosis for patients with Claudin-low tumors (Lim et al., 2009; Prat et al., 2010). The establishment of distinct breast cancer intrinsic subtypes has lead to molecular insights into the disease and more effective therapeutic strategies (Carey et al., 2007; Sorlie et al., 2001).

**Intratumor heterogeneity**

Heterogeneity exists not only between individual breast cancer patient tumors (intertumor heterogeneity), it occurs within tumors as well (intratumor heterogeneity), yet how tumor subpopulations interact to influence neoplastic cell invasion is not well understood. It is well known that this intratumor heterogeneity exists within a wide range of malignancies, containing populations of cells that differ in growth rate, differentiation state, invasiveness, and migratory ability (Heppner, 1984). Intratumor heterogeneity can be observed in human tumors by staining or sorting for molecular markers such as epithelial cell adhesion molecule (EpCAM), CD44 and CD24 and expression profiling of tumor cells and subpopulations (Fillmore and Kuperwasser, 2007; Navin et al., 2011; Park et al., 2010; Shipitsin et al., 2007). Sorting for $CD44^{+}$, $CD24^{-/low}$, and $EpCAM^{+}$ populations yields cells with greater tumorigenic potential than corresponding $CD44^{+}$, $CD24^{+}$, and $EpCAM^{+}$ subpopulations (Fillmore and Kuperwasser, 2007).

Striking evidence for tumor heterogeneity is observed during the therapeutic resistance that tumors undergo after anti-cancer treatment. A small subpopulation of multi-drug resistant cells may persist in a largely drug sensitive tumor after therapy, thereby causing relapse in the patient (Gerlinger et al., 2012; Shah et al., 2002). The

resulting tumor may be resistant to previously administered treatments or reconstitute the heterogeneity within the original tumor, causing relapse in the patient (Dean et al., 2005; Shah et al., 2002). Typically this is thought to be a process of selection pressure that allows for the acquisition and expansion of new traits (Dean et al., 2005). Thus, it is important to study heterogeneous populations within tumors to better understand cancer treatment and progression (Marusyk and Polyak, 2010).

**The tumor microenvironment can influence invasion and tumor progression**

Numerous studies from patients, animal models, and cell culture have shown that the tumor microenvironment can contribute to tumor initiation, progression, and metastasis (Hu and Polyak, 2008). A variety of cells in the tumor microenvironment influence tumor progression including the vasculature, immune and inflammatory cells, and fibroblasts (Tlsty and Coussens, 2006). In addition, extracullular matrix proteins can influence vascularization, tumor cell proliferation, and invasion (Tlsty and Coussens, 2006). Stromal cells have been shown to promote invasion via physical remodeling of the microenvironment and the secretion of paracrine factors which promote tumor cell proliferation, survival, migration and invasion (Friedl and Wolf, 2003a; Goswami et al., 2005; Jedeszko et al., 2009).

Fibroblasts secrete paracrine factors as well as synthesize, deposit, and remodel components of the extracellular matrix within the microenvironment to promote tumor progression and invasion (Bhowmick et al., 2004; Gaggioli et al., 2007). Fibroblasts can stimulate tumor cell proliferation by secreting growth factors including fibroblast growth

factor (FGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF), which may also serve as transforming chemoattractants for tumor cells (Bhowmick et al., 2004).

Fibroblasts also influence tumor cell invasion through physical reorganization of the ECM. Organotypic culture models have been used to show that stromal fibroblasts can lead the collective invasion of cancer cells by generating tracks within a matrix, which support neoplastic cell invasion (Dang et al., 2011; Gaggioli et al., 2007). Interestingly, when fibroblasts are co-cultured with basal breast cancer cell lines the tumor cells are induced to invade both *in vitro* and *in vivo*, however luminal breast cancer cell lines do not invade in the presence of fibroblasts suggesting that the role of the microenvironment may vary in response to intertumor heterogeneity (Dang et al., 2011; Hu et al., 2008).

The fibroblast-led invasion requires small RhoGTPases, Cdc42 for matrix remodeling and breast cancer cell invasion, and Rho function specifically in the leading fibroblast cells but not in following squamous cell carcinoma cells (Dang et al., 2011; Gaggioli et al., 2007). In support of these observations, when affinity probes binding activated Rho were used to analyze invasive squamous cell carcinoma clinical samples, significant binding was observed in tumor samples while adjacent non-tumor tissue bound weakly to the probes (Gaggioli et al., 2007). These data suggest an important role of small RhoGTPases in invasive cell behavior. The study of invasive cell behavior can be accomplished using a three-dimensional system, which mimics the *in vivo* ECM environment.

**Organotypic culture as a tool to study invasion *in vitro***

To study how tumor cells invade, we utilize an orgnotypic culture model. Using this organotypic culture system, a single-cell suspension of mammary epithelial cells is plated onto a semi-solid layer of reconstituted basement membrane known as Matrigel, which is a mixture of proteins that mostly consists of lamininI and collagenIV (Debnath et al., 2003). Over time, each cell proliferates and forms a clonal sphere of cells. Organotypic culture can be used to study the early steps of invasion because mammary cell lines form multicellular spheroids that reconstitute many features of the primary tumor (Figure 1-5).

Some advantages to using the organotypic culture model are that this model reconstitutes the architecture of the tissue of tumor origin by more closely resembling the three dimensional environment that cells normally encounter in the body as opposed to two dimensional, rigid plastic substrata that cells are routinely cultured on. Growing cells in this manner allows for the formation of crucial cell-cell contacts and provides a more accurate representation of the activated signaling pathways that occur in cells when they are arranged in a tissue (Bissell et al., 2002). Cells can invade into the surrounding ECM when plated in organotypic culture, allowing for the study of single and collective cell invasion. Invasive and noninvasive spheroids in organotypic culture are representative of benign and malignant lesions in the primary tumor, but because single cells grow to form clonal spheres, heterogeneity within invasive phenotypes can be explored (Figure 1-5).

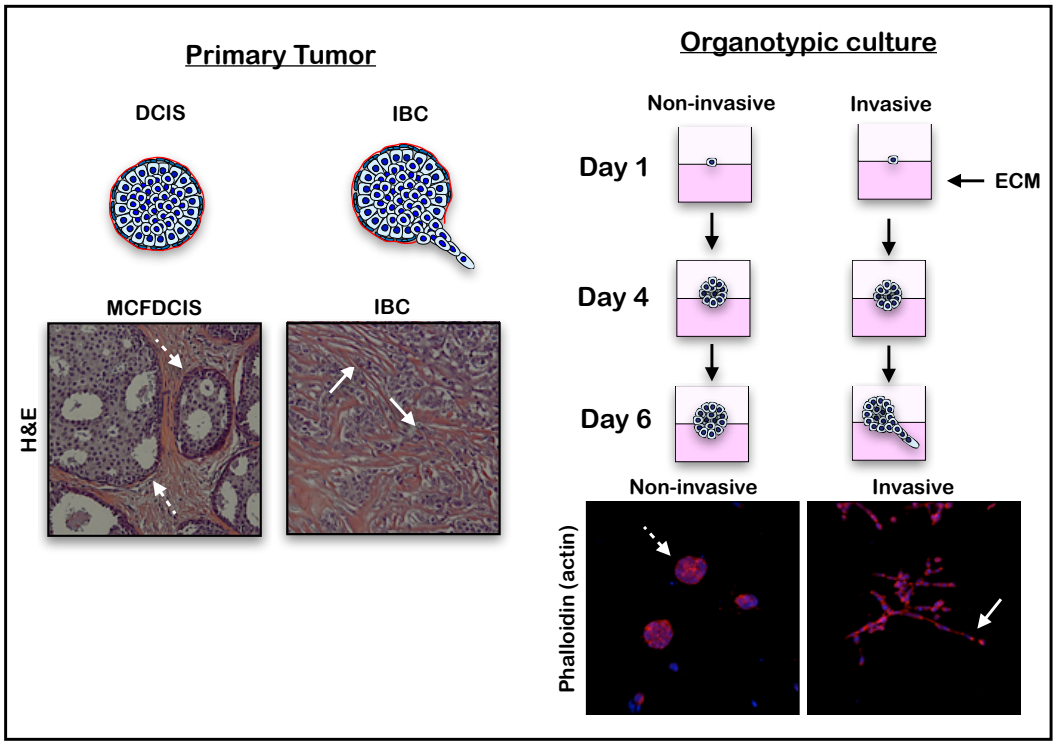



**Figure 1-5. Invasion can be studied *in vitro* using organotypic culture.** Organotypic culture models reconstitute the stroma, allowing cells to grow in a three-dimensional environment in which invasion can be studied. In this model, a single-cell suspension of mammary epithelial cells is plated onto a semi-solid layer of collagenI mixed with reconstituted basement membrane known as Matrigel, which is a mixture of proteins consisting of laminins and collagens. Over time, each cell proliferates and forms a clonal sphere of cells called a spheroid.

# CHAPTER TWO
# Heterogeneity Within Breast Cancer

## INTRODUCTION

### Overview

A pivotal step in the metastatic cascade is the early process of invasion. Once cells invade away from the boundaries of the tumor and into the extracellular matrix (ECM), they gain access to the vasculature allowing for dissemination of neoplastic cells throughout the body where they can form deadly metastatic lesions (Steeg, 2003). However, after decades of cancer research, relatively little is known about how cancer cells become invasive. Questions still remain in understanding what genes are necessary for invasion and how noninvasive cells within ductal carcinoma *in situ* (DCIS) tumors transition to invasive breast cancer cells (IBC). Here we identify isogenic invasive and noninvasive subpopulations within breast cancer cell lines to elucidate the requirements and interactions of subpopulations within tumors during invasion.

### Differential modes of invasion

Invasion occurs during the transition from noninvasive DCIS lesions to IBC (Burstein et al., 2004b). This transition can be observed in xenograft mouse models in which the breast cancer cell line, MCFDCIS, will form noninvasive DCIS-like lesions and over time progress to invasive breast cancer (Hu et al., 2008). There are multiple types of invasion, single cell invasion, collective invasion, and invasion mediated by components of the microenvironment (Rorth, 2009). Single cell invasion is generally characterized by individual cells that have undergone a transition, such as epithelial to

mesenchymal transition (EMT), that allows for invasion and migration away from the primary tumor (Thiery, 2002). By contrast, it is not known how collective cell invasion occurs, which is characterized by multiple cells invading away from the primary tumor.

Collective cell invasion has been observed in patient samples as well as in intravital imaging of human tumors grown orthotopically in mice (Conklin et al., 2011; Provenzano et al., 2006). We wondered what causes cells to collectively invade into the stroma. One possibility is that there may be distinct subpopulations within a tumor that have variable invasive capabilities. It is known that tumors can contain heterogeneous populations of cells so it is possible that stable subpopulations exist within a tumor that are capable of invasion. Thus for the remainder of this document, my studies will focus on collective cell invasion.

**Breast cancer cell lines recapitulate the heterogeneity observed in primary tumors**

To understand intratumoral heterogeneity, we utilized breast cancer cell lines, which retain much of the intertumoral heterogeneity observed in patient samples (Keller et al., 2010; Neve et al., 2006; Prat et al., 2010). Gene expression analysis of patient tumors classifies them into the breast cancer subtypes, Her2, Claudin-low, Basal-like, Normal breast-like and Luminal A and B (Prat et al., 2010). Gene expression profiling of breast cancer cell lines derived from patient tumors stratify into the breast cancer subtypes as well, suggesting that the heterogeneity observed between patient tumors is maintained in cell lines (Figure 2-1). The classification of breast cancer into intrinsic subtypes has provided valuable insight into tumor biology and treatment (Gupta et al., 2005; Neve et al., 2006; Padrick and Rosen, 2010). However, while heterogeneity within

primary tumors has been well characterized, the role tumor heterogeneity plays during invasion is not well understood. In this chapter we will investigate the invasive characteristics of heterogeneous subpopulations within breast cancer cell lines.

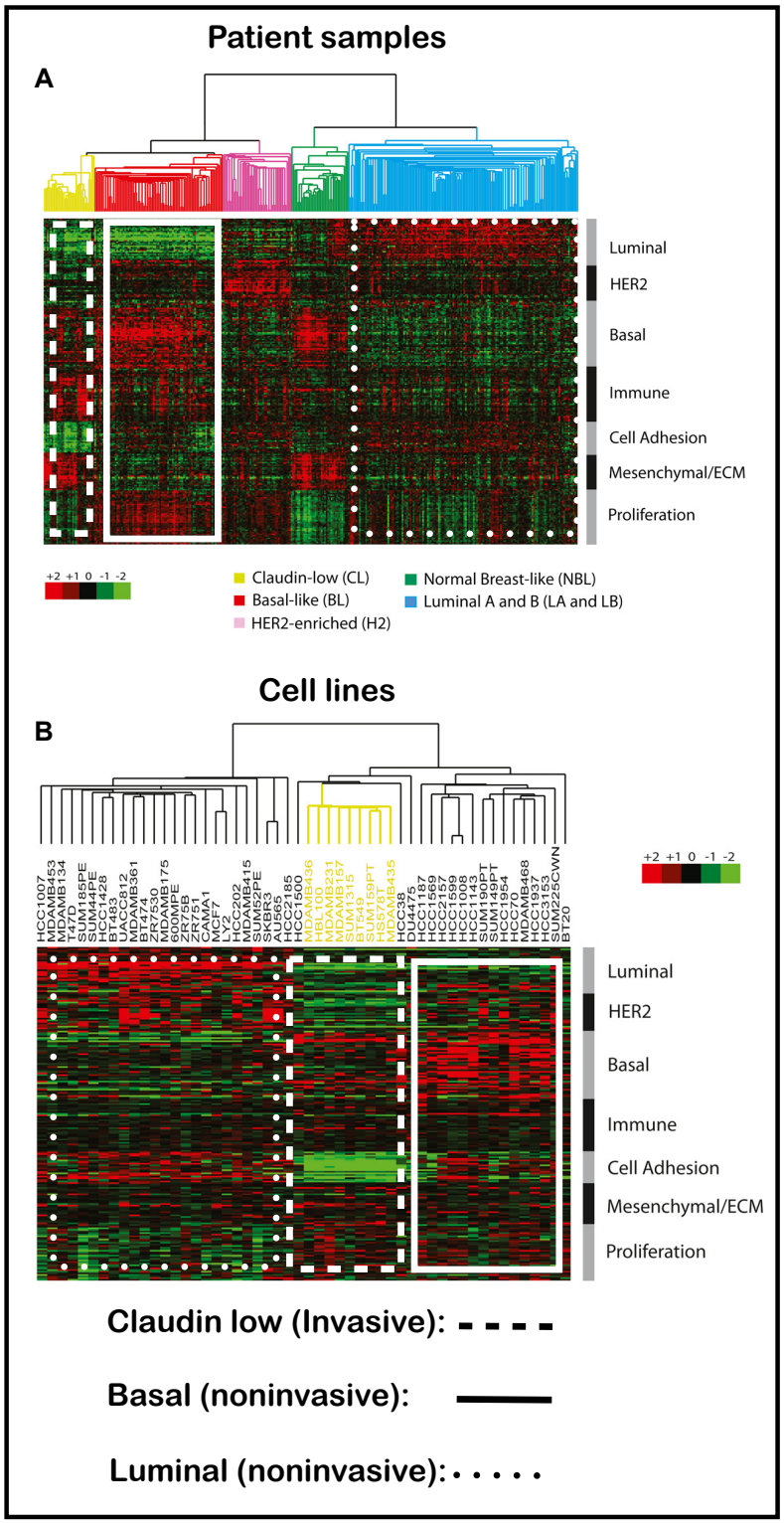

Patient samples
A
Luminal
HER2
Basal
Immune
Cell Adhesion
Mesenchymal/ECM
Proliferation
+2 +1 0 -1 -2
Claudin-low (CL)
Basal-like (BL)
HER2-enriched (H2)
Normal Breast-like (NBL)
Luminal A and B (LA and LB)
Cell lines
B
HCC1007
MDAMB453
MDAMB134
T47D
SUM185PE
SUM44PE
HCC1428
BT483
AU565
MDAMB361
BT474
ZR7530
MDAMB175
600MPE
ZR751
ZR75B
CAMA1
MCF7
LY2
HCC202
MDAMB415
SUM52PE
SKBR3
AU565
HCC1500
MDAMB436
HCC38
MDAMB231
MDAMB157
SUM1315
BT549
SUM159PT
HS578T
MDAMB435
HCC70
DU4475
HCC1187
HCC1569
HCC2157
HCC1599
HCC1008
HCC1143
SUM190PT
SUM149PT
HCC1954
MDAMB468
HCC1937
HCC3153
SUM225CWN
BT20
+2 +1 0 -1 -2
Luminal
HER2
Basal
Immune
Cell Adhesion
Mesenchymal/ECM
Proliferation
Claudin low (Invasive): - - - -
Basal (noninvasive): ——
Luminal (noninvasive): . . . . .

**Figure 2-1. Breast cancer cell lines retain the heterogeneity observed in patient samples.** Hierarchical clustering of genes from (**A**) human patient samples and (**B**) human derived breast cancer cell lines. Dotted and solid boxes highlight Claudin low, Basal and Luminal breast cancer subtypes within each panel, highlighting the similarities between patient and cell line data. Figure modified from (Prat et al., 2010).

## MATERIALS AND METHODS

### Cell Culture and reagents.

T47D, HCC1143, HCC1428, HCC1569 and HCC1954 cells were a gift from Michael Peyton and John Minna (UTSW). 4T1 cells were a gift from Fred Miller (Wayne State). HC-11 cells were a gift from Jeff Rosen (Baylor College of Medicine). T47D, HCC1143, HCC1428, HCC1569, HCC1954, 4T1 and MDA-MB-231 cells were cultured in a base medium of RPMI (Hyclone), 10% fetal bovine serum (FBS, Hyclone) and 1x penicillin streptomycin solution (Hyclone). HCC1143 medium was supplemented with 5 ng/ml EGF (Sigma), T47D medium was supplemented with 10 μg/ml insulin (Sigma) and HC-11 medium was supplemented with 10 μg/ml EGF and 5 μg/ml insulin and were cultured as described (Dang et al., 2011). SUM159 cells were cultured in Ham's F-12 medium containing 5% fetal bovine serum (FBS, Hyclone), 1x penicillin streptomycin solution (Hyclone), 5 μg/ml insulin (Sigma Aldrich), and 1mg/ml hydrocortisone (Sigma-Aldrich). SUM149 were grown in 5% FBS mammary epithelial growth medium (MEGM, Lonza). Human derived cell lines were validated by Powerplex analysis. Growth factor reduced Matrigel (BD Biosciences, 10-12 mg/ml stock concentration) and bovine collagen I (BD Biosciences) were used for organotypic culture experiments. Antibodies recognizing Collagen I (Abcam), Rac 1/2/3 (Cell Signaling), Cdc42 (Cell Signaling), N-WASP (Cell Signaling), E-cadherin (BD Biosciences), Vimentin (Cell

Signaling), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Calbiochem), β-Actin (Abcam), and Tubulin (Sigma-Aldrich) were used for immunofluorescence and Western blot analysis. Hoechst 33342, phalloidin, and secondary antibodies labeled with Alexa Fluor 488 nm or 546 nm, 680 nm (Invitrogen) and Infrared (IR) Dye 800CW (Li-Cor Biosciences) were used. Cell lines stably expressing pCLNRX-Histone 2B (H2B):GFP and PGK-H2B:mCherry were generated as described (Dang et al., 2011).

**Organotypic culture**

Single cells were plated in 8-well chamberslides (immunofluorescence staining, Falcon; live-cell imaging, Nunc) onto a base layer of Matrigel (5 mg/ml) and collagen I (1.5 mg/ml) and supplemented with a 2% Matrigel/growth medium mixture as described (Dang et al., 2011; Xian et al., 2005). All cultures were grown for 6-8 days except where indicated in the Figure Legends. For spheroid cluster experiments a 30 µl drop of a 500,000 cell/ml growth medium suspension was placed on a tissue culture dish lid and inverted onto the bottom dish containing sterile phosphate-buffered saline (PBS) to prevent sample desiccation. Drops were then incubated at 37˚C for 18-24 h and pipetted into a microfuge tube and pelleted. Spheroid clusters were resuspended in either 400 µl of growth media/2% Matrigel for "on top" cultures or 50 µl of Matrigel/collagen mix for embedded cultures and plated on 30 µl of a basement layer of Matrigel/collagen. Co-culture experiments were performed as follows. 2,000 4T1 cells were plated on top of a collagen-matrigel mixture with or without 4,000 MF4339 cells embedded in the collagen-matrigel mixture.

**Quantification of invasion**

A spheroid was classified as invasive if three or more cells invaded away from a primary

spheroid mass of ten or more cells. The number of spheroids counted per condition and replicates are indicated in the figure legends.

**Daughter Cell line Isolation**

Parental cell lines were plated in organotypic culture and allowed to grow for 8-20 days. To isolate the spheroids, 4˚C PBS was added to each well to dissolve the Matrigel/collagen. Spheroids were then isolated by pipetting into microfuge tubes based on phenotype using a phase contrast microscope. Spheroids with four or more projections of at least three cells were considered "invasive" and spheroids with no cellular projections were considered "noninvasive". Spheroids were then trypsinized into a single cell suspension and re-plated in organotypic culture and re-isolated. This process was repeated up to two additional times before expansion in monolayer culture to establish independent daughter cell lines.

**Immunoblot analysis and immunofluorescence staining**

Cells were lysed in Radioimmunoprecipiataion Assay (RIPA) buffer supplemented with a protease inhibitor cocktail (Calbiochem) as described (Pearson and Hunter, 2007). Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transfered to Immobilon-FL polyvinylidene fluoride (PVDF) transfer membrane (Millipore), and immunostained. Immunoblots were visualized using an Odyssey infrared scanner (LI-COR). Organotypic cultures were fixed and immunostained as described (Debnath et al., 2003; Pearson and Hunter, 2007). Images were acquired on Nikon and Zeiss LSM510 confocal microscopes in TIFF format. Images were arranged using Adobe Photoshop CS3 and Keynote, and are representative of three independent experiments.

### Real-time imaging of organotypic cultures

Imaging was performed using a Perkin Elmer Ultraview ERS spinning disk confocal microscope enclosed in a 37˚C chamber supplemented with humidified $CO_2$ (Solent) and a CCD camera (Orca AG; Hamamatsu). Images were acquired with a 20x (Zeiss) objective using Volocity software (Perkin Elmer) and analyzed with Imaris software (Bitplane). At least six to ten different x,y coordinates with six to ten or more z -slices over 60-100 μm span for each condition were imaged in parallel.

## RESULTS

### Breast cancer cell lines can contain subpopoulations of invasive and noninvasive cells

Breast cancer cells plated in organotypic culture grow over time to form clonal, multicellular spheroids, which can display a range of morphologies (Figure 2-2 A, B, and C). Spheroids typically contain 20-100 cells varying from one sphere to another within the same cell line. Phalloidin labels F-actin, thus allowing for the visualization of cell organization and morphology within spheroids to determine if invasion, which we define as three or more cells invading away from the primary mass, is occurring. Cell lines such as HCC1954 are composed of noninvasive spheroids, exhibiting a benign-like architecture without cells projecting outward into the surrounding ECM, characteristic of early stage breast cancer (Figure 2-2 A and B). Yet other cell lines such as MDAMB-231 are composed of primarily invasive spheroids, which display projections that are three or more cells in length and invade away from the primary mass and into the surrounding model stroma (Figure 2-2 A and B).

We found that breast cancer cell lines can contain both invasive and noninvasive spheroids when plated in organotypic culture, reminiscent of the molecular intratumoral heterogeneity observed in patient tumors (Figure 2-2 A, B, and C) (Park et al., 2010). The cell lines that contained both invasive and noninvasive spheroids when plated in organotypic culture included 4T1, SUM159, SUM149, and HCC1143 (Figure 2-2 A, B, and C). 4T1 cells are a mouse mammary carcinoma cell line that spontaneously metastasize from an orthotopic site in syngeneic Balb/c mice (Fidler and Kripke, 1977; Tao et al., 2008). HCC1143, a human breast cancer cell line derived from an invasive ductal carcinoma and SUM159, a human breast cancer cell line derived from a primary tumor, are triple negative, lacking HER2 and estrogen and progesterone receptors (Figure 2-2 A and B) (Flanagan et al., 1999; Gazdar et al., 1998). We did not observe invasive populations in human breast cancer cell lines T47D, HCC1954, HCC1569, and HCC1428 however we cannot rule out the possibility that an invasive population was lost during expansion in tissue culture (Figure 2-2 A and B).

The invasive spheroids within the parental population produced projections that invaded into the surrounding model stroma, causing the lattice-like architecture of collagen to linearize and accumulate around the invasive projections, forming tracks (Figure 2-2 D). These results are consistent with collagen reorganization that observed around collectively invading cells in patient tumors and transgenic mice (Conklin et al., 2011; Provenzano et al., 2006). By contrast, a barrier of collagen was present around the perimeter of the noninvasive spheroids, with the cells unable to penetrate through the barrier of collagen surrounding the spheroid (Figure 2-2 D).

The ability of a cell to invade has been linked to the ability of a cell to move, but previous work in our lab has shown that movement can be induced in cells without causing invasion (Pearson and Hunter, 2007). To determine if this difference in invasive potential was linked to the ability of a cell to move, we used live-cell imaging which allows for the investigation of cell behavior in real time at a single cell resolution (Pearson and Hunter, 2007). Our live-imaging experiments were performed using the Perkin Elmer Ultraview ERS spinning disk confocal microscope, which acquired images at 10 frames per second on a multi-dimensional x-y-z plane stage in an environmentally controlled chamber. Confocal slices were acquired allowing for three-dimensional reconstruction of images and Imaris cell tracking software allowed for the quantification of multiple cell parameters including cell velocity and displacement. Using live-imaging we determined that SUM159 invasive and noninvasive populations within the parental cell line were motile (Figure 2-2 E). Cells extended in both single file and multiple cell width projections (Figure 2-2 E). Cells within the projections were capable of moving forward and backward, independent of the cells surrounding them, and through and around the main spheroid switching contacts with cell partners, which we term "intraspheroid movement" (Dang et al., 2011). Spheroid projections were dynamic, capable of extending forward to form long, filopodia-like structures and retracting short distances in the direction of the main spheroid (Figure 2-2 E). Treating cells with cytochalasin D, a commonly used reagent that disrupts actin polymerization, suppressed cell motility serving as a baseline control for lack of cell movement (Figure 2-2 E) (Bruijns and Bult, 2001). This movement of cells within spheroids is not exclusive to SUM159 cells, intraspheroid movement was also observed in noninvasive human breast

cancer cell lines HCC1806 and HCC1954 which are derived from a primary tumor and are non-invasive yet motile when plated in organotypic culture (Dang et al., 2011).

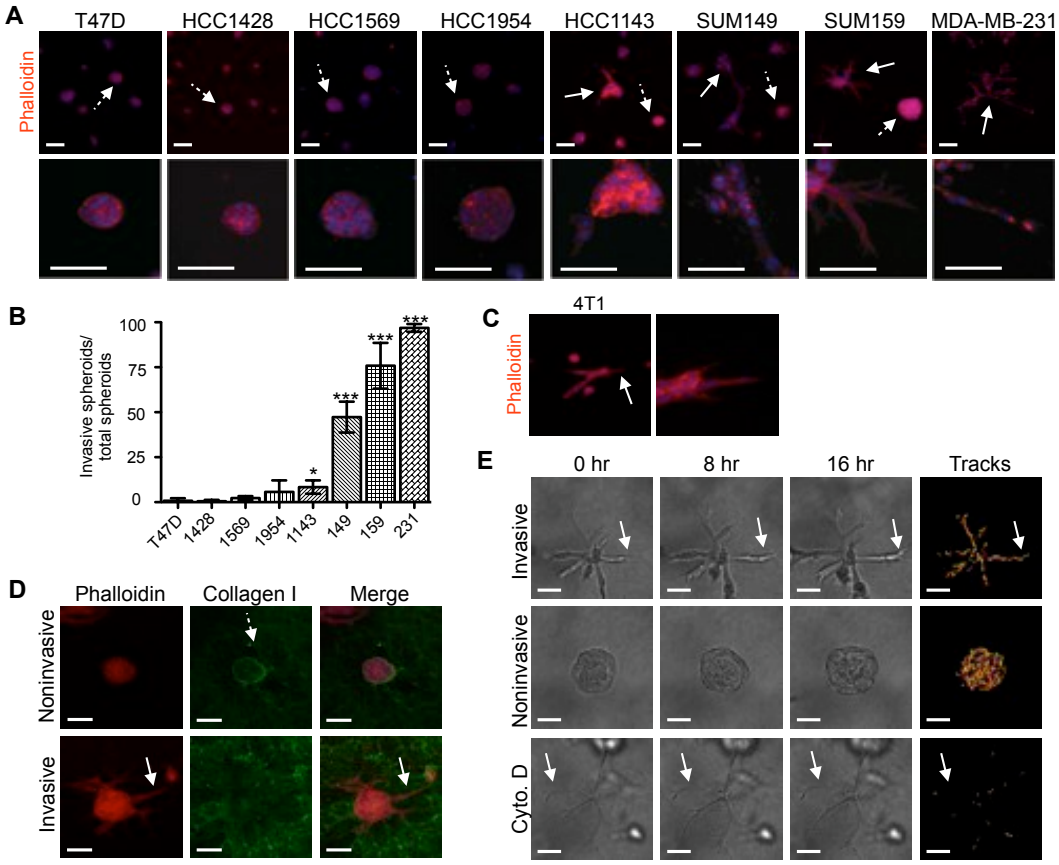



**Figure 2-2. Breast cancer cell lines contain subpopulations of invasive and noninvasive cells.** (**A**) Breast cancer cells grown in organotypic culture for five or more days were stained with phalloidin (red, F-actin) and Hoechst (blue, nuclei). Solid arrows identify invasive spheroids and dotted arrows identify noninvasive spheroids. Bar, 50 μm. (**B**) Quantification of invasion. Data are the mean +/- standard deviation (S.D.) of at least 50 spheroids analyzed in three independent experiments. HCC1143, p=0.0298; SUM149, p=0.0008; SUM159, p=0.0005; MDA-MB-231, p<0.0001 (Student's t-test, compared to T47D). (**C**) 4T1 mouse mammary carcinoma cells were grown in organotypic culture for three days and stained with phalloidin (red, F-actin) and Hoechst (blue, nuclei). (**D**) Confocal slices of day four SUM159 spheroids immunostained with collagen I (green) and counterstained with phalloidin (red, F-actin), and Hoechst (blue, nuclei). Bar, 50 μm. **(E)** Quantification of mean cell speed and displacement length. Data are the mean +/- S.D. of five spheroids analyzed per

condition in three independent experiments. Speed p=0.0037 and displacement p=0.0045 (Student's t-test).

**Invasive and noninvasive subpopulations are stable**

To determine if the invasive and noninvasive phenotypes displayed by the subpopulations within the 4T1, SUM159, and HCC1143 cell lines were heritable traits, we isolated daughter subpopulations of invasive (INV) and noninvasive (NON) cells. Previous work investigating invasive heterogeneity within tumor cell populations have not focused on determining the heritability of invasive phenotypes or have found that they are transient (Sanz-Moreno et al., 2008; Wang et al., 2004). To determine if the invasive and noninvasive phenotypes were transient or if the cells retained a heritable invasive or noninvasive phenotype, I separated the 4T1, SUM159, and HCC1143 invasive spheroids from the noninvasive spheroids in orgnotypic culture (Figure 2-3).

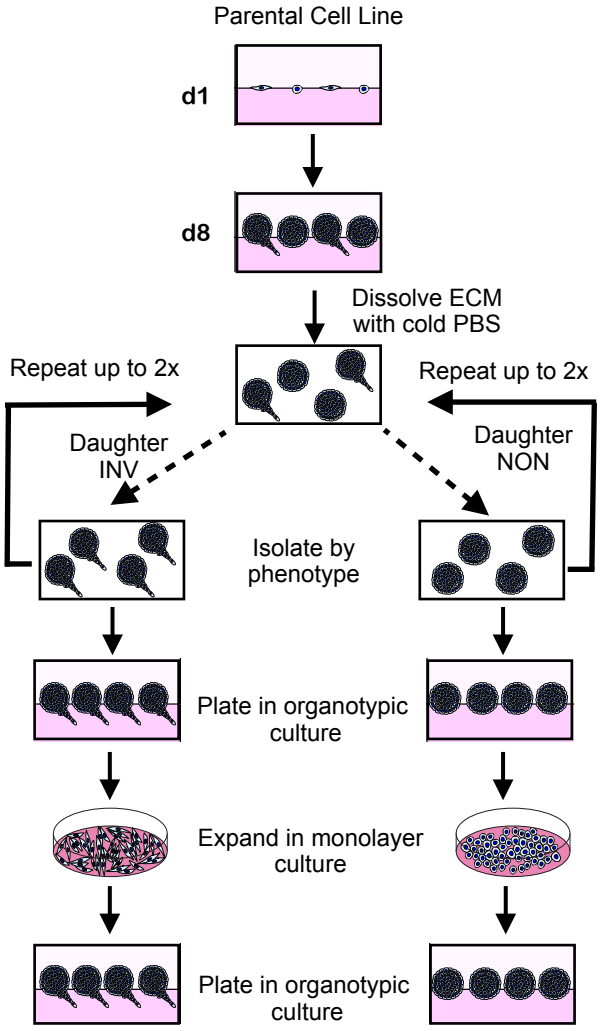



**Figure 2-3. Cell line separation.** Model figure depicting the methodology for generating the daughter invasive (INV) and noninvasive (NON) subpopulations. Parental cells were allowed to grow in organotypic culture then the basement membrane was dissolved using cold PBS. Invasive and noninvasive spheroids were then manually isolated, dissociated and re-plated in organotypic culture. This process was repeated one to two times then the isolated lines were plated in tissue culture and expanded. When the expanded cell lines were plated in organotypic culture, they retained their respective phenotype up to 30 population doublings. Also see Materials and methods.

Once separated, I established daughter INV and NON cell lines in monolayer culture and expanded the populations. When the populations were re-plated in organotypic culture, the INV and NON populations formed spheroids that recapitulated the isolated phenotype (Figure 2-4 A and B). The INV subpopulation was enriched in spheroids that were invasive and the NON population was enriched for spheroids that were noninvasive (Figure 2-4 A and B). We found that the INV and NON cell lines retained their respective phenotypes up to 30 population doublings in culture, suggesting that the INV and NON cell line phenotypes were heritable.

The heterogeneity in organotypic culture phenotype within breast cancer cell lines was not restricted to 4T1, SUM159 and HCC1143 cells. MDAMB157 (data not shown) and SUM149 human breast cancer cell lines also displayed the INV and NON populations when plated in organotypic culture (Figure 2-2 A and B). However, I was not able to separate the invasive and noninvasive populations from the MDAMB157 and SUM149 cell lines because the MDAMB157 cells could not withstand the separation process, and the SUM149 cells produced dispersed, invasive spheroids with cell-cell contacts that were not stable enough to isolate using our separation procedure.

**Both INV and NON cells are motile**

To determine why the INV cells could migrate away from the primary spheroid and invade into the surrounding matrix while the NON subpopulation could not, we performed live imaging on the cells plated in organotypic culture. It was unlikely but possible, that the separated NON cells were simply not capable of motility while the INV subpopulation was, allowing only the INV subpopulation to migrate and invade away from the primary spheroid. Live imaging analysis revealed that the NON population was

not only motile, but more motile than the INV subpopulation (Figure 2-4 C). Using Imaris tracking software, we tracked the nuclei of cells within each spheroid, demonstrating that the NON cells within spheroids displayed greater speed and displacement than the INV cells (Figure 2-4 C). While the NON cells were more motile than the INV cells, they could not invade into the surrounding model stroma, suggesting that motility is not the rate limiting step in invasion (Figure 2-4 C).

**INV cells remodel the microenvironment to promote invasion**

We immunostained the INV and NON cells plated in organotypic culture, showing the collagen fibers embedded within the basement membrane (Figure 2-4 D). CollagenI staining revealed that the NON spheroids did not disrupt the surrounding collagen, the extent of remodeling was limited to a collagen ring formed around the NON spheroid caused by collagen displacement as the spheroid grew over time (Figure 2-4 D). However, the INV spheroids disrupted the collagen pattern surrounding the spheroids, causing collagen linearization and forming tracks around the invasive projections (Figure 2-4 D). The tracks that are formed by the INV cells are consistent with collagen remodeling that is observed around collectively invading cells in patient tumors and transgenic mice (Conklin et al., 2011; Provenzano et al., 2006). INV and NON populations were capable of motility but the collagen fibers adjacent to the INV spheres were remodeled, possibly allowing them to invade. Second harmonic imaging of breast cancer patient samples has shown that invasive tumors with increased amounts of remodeling are associated with poor patient outcome (Conklin et al., 2011). This remodeling may occur by stromal cells such as fibroblasts, or an invasive tumor cell type such as the INV subpopulation.

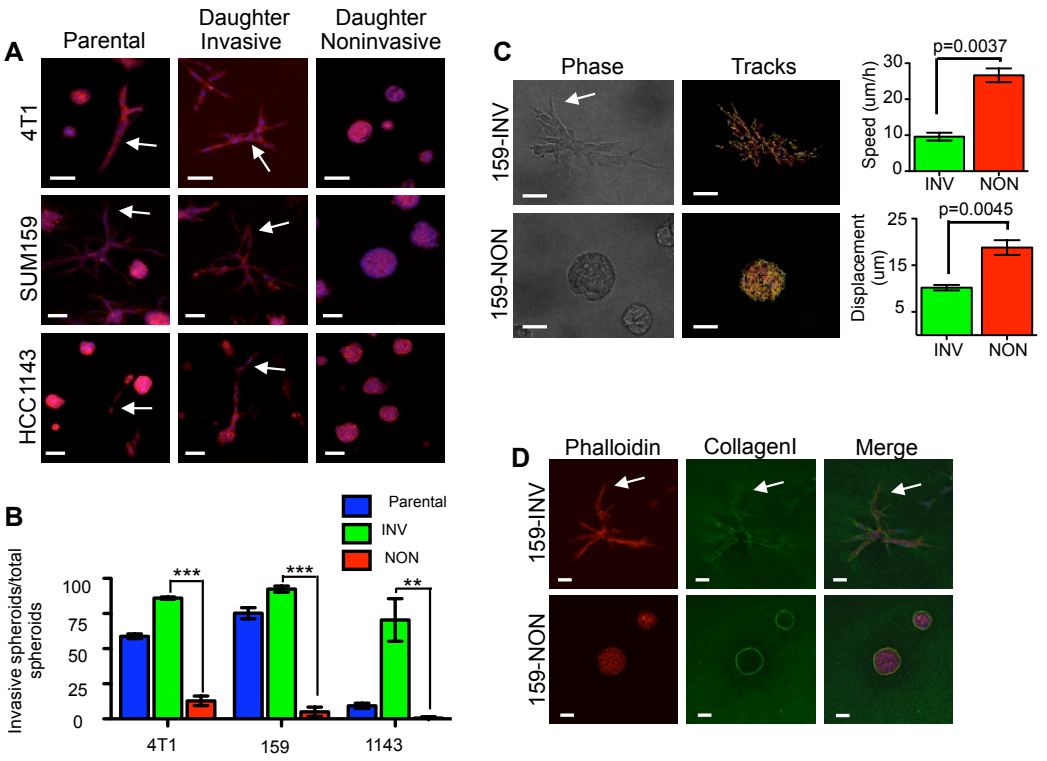


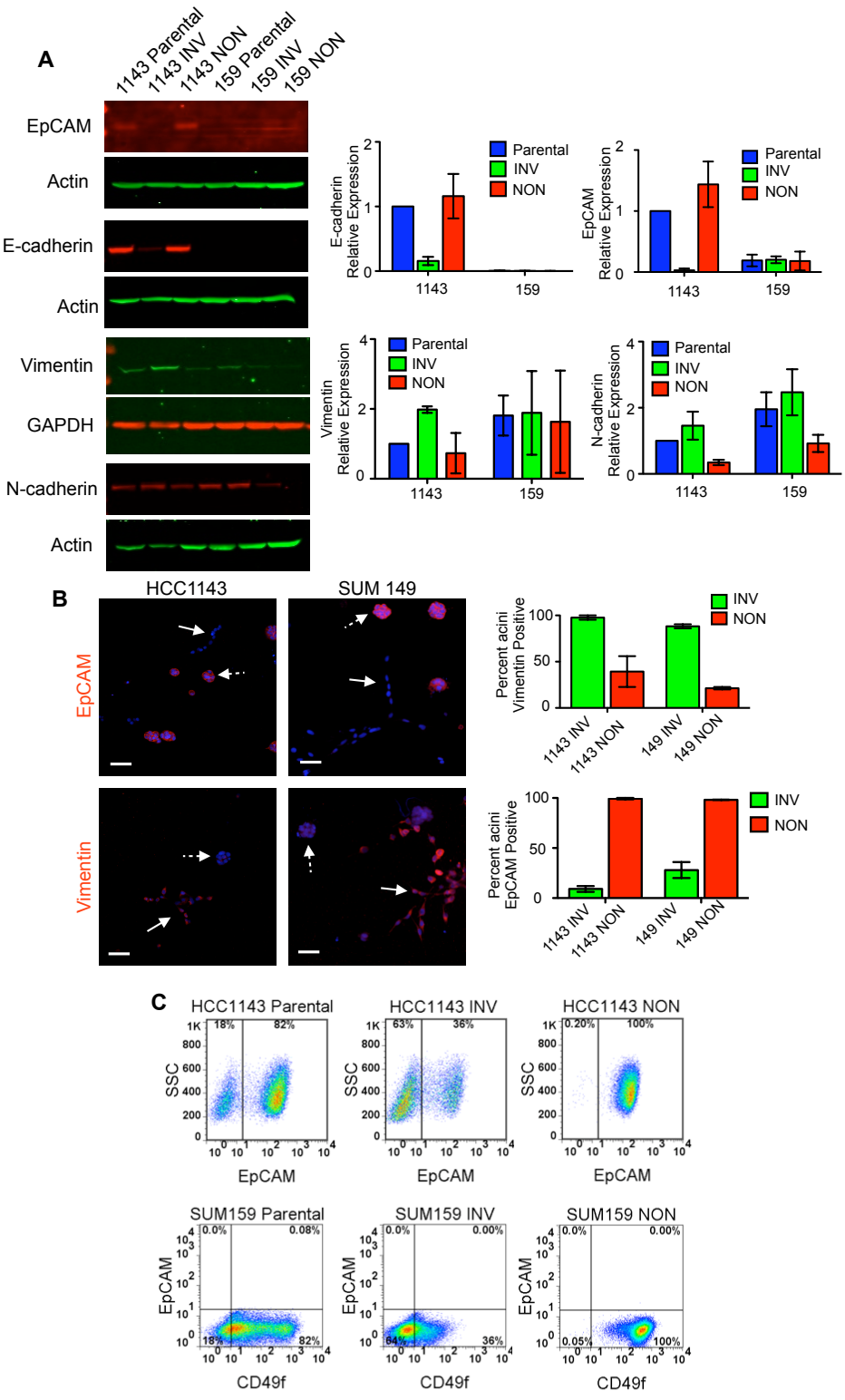

**Figure 2-4. INV and NON subpopulations are stable and motile.** (**A**) 4T1 (day three), SUM159 (day four), and HCC1143 (day six) parental and daughter INV and NON subpopulations grown in organotypic culture were fixed and stained with phalloidin (red, F-actin). Bar, 50 µm. (**B**) Quantification of invasion for the parental and daughter subpopulations. Data are the mean +/- S.D. of at least 50 spheroids analyzed in three independent experiments. 4T1-INV, p= 0.0005; SUM159-INV, p=0.0010; HCC1143-INV, p=0.0013 (Student's t-test). (**C**) Real time imaging and tracking of cell movement. Phase images of the SUM159-INV and SUM159-NON populations plated in organotypic culture. Right panels show tracks of cell movement over time. Scale bar, 50 µm. Right panels, quantification of mean cell speed and displacement length. Data are the mean +/- S.D. of 5 spheroids analyzed per condition in three independent experiments. Speed p= 0.0037 and displacement p= 0.0045 (Student's t-test). (**D**) Confocal slices of day four SUM159-INV and SUM159-NON subpopulations grown in organotypic culture immunostained with collagen I (green) and counterstained with phalloidin (red, F-actin), and Hoechst (blue, nuclei). Scale bar, 50 µm.

**INV cells are more mesenchymal than NON cells**

Neoplastic cells that have undergone EMT have been shown to display enhanced invasive and migratory properties, suggesting that the INV population was more mesenchymal than the NON population (Thiery, 2002). The HCC1143 INV cell line displayed higher levels of mesenchymal markers Vimentin and N-cadherin which has been shown to promote invasion, than the NON line, suggesting that the HCC1143 INV cells were more mesenchymal than the HCC1143 NON cells (Figure 2-5 A) (Kalluri and Weinberg, 2009; Tanaka et al., 2010). The presence of the epithelial cell adhesion molecule, E-cadherin, is indicative of an epithelial cell type (Gumbiner, 2005; Kalluri and Weinberg, 2009). Similarly, the epithelial cell adhesion molecule EpCAM has been used to isolate epithelial (EpCAM-high) populations from more mesenchymal (EpCAM-low) populations (Prat et al., 2010). The HCC1143 NON line displayed higher levels of epithelial markers E-cadherin and EpCAM, suggesting that the NON subpopulation was more epithelial than the INV subpopulation (Figure 2-5 A).

Similarly, SUM159 INV cells displayed higher levels of mesenchymal markers N-cadherin and Vimentin than the NON cells, suggesting the SUM159 INV cells were more mesenchymal than the NON cells (Figure 2-5 A). However, both populations lacked epithelial marker expression of E-cadherin and EpCAM, which suggests that SUM159 NON cells may have undergone an incomplete EMT, suggesting that EMT is necessary but not sufficient for invasion (Figure 2-5 A). Consistent with these data, the NON spheroids within the HCC1143 and SUM149 parental cells plated in organotypic culture expressed EpCAM and did not express Vimentin while the INV spheroids within the parental populations expressed Vimentin and not EpCAM (Figure 2-5 B).

Additionally, fluorescent activated cell sorting (FACS) of HCC1143 parental, INV, and NON populations showed that the NON population was enriched for EpCAM-high cells, while the INV population was enriched for EpCAM-low cells, suggesting that the HCC1143 INV and NON subpopulations were epigenetically distinct (Figure 2-5 C). Both SUM159 INV and NON populations were EpCAM-low but the differential expression of CD49f (α6 integrin) supported the conclusion that SUM159 INV and NON cells were also epigenetically distinct (Figure 2-5 C)(Prat et al., 2010). These data support the conclusion that the loss of epithelial characteristics are necessary but not sufficient for invasive cell behavior.


A
1143 Parental
1143 INV
1143 NON
159 Parental
159 INV
159 NON
EpCAM
Actin
E-cadherin
Actin
Vimentin
GAPDH
N-cadherin
Actin
E-cadherin Relative Expression
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Vimentin Relative Expression
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1143
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B
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SUM 149
EpCAM
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1143 INV
1143 NON
149 INV
149 NON
C
HCC1143 Parental
HCC1143 INV
HCC1143 NON
SUM159 Parental
SUM159 INV
SUM159 NON
SSC
EpCAM
CD49f

**Figure 2-5. INV and NON subpopulations are epigenetically distinct.** (**A)** HCC1143 and SUM159 parental, INV, and NON lysates were immunoblotted with anti-EpCAM, anti-E-cadherin, anti-N-cadherin, and anti-Vimentin antibodies. Immunoblotting of Actin and GAPDH from the same lanes is shown as a loading control. Quantification of EpCAM, E-cadherin, Vimentin, and N-cadherin expression (right panel). Data are the mean +/- range of two independent experiments (EpCAM, E-cadherin, and Vimentin) and mean +/- S.E.M for N-cadherin. Each set of values was first normalized to the loading control, shown are values normalized to 1143 parental. (**B)** Day six HCC1143 plated in organotypic culture. Immunofluorescence was performed using antibodies towards EpCAM (red) or Vimentin (red) and counterstained with hoechst (blue). Solid arrows identify INV spheroids and dotted arrows identify NON spheroids. Scale bar, 50 μm. (**C**) Fluorescent activated cell sorting (FACS) analysis of SUM 159-parental, -INV, and -NON cells grown in monolayer culture and stained with anti-CD24 and CD44 antibodies. Results are representative of four independent experiments. FACS analysis of HCC1143- parental, -INV, and -NON cells grown in monolayer culture and stained with an anti-EpCAM antibody. Results are representative of two independent experiments.

**INV and NON populations are isogenic**

The INV and NON populations were derived from individual cell lines, suggesting that they were isogenic, but it was possible that contaminating cell lines might explain the presence of invasive and noninvasive subpopulations. To determine if the INV and NON populations were in fact isogenic and derived from the same patient tumor cell line, we fingerprinted the SUM159 and HCC1143 parental, INV, and NON cell lines (Figure 2-6). Powerplex analysis of the parental, INV, and NON populations supported the conclusion that the parental and daughter populations were isogenic and thus were derived from the same patient (Figure 2-6).


**Figure 2-6. INV and NON subpopulations are isogenic.** Powerplex fingerprinting of the HCC1143 and SUM159 parental, INV, and NON cells. Small variations that are seen between the SUM159 INV and NON lines are considered insignificant and can be caused for example by the loss of a chromosome during the process of culturing cells or small detection differences in the Powerplex system. Powerplex analysis performed by Luc Girard.

# DISCUSSION

Here, we show that multiple breast cancer cell lines contain subpopulations of cells that display invasive and noninvasive phenotypes. Furthermore, these subpopulations can be isolated and stably maintained in culture for multiple passages, providing a unique opportunity to study heterogeneity within breast cancer cell lines. In the past, heterogeneity has been investigated using isolation methods based on cell surface markers such as CD24 and CD44 using FACS or immunomagnetic bead separation (Al-Hajj et al., 2003; Biddle et al., 2011). To our knowledge, this is the first study to separate cell lines based on an invasive phenotype in organotypic culture. Previously, the isolation of clones has also been used to study heterogeneity, but these studies are limited to a population of cells that may contain highly specific and divergent phenotypes. Our study addresses this problem because the INV and NON populations were derived from multiple spheroids, thus a large population sampling was used to produce the daughter cell lines. In the subsequent chapters, the INV and NON cell lines will be used to understand how tumor cells interact and invade.

We used Powerplex analysis to determine that the INV and NON populations are not cross-contaminating cell lines, suggesting that the separated populations are isogenic (Gazdar et al., 2010). However, the Powerplex kit amplifies polymorphic short tandem repeats of specific loci in the genome, thus the resolution is not specific enough to perform individual base pair analysis of DNA within a cell line (Masters et al., 2001). Therefore, it is possible that there may be individual point mutations between the INV and NON populations that confer a genetic distinction between the two populations. To determine if the INV and NON populations are truly isogenic, DNA sequencing of the

INV and NON cell lines can be performed, but a drawback of this technique is that at this time, DNA sequencing is expensive. Isogenic invasive and noninvasive cancer cell populations can be utilized to study multiple aspects of invasion.

# CHAPTER THREE
# Cooperation Between Tumor Subpopulations

## INTRODUCTION

### Overview

I have shown that stable subpopulations of invasive (INV) and noninvasive (NON) cells exist within breast cancer cell lines. The INV subpopulation displays mesenchymal characteristics and are capable of autonomous invasion when plated in organotypic culture. The NON subpopulation is less mesenchymal than the INV subpopulation and is not capable of autonomous invasion. However, because the two populations were derived from the same breast cancer cell line, it is likely that the INV and NON populations would exist within the same tumor. Tumors are heterogeneous (Heppner, 1984), therefore it is possible that the INV and NON subpopulations might interact during invasion if clustered together (Figure 3-1).

### Tumor Heterogeneity

Heterogeneity within tumors is observed in a wide range of malignancies, including breast cancer (Al-Hajj et al., 2003; Hanahan and Weinberg, 2000). Heterogeneous tumors can contain populations of cells with differential morphologies, motility, proliferation, and expression of cell surface markers (Krugmann et al., 2001; Ponti et al., 2005). Evidence of intratumor heterogeneity is observed in human tumors by staining or sorting for molecular markers such as EpCAM, CD44 and CD24 and expression profiling of tumor cells and subpopulations (Fillmore and Kuperwasser, 2007; Navin et al., 2011; Park et al., 2010; Shipitsin et al., 2007).

The heterogeneity that develops within tumors can be due to epigenetic variation between tumor cells, or the presence of genetically distinct, independent clones within a tumor (Hanahan and Weinberg, 2000). Such heterogeneity within a tumor can have a large impact on patient survival, thus it is important to study heterogeneous populations within tumors to better understand cancer treatment and progression. Because tumors are heterogeneous, it is possible that subpopulations within a tumor may interact and potentially cooperate during invasion.

**Cooperation between cells**

How heterogeneous subpopulations within a tumor interact during invasion has not been well studied but cooperation between cells has been observed in tumorigenesis (Bidard et al., 2008). Cooperation between tumor cells and the microenvironment has been documented previously; tumor cells as well as fibroblasts have been shown to secrete growth factors that promote both tumor cell proliferation and angiogenesis (Barbera-Guillem et al., 2002; Coppe et al., 2006; Krtolica et al., 2001; Tyan et al., 2011). Fibroblasts also physically remodel the tumor microenvironment to promote invasion of autonomously noninvasive breast cancer cells (Dang et al., 2011; Gaggioli et al., 2007). While cooperation has been observed in these systems, a limited number of studies have investigated the cooperative role of tumor subpopulations in invasion. In this chapter, we will investigate the interaction between INV and NON breast cancer subpopulations to determine if cooperation occurs during collective invasion.

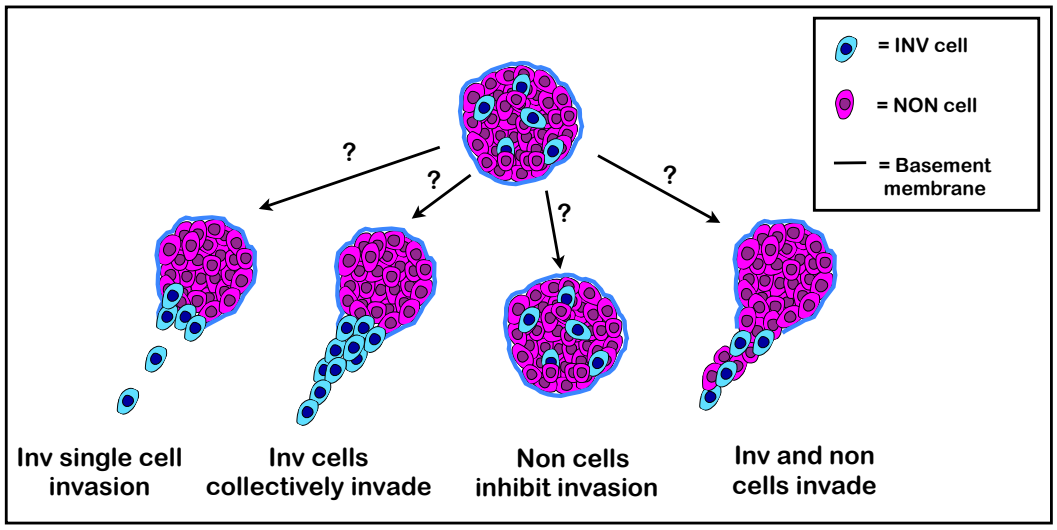



**Figure 3-1. Breast cancer cell lines may contain populations with differential invasive potential.** To determine how invasive and noninvasive populations might interact within a heterogeneous tumor, we clustered the INV and NON populations. There were four possibilities that could have resulted from the clustering of INV and NON cells. INV cells might be the only cells that could invade away from the primary mass; this could happen as single or collective cell invasion. It is also formally possible that the NON cells might prevent the invasion of the INV population. An interesting possibility is that the INV and NON cells might cooperate, with the INV cells allowing the autonomously NON cells to cooperate. We hypothesized that the INV subpopulation would allow the NON subpopulation to invade.

## MATERIALS AND METHODS

### Organotypic culture

Single cells were plated in 8-well chamberslides (immunofluorescence staining, Falcon; live-cell imaging, Nunc) onto a base layer of Matrigel (5 mg/ml) and collagen I (1.5 mg/ml) and supplemented with a 2% Matrigel/growth medium mixture as described (Dang et al., 2011; Xian et al., 2005). All cultures were grown for 6-8 days except where indicated in the Figure Legends. For spheroid cluster experiments a 30 µl drop of a 500,000 cell/ml growth medium suspension was placed on a tissue culture dish lid and

inverted onto the bottom dish containing sterile phosphate-buffered saline (PBS) to prevent sample desiccation. Drops were then incubated at 37˚C for 18-24 h and pipetted into a microfuge tube and pelleted. Spheroid clusters were resuspended in either 400 µl of growth media/2% Matrigel for "on top" cultures or 50 µl of Matrigel/collagen mix for embedded cultures and plated on 30 µl of a basement layer of Matrigel/collagen.

**Quantification of invasion**

A spheroid was classified as invasive if three or more cells invaded away from a primary spheroid mass of ten or more cells. The number of spheroids counted per condition and replicates are indicated in the figure legends.

**Transfection**

Cells were transfected with 50-100nM of small interfering ribonucleic acid (siRNA) using RNAiMax transfection reagent for 24-48 h. OnTargetplus siRNAs were used except where indicated when siGenome pools (Dharmacon) were used. Cells were then either harvested for lysates or plated in organotypic culture for 24-48 h before fixing, see figure legends. Target sequences for siRNA knockdown sense strands were:

Non-Targeting OnTargetplus pool (UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA).

DOCK10 OnTargetplus pool (DOCK10-05: GGACCUGACUAAGCGUAUA, DOCK10-06: CAAUAUAGCUACGGAGGUU, DOCK10-07: CAACAUUCGCUUGCAAUUA, DOCK10-08: CCAGACAGCUAUCAAACAU).

Cdc42 OnTargetplus pool (Cdc42-05: CGGAAUAUGUACCGACUGU, Cdc42-06: GCAGUCACAGUUAUGAUUG, Cdc42-07: GAUGACCCCUCUACUAUUG, Cdc42-

08: CUGCAGGGCAAGAGGAUUA).

N-WASP OnTargetplus pool (N-WASP-07: CAGCAGAUCGGAACUGUAU, N-WASP-08: UAGAGAGGGUGCUCAGCUA, N-WASP-09: GGUGUUGCUUGUCUUGUUA, N-WASP-10: CCAGAAAUCACAACAAAUA).

GAPDH siGenome SMARTpool (CAACGGAUUUGGUCGUAUU, GACCUCAACUACAUGGUU, UGGUUUACAUGUUCCAAUA, GUCAACGGAUUUGGUCGUA).

CDC42 siGenome SMARTpool (GGAGAACCAUAUACUCUUG, GAUUACGACCGCUGAGUUA, GAUGACCCCUCUACUAUUG, CGGAAUAUGUACCGACUGU).

Rac1 SiGenome SMARTpool (UAAGGAGAUUGGUGCUGUA, UAAAGACACGAUCGAGAAA, CGGCACCACUGUCCCAACA, AUGAAAGUGUCACGGGUAA).

**Flow cytometry and antibodies**

Antibodies to the following human antigens were used for flow cytometry analyses: CD44-FITC, CD24-AlexaFluor647 (BD Biosciences), and EpCAM (VU1D9)-FITC (Stem Cell Technologies). Analyses were conducted following standard flow cytometry procedures. For each sample, 5 x $10^5$ cells were washed and resuspended in PBS supplemented with 2% FBS. Cells were mixed with antibody diluted in 2% FBS PBS and incubated for 20 min in the dark at 4 °C. Samples were then washed and resuspended in propidium iodide diluted in 2% FBS/PBS. At least 50,000 events were collected on an LSRII flow cytometer (Becton Dickinson), and analyzed using Flow Jo software (Tree Star Technologies).

**Single cell assay**

Cells were transfected for 24-48 h in 96 well plates and then plated in organotypic culture and harvested 24 h later.

**Immunoblot analysis and immunofluorescence staining**

Cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Calbiochem) as described (Pearson and Hunter, 2007). Equal amounts of protein were separated by SDS-PAGE, transfered to Immobilon-FL polyvinylidene fluoride (PVDF) transfer membrane (Millipore), and immunostained. Immunoblots were visualized using an Odyssey infrared scanner (LI-COR). Organotypic cultures were fixed and immunostained as described (Pearson and Hunter, 2007). Images were acquired on Nikon and Zeiss LSM510 confocal microscopes in TIFF format. Images were arranged using Adobe Photoshop CS3 and Keynote, and are representative of three independent experiments.

**Real-time imaging of organotypic cultures**

Imaging was performed using a Perkin Elmer Ultraview ERS spinning disk confocal microscope enclosed in a 37˚C chamber supplemented with humidified $CO_2$ (Solent) and a CCD camera (Orca AG; Hamamatsu). Images were acquired with a 20x (Zeiss) objective using Volocity software (Perkin Elmer) and analyzed with Imaris software (Bitplane). At least 6-10 different x,y coordinates with 6-10 or more z -slices over 60-100 μm span for each condition were imaged in parallel.

# RESULTS

## INV cells can induce the invasion of NON cells

It is likely that the INV and NON populations would likely co-exist within the same tumor, thus we hypothesized that the INV population would cooperate with the NON population to allow the autonomously noninvasive population to invade, which would be a new concept for tumor cell invasion. To reconstitute heterogeneity within our organotypic culture system, we had to devise a method to mix the SUM159 INV and NON cells to form mosaic clusters. Traditionally, single cells are plated in organotypic culture, which grow into clonal multicellular spheroids. To generate multicellular spheroids, we plated a single cell suspension in hanging drops overnight, which allowed them to aggregate into clusters of cells, which we then plated in organotypic culture (see Methods). Each cluster was composed of approximately 50-100 cells and was noninvasive at the time of plating in organotypic culture (Figure 3-2 A). Over a period of five days, clusters that contained INV cells became invasive, displaying projections that invaded away from the primary spheroid, while NON clusters did not invade (Figure 3-2 B). Interestingly, NON cells were induced to invade by INV cells within heterogeneous clusters composed of 25% INV cells and 75% NON cells (Figure 3-2 A and B).

As mentioned previously, the 4T1 cell line contained INV and NON populations as well that could be stably separated and maintained in cell culture (Figure 2-4 A). Similar to the SUM159 cells, 4T1 INV and NON populations were clustered and plated in organotypic culture. Over time, the clusters composed entirely of INV cells invaded into the surrounding matrix while the clusters composed of NON cells remained noninvasive (Figure 3-2 C). Heterogeneous clusters composed of 20% 4T1-INV and

80% 4T1-NON cells displayed invasive projections, the majority of which contained both INV and NON cells (Figure 3-2 C). The invasive projections always contained at least one INV cell, which was positioned at the leading edge of a majority of the projections and the cells collectively invaded, remaining in direct physical contact during invasion (Figure 3-2 B and C). These data suggest that one subpopulation of tumor cells can induce the invasion of another.

**Secreted factors are not sufficient to induce invasion of NON cells**

Cooperation between INV and NON cells is reminiscent of cooperation that takes place between tumor cells and components of the microenvironment, such as fibroblasts. It has been shown that tumor cells can be induced to invade by fibroblasts (Dang et al., 2011; Gaggioli et al., 2007). Typically, this cooperation between tumor cells and the microenvironment is driven by paracrine factors secreted by either the tumor cells or the microenvironment (Goswami et al., 2005; Iwazawa et al., 1996). To determine if secreted factors played a role in the INV and NON cell cooperative invasion, we co-cultured SUM159 INV and NON spheroids plated as single cells, in organotypic culture. When co-cultured, the SUM159 INV and NON cells maintained their respective phenotypes, suggesting that secreted factors are not sufficient to induce invasion of the NON population (Figure 3-2 D). These results are consistent with our prior observation that the SUM159 parental cell line maintained distinct INV and NON spheroids when plated in organotypic culture and suggest that paracrine signals are not sufficient to induce invasion.

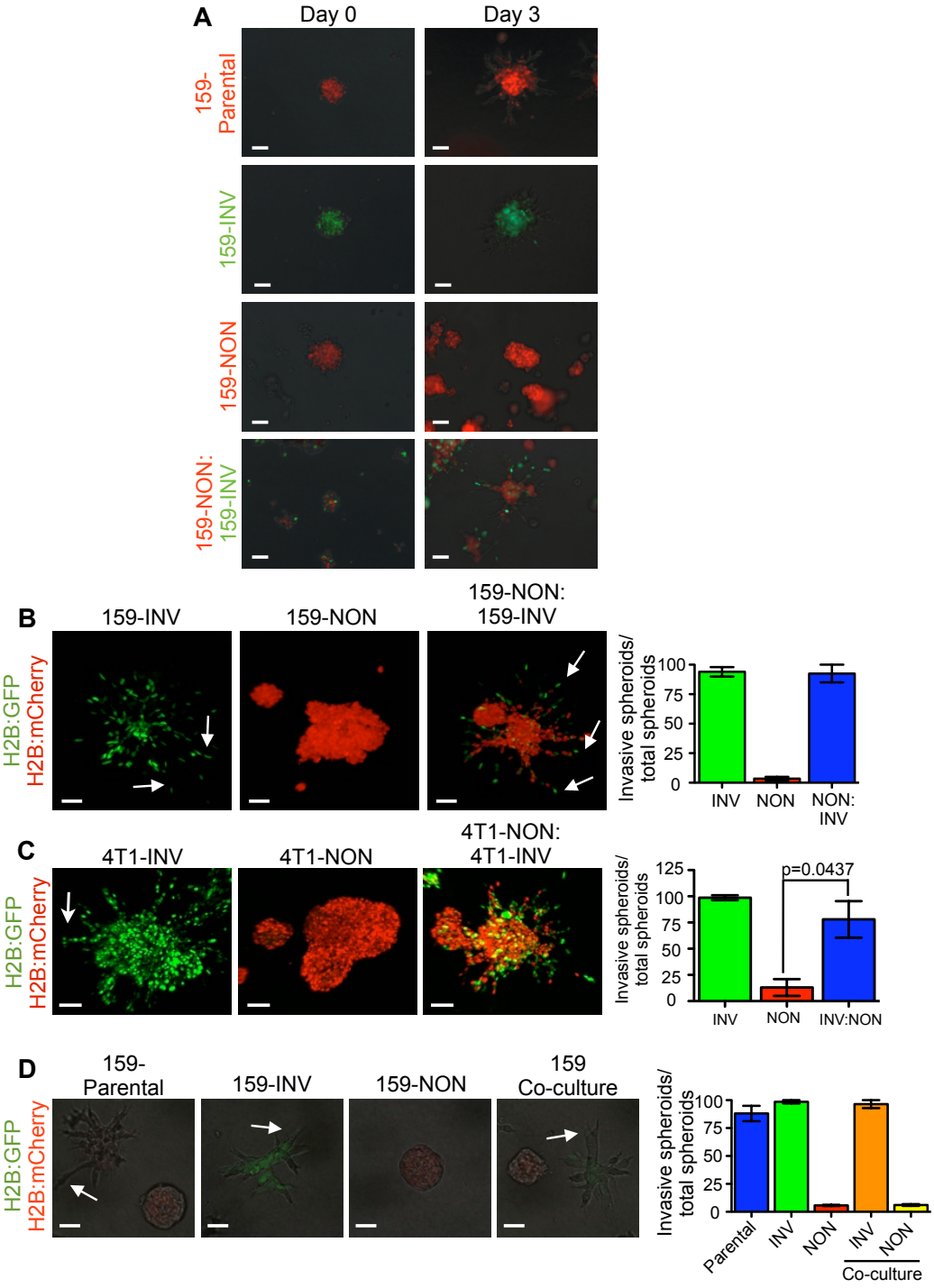

A
Day 0
Day 3
159-Parental
159-INV
159-NON
159-NON: 159-INV
B
159-INV
159-NON
159-NON: 159-INV
H2B:GFP
H2B:mCherry
Invasive spheroids/ total spheroids
100
75
50
25
0
INV
NON
NON: INV
C
4T1-INV
4T1-NON
4T1-NON: 4T1-INV
H2B:GFP
H2B:mCherry
Invasive spheroids/ total spheroids
125
100
75
50
25
0
p=0.0437
INV
NON
INV:NON
D
159-Parental
159-INV
159-NON
159 Co-culture
H2B:GFP
H2B:mCherry
Invasive spheroids/ total spheroids
100
75
50
25
0
Parental
INV
NON
INV
NON
Co-culture

**Figure 3-2. INV cells induce the migration of NON cells into the stroma.** (**A**) Representative images show that all cell clusters start as noninvasive spheroids that invade over time when the INV subpopulation is present. Phase contrast and fluorescent overlay images of homogenous SUM159-parental (H2B:mCherry, red, nuclei), -INV (H2B:GFP, green, nuclei), -NON (H2B:mCherry, red, nuclei), and heterogeneous (25% SUM159-INV, 75% SUM159-NON) spheroid clusters 8 h after plating (day 0) and on day three are shown. Scale bar, 100 μm. (**B**) The indicated SUM159 subpopulations were clustered together in hanging drops to generate heterogeneous spheroids before plating in organotypic culture. Homogenous and heterogeneous spheroids (75% NON, 25% INV) five days after plating in organotypic culture are shown. Solid arrows identify invasive projections. Quantification of invasion is shown in the bar graph on the right (See Methods for details). The results are the mean +/- range of 30 spheroids analyzed per condition in two independent experiments. Scale bar, 50 μm. (**C**) The indicated 4T1 subpopulations were clustered together in hanging drops to generate heterogeneous spheroids before plating in organotypic culture. Shown are homogenous and heterogeneous spheroids (80% NON-red, 20% INV-green) eight days after plating in organotypic culture are shown. Solid arrows identify invasive projections. Scale bar, 50 μm. Quantification of invasion of homogenous and heterogenous 4T1 spheroid clusters is shown in the right panel. Generation, plating and analysis of the homogenous and heterogenous 4T1 subpopulation clusters was performed as described in Figure 3-2 A. The invasive projections in the heterogenous spheroids had to contain at least one SUM159-NON cell to be considered invasive. Data are the mean +/- S.E.M of three independent experiments. Statistical significance was determined by Student's t-test. (**D**) Day four culture of SUM159-parental, SUM159-INV and SUM159-NON cells cultured alone or as 50:50 mix (not clustered together) of SUM159-INV and SUM159-NON cells. Quantification of invasion is shown in bar graph on the right. Data are the mean +/- range of 50 spheroids analyzed per condition in two independent experiments. Solid arrows identify invasive projections. Scale bar, 50 μm.

**Motile NON cells actively migrate into projections behind INV cells**

To determine how invasive subpopulations might influence the growth and progression of heterogeneous primary tumors, we performed live-imaging on clusters plated in organotypic culture. Homogeneous NON cell spheroids displayed intraspheroid movement within the clusters, yet they failed to form actin rich projections that protruded into the surrounding matrix (Figure 3-3 A). However, 25% INV cells within the mosaic cluster was sufficient to induce the invasion of NON cells (Figure 3-3 A). The NON cells followed directly behind the INV cells into the invasive projections as if following a

path created by the INV cells (Figure 3-3 A), consistent with the tracks formed in the ECM by the INV cells (Figure 2-4 D). Similarly, live imaging analysis of the 4T1 heterogeneous clusters revealed that the 4T1 INV population actively led the invasive projection as the 4T1 NON cells actively followed behind the path created by the INV cell (Figure 3-3 B). Both INV and NON cells were capable of moving away from the spheroid and returning along the projections toward the spheroid, occasionally exchanging positions at the leading edge of the projection. However, only the INV cells were capable of advancing the leading edge of the invasive projection.

Cooperation between tumor subpopulations during invasion has not previously been shown. Molecular and genetic heterogeneity is observed early in tumor progression, so it is possible that this cooperation could occur during the transition from DCIS to invasive breast cancer as the invasive population would lead the noninvasive population out of the duct. Traditionally it was thought that tumor cell invasion occurred autonomously, but we suggest an alternative model of invasion in which one tumor cell can cooperate to induce the invasion of another.

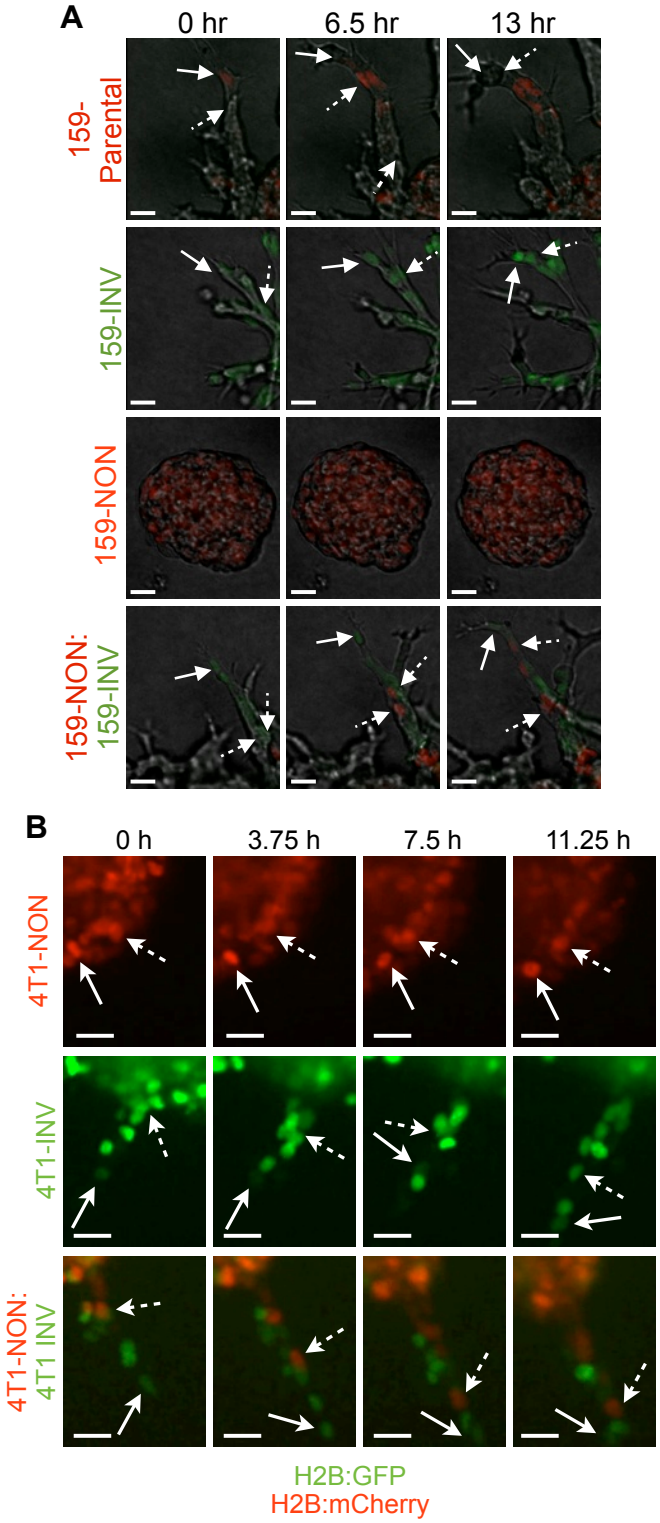

A
0 hr
6.5 hr
13 hr
159-Parental
159-INV
159-NON
159-NON: 159-INV
B
0 h
3.75 h
7.5 h
11.25 h
4T1-NON
4T1-INV
4T1-NON: 4T1 INV
H2B:GFP
H2B:mCherry

**Figure 3-3. NON cells actively migrate behind the INV cells.** (**A**) Real time imaging of SUM150-parental, -INV, -NON and heterogeneous (75% NON, 25% INV) spheroid clusters. Images were acquired at 30 min intervals starting 48 h after embedding in organotypic culture. Shown are representative images over 13 h. Solid arrows indicate areas of invasion and dotted arrows indicate cells following into invasive areas. Scale bar, 25 μm. (**B**) Real-time imaging of day six 4T1-INV (H2B:GFP, green, nuclei), 4T1-NON (H2B:mCherry, red, nuclei), and heterogeneous (80% 4T1-NON, 20% 4T1-INV) spheroid clusters. Images were acquired at 15 min intervals over a period of 11.25 h total. Solid and dotted arrows track individual cell movement over time. Scale bar, 25 μm.

### 4T1 INV cells can promote invasion of non-tumorigenic cells

We have shown that one subpopulation of invasive tumor cells can induce the invasion of an autonomously noninvasive tumor cell subpopulation. To determine if this cooperative invasion was restricted to subpopulations derived from the same tumor, we created mosaics using a mouse mammary carcinoma cell line, HC-11, and the invasive population of the 4T1 cell line. When clustered, the 4T1 INV cells induced the invasion of autonomously noninvasive and non-tumorigenic HC-11 cells, suggesting that the requirement to be a follower cell is not limited to isogenic tumor cell types and that potentially any motile cell may be induced to invade (Figure 3-4). These data suggest that tumors may contain subpopulations of invasive cells that can endow invasive capabilities upon potentially any noninvasive cell type.

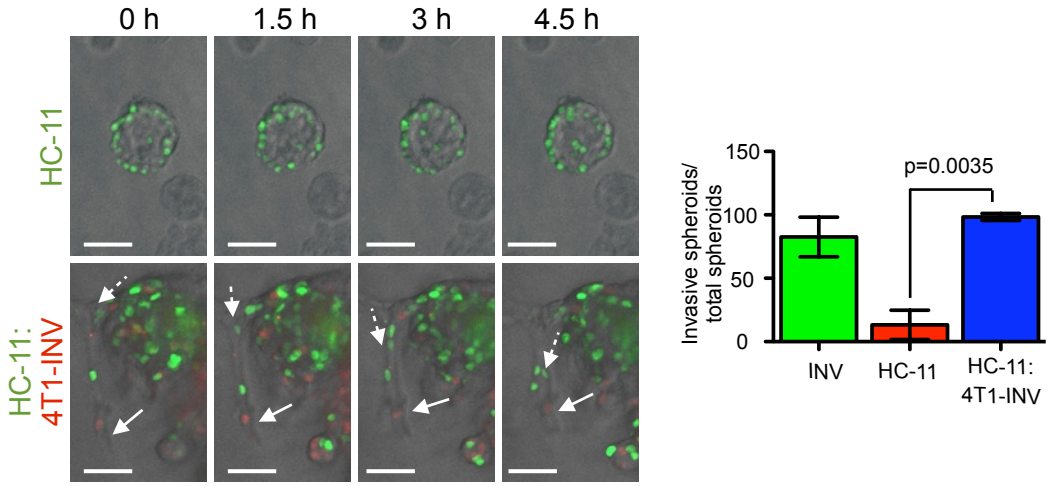



**Figure 3-4. INV cells induce the migration of NON as well as non-tumorogic cells into the stroma.** Real time imaging of day six 4T1-INV, HC-11 and heterogeneous 4T1-INV:HC-11 (90% HC-11-green, 10% 4T1-red) spheroids in organotypic culture. Spheroids were imaged at 15 min intervals over a span of 4.5h. Solid arrows indicate areas of invasion and dotted arrows indicate cells migrating into invasive projections. Bar, 50 μm. Quantification of invasion is shown in the right panel. Spheroids composed of at least 30 cells and heterogeneous spheroid clusters that contained 10% or more 4T1 cells were quantified. The mean +/- S.D. of ten spheroids analyzed per condition in three independent experiments is shown, p=0.0035 (Student's t-test).

**Members of the Cdc42 pathway are necessary for INV cell extension**

To identify and potentially target invasive and noninvasive populations in patients, we sought to molecularly characterize the INV and NON populations. Invasion usually requires the formation of invasive projections such as filopodia, which are regulated by small Rho GTPases such as Cdc42 through the activation of N-WASP and ARP2/3 (Jaffe and Hall, 2005). Filopodia are actin-based projections, which contribute to cell motility and invasion. Filopodia are thin, plasma membrane projections and are often found at the leading edge of broad, sheet-like projections called lamellipodia (Mattila and Lappalainen, 2008). Filopodia are involved in a number of processes including probing the environment around the cell for sites at which to form attachments

(Vasioukhin et al., 2000). Generally, invasive mesenchymal cells such as fibrobalsts and invasive cancer cells display filopodial projections, thus highlighting the role of filopodia during invasion (Machesky, 2008). Filopodia dynamics are regulated by the small Rho GTPase, Cdc42 which activates N-WASP, thus promoting Arp2/3 mediated actin nucleation and branching (Cerione, 2004; Nobes and Hall, 1995). However, direct regulators of invasion via the Cdc42 pathway have not been identified.

The small Rho GTPase Cdc42 is associated with promoting an invasive phenotype through the regulation of filopodial projections (Cerione, 2004; Hall, 1998; Krugmann et al., 2001; Nobes and Hall, 1995; Stengel and Zheng, 2011). The SUM159, HCC1143, and 4T1 INV populations all display thin, filopodial projections at the leading edge of the invasive projections, thus we hypothesized that Cdc42 would be necessary for INV cell invasion. To determine if Cdc42 was necessary for invasion we used transient siRNA mediated knockdown to decrease Cdc42 expression in the SUM159 INV population. To rapidly determine which genes were required for invasion, we developed a single cell assay in which we transfected cells in monolayer then plated them in orgnaotypic culture and assessed their single cell phenotype after 24 hours. INV cells plated in organotypic culture for 24 hours display an elongated phenotype with filopodial projections extending from the cell, while NON cells exhibit a short phenotype lacking projections (Figure 3-5 A). When SUM159 INV cells were transfected with siRNAs targeting Cdc42 and N-WASP, a member of the Cdc42 pathway that regulates actin polymerization (Padrick and Rosen, 2010; Rohatgi et al., 2000), cell length was reduced (Figure 3-5 B). Interestingly, knockdown of Rac1, a small GTPase that regulates lamellipodial dynamics, expression did not reduce cell elongation, suggesting that not all

small Rho GTPases that regulate membrane projections are necessary for invasion (Figure 3-5 C) (Nobes and Hall, 1995; Wu et al., 2009). These data are consistent with the known function of Cdc42 as a regulator of filopodia (Nobes and Hall, 1995). Cdc42 activates multiple downstream effector molecules but the requirement of N-WASP expression for elongation suggests that Cdc42 is regulating invasion specifically through N-WASP activation and actin polymerization in the 159 INV cells (Ahmed et al., 2010; Stengel and Zheng, 2011).

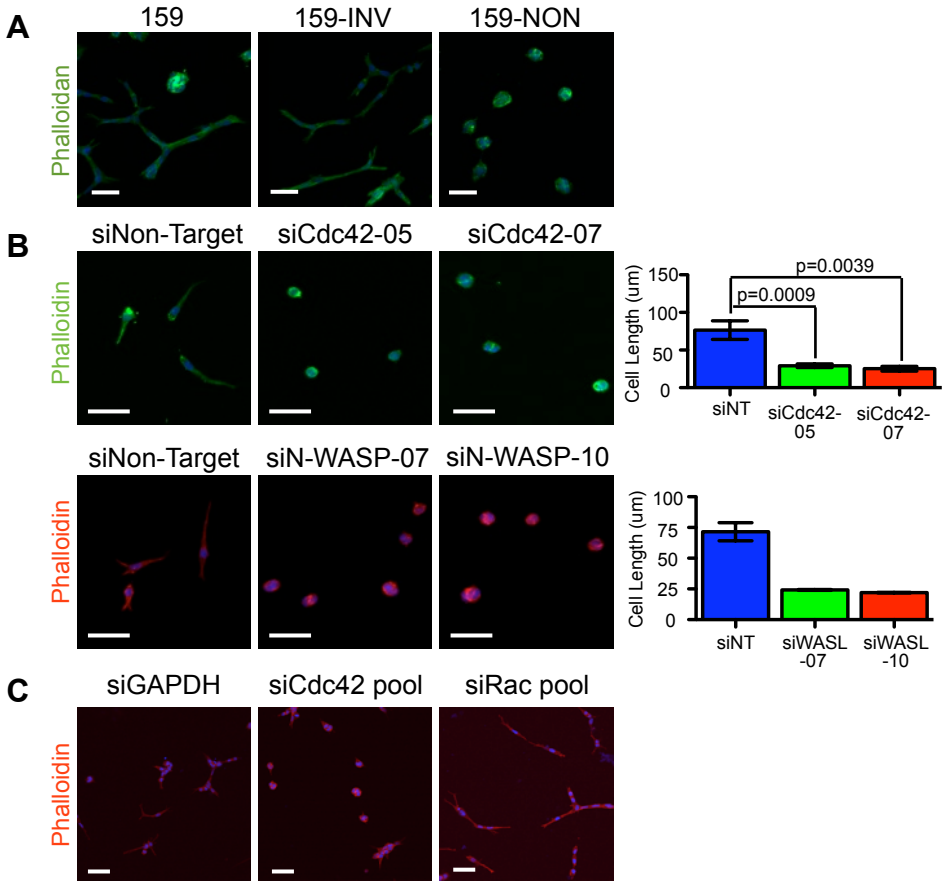



**Figure 3-5. Components of the Cdc42 pathway are required for non-cell autonomous control of invasion.** (**A**) SUM159 Parental, INV, and NON populations were plated in organotypic culture for 48 hours then fixed and stained with

phalloidin (green, actin). Scale bar, 50 μm. (**B**) SUM159-INV cells were transfected with the indicated siRNAs for 24 h (siCdc42) or 48 h (siN-WASP) and then plated in organotypic culture for 24 h. Transfected cultures were stained with phalloidin (green, F-actin) and Hoechst (blue, nuclei). Quantification of the length of the longest edge of individual cells is shown. Data are the mean +/- range (siN-WASP, two independent experiments**)** or S.D. (siCdc42, three independent experiments**)** of 50 cells analyzed per condition. Statistical significance was determined by Student's t-test. Scale bar, 50 μm. (**C**) SUM159 parental cells transfected with the indicated siGenome SMARTpool siRNAs for 24 h were then plated in organotypic culture for 24 h and stained with phalloidin (red, F-actin) and Hoechst (blue, nuclei). Scale bar, 50 μm.

To determine if Cdc42 and N-WASP expression was specifically required for invasion in SUM159 INV cells we tested the highly invasive human breast cancer cell line HS.578T. Knockdown of Cdc42 and N-WASP protein expression did suppress elongation in the 578T cells similar to the SUM159 INV results, demonstrating that Cdc42 regulates invasion across multiple invasive cell lines (Figure 3-6 A). Protein knockdown was confirmed by western blot (Figure 3-6 B).

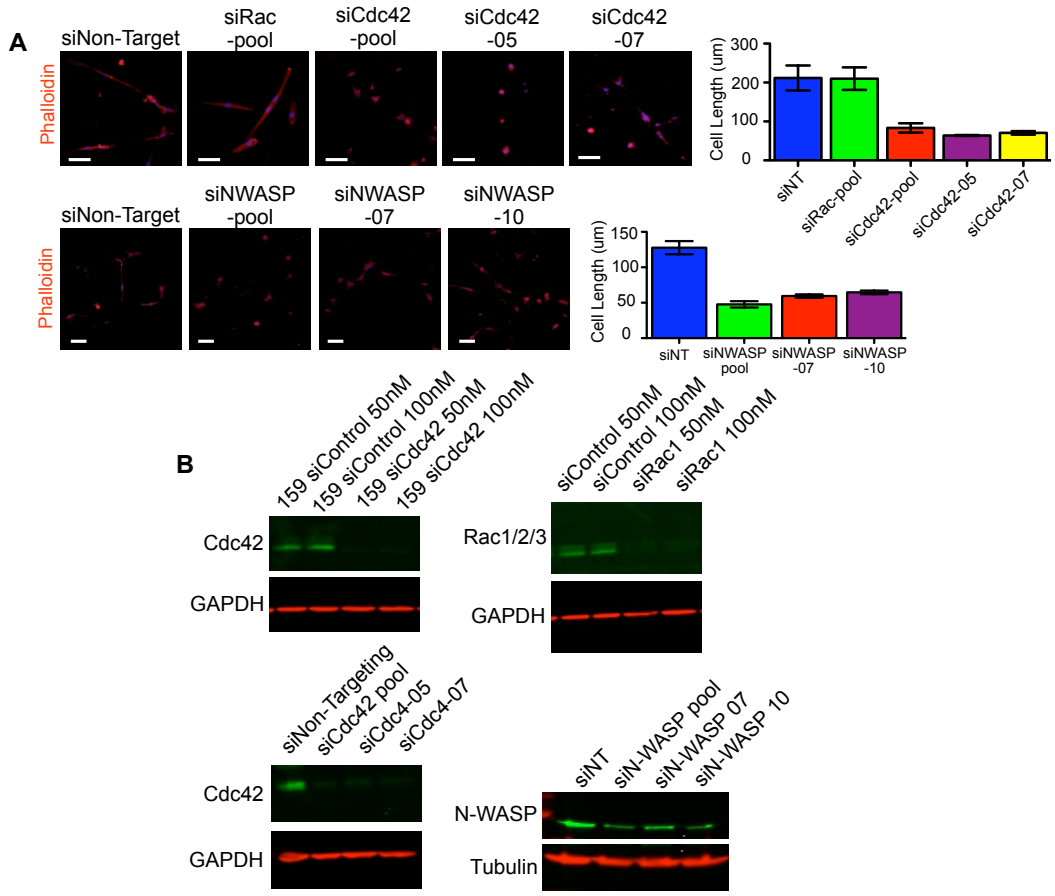



**Figure 3-6. Components of the Cdc42 pathway are required for HS.578T cell invasion.** (**A**) 578T cells were transfected with the indicated individual or pooled siRNAs for 48 h. The transfected cells were then plated in organotypic culture for 24 h stained with phalloidin (red, F-actin) and Hoechst (blue, nuclei). Quantification of average cell length is shown in the bar graph on the right. Data are the mean +/- range of 30 spheroids analyzed per condition in two independent experiments. Experiment performed by Mary Topolovski. Scale bar, 50 μm. (**B**) Western blot validation of siRNA mediated knockdown. Lysates of SUM159-parental (siCdc42- top panel and siRac) and SUM159-INV (siCdc42- bottom panel and siN-WASP) cells transfected for 48 h with siRNAs were immunoblotted with the indicated antibodies.

### Cdc42 is necessary for invasion but not cell motility

It was possible that Cdc42 was simply causing a reduction in cell motility, thus preventing the cells from migrating away from the primary spheroid, so to determine how an integrator of this pathway, Cdc42, was regulating INV cell behavior, we clustered

SUM159 cells transfected with siRNA targeting Cdc42 and plated them in organotypic culture. Knockdown of Cdc42 prevented SUM159 INV collective cell invasion from occurring, as cells transfected with siRNAs targeting Cdc42 did not form invasive projections into the surrounding matrix (Figure 3-7 A). To determine if knockdown of Cdc42 was regulating motility, we performed live imaging experiments on clustered SUM159 INV cells transfected with siRNA targeting Cdc42 in organotypic culture. While Cdc42 expression was necessary for invasion, reduced Cdc42 expression did not reduce cell motility in the 159 INV cells (Figure 3-7 B). INV cells that were transiently transfected with siRNA against Cdc42 and then plated as clusters in organotypic culture were capable of intraspheroid movement, with speed and displacement rates comparable to control cells (Figure 3-7 B). Reduction of Cdc42 expression in INV cells did not reduce cell motility, yet cells were not capable of invading away from the primary mass, suggesting that Cdc42 is necessary for invasion but not motility.

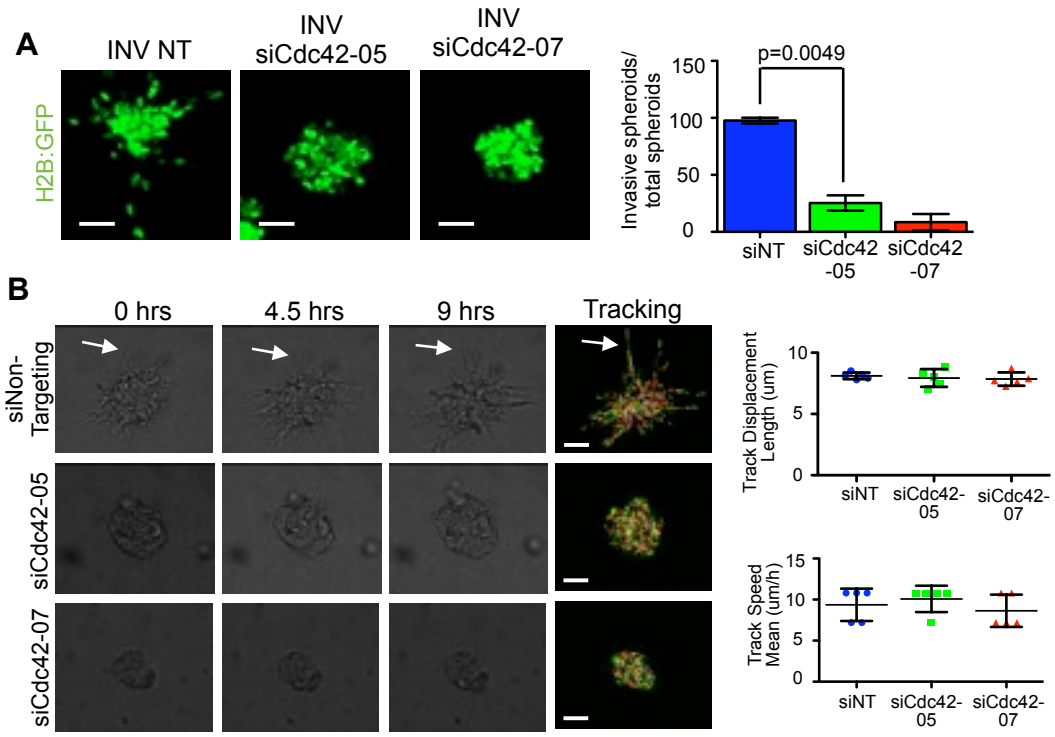



**Figure 3-7. Components of the Cdc42 pathway are required for invasion but not motility.** (**A**) SUM159-INV cells were transfected for 24 h with the indicated siRNAs and then clustered into spheroids for 24 h. Spheroid clusters were embedded in organotypic culture for 48 h. The quantification of invasion is shown in the bar graph below. Data are the mean +/- SD of three independent experiments. Scale bar, 50 µm. (**B**) Time lapse images and tracking of SUM159-INV cells after transfection with the indicated siRNAs for 24 h, clustering into spheroids for an additional 24 h and then embedding in organotypic culture for 24 h (72 h total). Images were acquired at 15 min intervals starting 24 h after embedding in organotypic culture. Shown are representative images over 9 h time period. Solid arrows identify invasive projections. Tracking of cell movement is shown in the right-hand panels. The quantification of mean track speed (µm/h) and mean track displacement length (µm) is shown in scatter plots on the right. The mean +/- S.D. of five spheroids analyzed per condition in one experiment using Imaris tracking software is shown. Results and quantification are representative of three independent experiments. Scale bar, 50 µm.

**Remodeling of the microenvironment is necessary for NON cell invasion**

As a regulator of filopodial dynamics, Cdc42 and N-WASP were used to determine if remodeling of the microenvironment was necessary for NON cell invasion. Cdc42 or N-WASP expression was transiently knocked down in the INV cell population, then clustered with untransfected NON cells, and plated in organoytpic culture (Figure 3-8 A-C). While the control non-targeting heterogeneous clusters collectively invaded, the heterogeneous clusters with the knockdown of Cdc42 or N-WASP in the INV cells did not form invasive projections, thus preventing cooperative invasion from occurring (Figure 3-8 A-C). These data suggest that remodeling of the microenvironment is necessary for cooperative NON cell invasion.

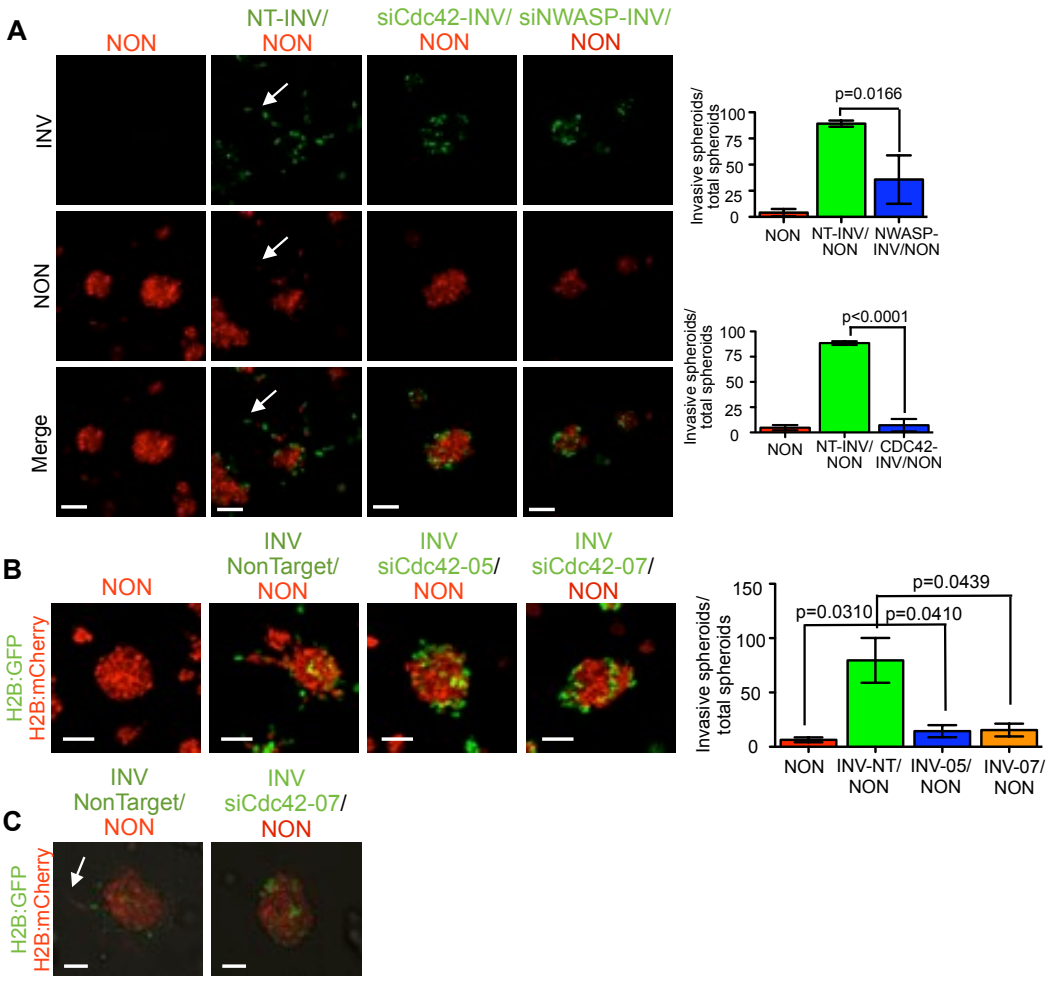



**Figure 3-8. Remodeling of the microenvironment is necessary for cooperative invasion.** (**A**) SUM159-INV cells were transfected for 24 h with the indicated siRNAs before clustering with untransfected SUM159-NON cells in spheroids at a ratio of 25% SUM159-INV:75% 159-NON cells. Spheroid clusters were then embedded in organotypic culture for 48 h. Solid arrows identify invasive projections. The quantification of invasion is shown in the bar graphs to the right. Data are the mean +/- S.D. of 50 spheroids analyzed per condition in three independent experiments. Statistical significance was determined by Student's t-test. Scale bar, 50 µm. (**B**) Heterogenous clusters showing that identical results are obtained when using two distinct individual siRNAs to reduce Cdc42 expression in the SUM159-INV subpopulation. SUM159-INV (H2B:GFP, green, nuclei) cells were transfected for 24 h with individual siRNAs targeting Cdc42, clustered into spheroids with SUM159-NON cells (H2B:mCherry, red, nuclei) and plated in organotypic culture for 48 h. The quantification of invasion is shown in the bar graph on the right. Data are the mean +/- S.D. of three independent experiments. Scale bar, 50 µm. (**C**) Phase and fluorescence

overlay showing that SUM159-INV (H2B:GFP, green, nuclei) cells transfected with an siCdc42 pool do not form filopodia when clustered into heterogenous spheroids with SUM159-NON (H2B:mCherry, red, nuclei) cells in organotypic culture. SUM159-INV cells transfected for 24 h with the indicated pooled siRNAs and were clustered into spheroids with untransfected SUM159-NON cells at a ratio of 25% SUM159-INV:75% 159-NON cells and embedded in organotypic culture for 48 h. Solid arrows identify invasive projections. Scale bar, 50 μm.

## DISCUSSION

Here we show that invasive breast cancer subpopulations can cooperate to induce the invasion of autonomously noninvasive breast cancer subpopulations. Cooperation during invasion is observed between tumor cells and the microenvironment (Gaggioli et al., 2007). However, we have shown that a subpopulation within a tumor can cooperate to induce the invasion of another subpopulation that is not capable of autonomous invasion. Our results are consistent with recent work by Calbo et al. who showed a role for *in vivo* cooperation during metastasis in a murine small cell lung cancer model. They found that tumor cells metastasized more efficiently in the presence of two distinct subpopulations, than either alone, however, they credited the cooperation to secreted factors (Calbo et al., 2011). Although it is not clear which step cooperation plays a role in during the metastatic cascade in the Calbo study, the data suggests a functional role for cooperation between tumor subpopulations in cancer types other than breast cancer. Thus, it is possible that we would find invasive and noninvasive subpopulations in other types of cancer cell lines, such as lung and pancreatic cancer, using our model.

A role for small Rho GTPases during invasion is supported by previous work (Gaggioli et al., 2007). Gaggoili et al. found the small GTPase, Rho to be necessary for invasion in leading fibroblast cells while we found that the small GTPase Cdc42

expression was necessary in leading INV cells. In the work of Gaggioli et al. Cdc42 expression was found to be necessary for follower cell invasion, thus it would be interesting to determine if Cdc42 expression is required for cooperative follower cell behavior in our system.

Intratumor heterogeneity has been well documented in multiple cancer types, ranging from leukemia to breast cancer (Fillmore and Kuperwasser, 2007; Marusyk and Polyak, 2010; Roche-Lestienne et al., 2002). While the clinical consequences of heterogeneity in tumor response to drug treatments are beginning to be appreciated, how intratumor heterogeneity contributes to tumor invasion is not well understood. Our data provides a functional role for tumor heterogeneity during invasion.

**Cooperative invasion model**

We propose a new model in which tumor cell subpopulations cooperate to invade and metastasize (Figure 3-9). In this model, invasive cells are capable of remodeling the microenvironment, allowing autonomously noninvasive cells to invade away from the ductal epithelium and gain access to the vasculature and lymphatic system. Once out of the duct, both invasive and noninvasive tumor cell subpopulations may have the potential to disseminate throughout the body and seed vital organs. Both populations may be capable of colonizing foreign tissues, but it is possible that the autonomously noninvasive cells may have an enhanced ability to colonize foreign tissues and form metastatic nodules that are responsible for the patient's death.

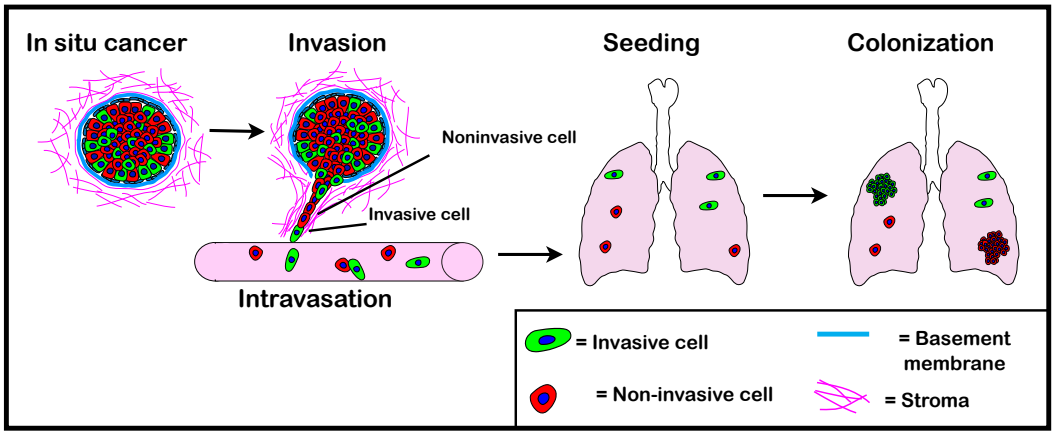



**Figure 3-9. Model of non-cell autonomous control of invasion.** Heterogeneous tumors contain subpopulations of invasive (green) and noninvasive (red) cells. Over time the invasive subpopulation can invade, creating a path in which the noninvasive cells can invade away from the primary tumor. Once in the blood stream, both populations may have the potential to seed organs, such as the lung, and form metastatic lesions.

# CHAPTER FOUR
# Identification and characterization of enhancers of invasion

## INTRODUCTION

### Overview

The prevailing model of breast cancer progression outlines the gradual acquisition of genetic alterations, which eventually lead to the development of invasive properties (Little, 2010). Studies attempting to identify genes necessary for invasion have used global gene expression analysis to compare DCIS and IBC samples, however distinct signatures for invasive stages of breast cancer were not found, perhaps outlining the heterogeneity that exists within tumors (Ma et al., 2003; McSherry et al., 2007; Schuetz et al., 2006). To circumvent this problem, we separated the isogenic invasive and noninvasive subpopulations derived from breast cancer cell lines and performed gene expression analysis to identify genes necessary for invasion.

## MATERIALS AND METHODS

### Cell Culture and reagents

T47D, HCC1143, HCC1428, HCC1806, HCC1569 and HCC1954 cells were a gift from from Michael Peyton and John Minna (UTSW). HC-11 cells were a gift from Jeff Rosen (Baylor College of Medicine). T47D, HCC1143, HCC1428, HCC1569, HCC1954, and MDA-MB-231 cells were cultured in a base medium of RPMI (Hyclone), 10% fetal bovine serum (FBS, Hyclone) and 1x penicillin streptomycin solution (Hyclone). HCC1143 medium was supplemented with 5ng/ml EGF (Sigma), T47D medium was

supplemented with 10 μg/ml insulin (Sigma) and HC-11 medium was supplemented with 10 μg/ml EGF and 5 μg/ml insulin and were cultured as described (Dang et al., 2011). SUM159 cells were cultured in Ham's F-12 medium containing 5% fetal bovine serum (FBS, Hyclone), 1x penicillin streptomycin solution, 5 μg/ml insulin (Sigma Aldrich), and 1mg/ml hydrocortisone (Sigma-Aldrich). SUM149 were grown in 5% FBS mammary epithelial growth medium (MEGM, Lonza). MCFDCIS cells were cultured as described (Dang et al., 2011). Human derived cell lines were validated by Powerplex analysis. Growth factor reduced Matrigel (BD Biosciences, 10-12 mg/ml stock concentration) and bovine collagen I (BD Biosciences) were used for organotypic culture experiments. Antibodies recognizing Collagen I (Abcam), Rac 1/2/3 (Cell Signaling), Cdc42 (Cell Signaling), N-WASP (Cell Signaling), PDGFRA (Cell Signaling), DOCK10 (Bethyl Laboratories), GAPDH (Calbiochem), and Tubulin (Sigma-Aldrich) were used for immunofluorescence and western blot analysis. Hoechst (Invitrogen), phalloidin (Invtirogen), and secondary antibodies labeled with Alexa fluor 488 nm or 546 nm, 680 nm (Invitrogen) and IR Dye 800CW (Li-Cor Biosciences) were used. Cell lines stably expressing pCLNRX-H2B:GFP and PGK-H2B:mCherry were generated as described (Dang et al., 2011).

**Organotypic culture.** Single cells were plated in 8-well chamberslides (immunofluorescence staining, BD Biosciences; live-cell imaging, Nunc) onto a base layer of Matrigel (5 mg/ml) and collagen I (1.5 mg/ml) and supplemented with a 2% Matrigel/growth medium mixture as described (Dang et al., 2011; Xian et al., 2005). All cultures were grown for 6-8 days except where indicated in the figure legends.

**Single cell assay**

Cells were transfected for 24-48 h in 96 well plates and then plated in organotypic culture and harvested 24 h later.

**Transfection**

Cells were transfected with 50-100nM of siRNA using RNAiMax transfection reagent for 24-48 h. OnTargetplus siRNAs were used except where indicated when siGenome pools (Dharmacon) were used. Cells were then either harvested for lysates or plated in organotypic culture for 24-48 h before fixing, see figure legends. Target sequences for siRNA knockdown sense strands were:

Non-Targeting OnTargetplus pool (UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA).

DOCK10 OnTargetplus pool (DOCK10-05: GGACCUGACUAAGCGUAUA, DOCK10-06: CAAUAUAGCUACGGAGGUU, DOCK10-07: CAACAUUCGCUUGCAAUUA, DOCK10-08: CCAGACAGCUAUCAAACAU).

Cdc42 OnTargetplus pool (Cdc42-05: CGGAAUAUGUACCGACUGU, Cdc42-06: GCAGUCACAGUUAUGAUUG, Cdc42-07: GAUGACCCCUCUACUAUUG, Cdc42-08: CUGCAGGGCAAGAGGAUUA).

N-WASP OnTargetplus pool (N-WASP-07: CAGCAGAUCGGAACUGUAU, N-WASP-08: UAGAGAGGGUGCUCAGCUA, N-WASP-09: GGUGUUGCUUGUCUUGUUA, N-WASP-10: CCAGAAAUCACAACAAAUA).

GAPDH siGenome SMARTpool (CAACGGAUUUGGUCGUAUU, GACCUCAACUACAUGGUU, UGGUUUACAUGUUCCAAUA,

GUCAACGGAUUUGGUCGUA).

CDC42 siGenome SMARTpool (GGAGAACCAUAUACUCUUG, GAUUACGACCGCUGAGUUA, GAUGACCCCUCUACUAUUG, CGGAAUAUGUACCGACUGU).

**Real-Time QPCR**

Cells were transfected with 50nM of siRNA using RNAiMax transfection reagent. Total RNA was harvested using the GenElute Mammalian Total RNA Mimiprep Kit (Sigma) after 48 h and converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Knockdown of mRNA transcripts were validated using TaqMan Gene Expression Assays (Applied Biosystems) and Applied Biosystems 7500 Real-Time PCR System was used to measure samples in triplicate. GAPDH was used as a loading control and relative gene expression in test conditions were compared to the Non-targeting control.

**Immunoblot analysis and immunofluorescence staining**

Cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Calbiochem) as described (Pearson and Hunter, 2007). Equal amounts of protein were separated by SDS-PAGE, transfered to Immobilon-FL polyvinylidene fluoride (PVDF) transfer membrane (Millipore), and immunostained. Immunoblots were visualized using an Odyssey infrared scanner (LI-COR). Organotypic cultures were fixed and immunostained as described (Pearson and Hunter, 2007). Images were acquired on Nikon and Zeiss LSM510 confocal microscopes in TIFF format. Images were arranged using Adobe Photoshop CS3 and Keynote, and are representative of three independent experiments.

**RNA isolation procedure**

RNA from biological replicates was harvested using the RNeasy Plus Mini Kit (Qiagen).

**Statistical Methods**

The mRNA expression data (Human HT-12 v4 Expression BeadChip, Illumina Inc.) for cell lines was processed with a model-based background correction approach (Xie et al., 2009), quantile-quantile normalization and log2 transformation. Unsupervised hierarchical clustering was implemented with complete linkage and Euclidean distance. Statistical Analysis of Microarray (SAM) analysis (Tusher et al., 2001) was used to identify differentially expressed genes between the INV and NON HCC1143 and SUM159 subpopulations. Median values of replicate probe sets for the same genes were used to summarize expression values for each gene.

**Patient sample analysis**

To test whether the invasive gene signature was associated with clinical outcome in breast cancer patients, we used a breast cancer dataset (GSE18229 with microarray platform GPL1390) (Prat et al., 2010) containing 161 primary tumor samples with microarray data (Agilent Human 1A Oligo Custom Microarray) and clinical outcomes. Due to platform differences (Illumina vs. Agilent) and missing data in the patient cohort, only 29 genes had complete expression data in all patients. Quantile-quantile normalization was used to normalize cell line data and primary tumor data. The expression of these 29 genes in the invasive HCC1143 and SUM159 subpopulations and seven noninvasive cell lines was used to develop a prediction model using the Random Forest approach (Breiman, 2001). The model was then used to classify each primary tumor as “invasive” or “noninvasive”. Kaplan Meier survival curves were drawn for both

the “invasive” and “noninvasive” groups and the survival differences between the groups were compared using the log-rank test.

## RESULTS

### EMT signatures

One mechanism by which cells have been known to become invasive is through the process of epithelial to mesenchymal transition (EMT) (Tomaskovic-Crook et al., 2009). EMT is a process by which cells loose their epithelial characteristics, distinguished by a loss of cell adhesion, and acquire mesenchymal characteristics, such as the ability to invade and migrate (Thiery, 2002). Cells that have undergone EMT show characteristic expression changes in markers of EMT. Decreases in epithelial cell markers such as E-cadherin, a cell adhesion protein, are characteristic of cells that have undergone EMT (Kalluri and Weinberg, 2009). Similarly, increases in mesenchymal cell markers such as N-cadherin and Vimentin, an intermediate filament, are characteristic of cells that have undergone EMT (Figure 4-1). Cells that display both sets of markers have undergone a partial EMT, having not fully transitioned to a mesenchymal state (Kalluri and Weinberg, 2009).

During development, EMT is crucial for accurate embryonic cell migration and patterning, but when cancer cells undergo EMT, malignancy can occur (Kalluri, 2009). EMT is widely thought to be an important cause of cancer cell invasion but EMT profiles alone cannot determine which patients will or will not relapse (Chikaishi et al., 2011). While EMT has been widely studied, it is still unclear how invasion is regulated. Here we describe the identification of an invasive cell signature that is predictive of poor

patient outcome and is distinct from a purely EMT signature.

**Gene expression classification of INV and NON subpopulations**

Breast cancer cell lines contain invasive and noninvasive subpopulations that can be stably isolated and maintained in culture (see Chapter Two). Utilizing the isogenic INV and NON subpopulations, we performed gene expression analysis on the SUM159 and HCC1143 parental, INV, and NON cell lines and six breast cancer cell lines that are noninvasive when plated in organotypic culture, HCC1954, HCC1806, MCF7, MCF-DCIS, HCC1428, and T47D to gain insight into the epigenetic changes that cause a cell to become invasive.

Previously, we found that the INV populations displayed mesenchymal characteristics and markers, thus to further examine the EMT profiles of the isogenic populations, we compared the expression profiles of the invasive and noninvasive cell lines against many of the traditionally used markers of EMT (Figure 4-1 A). The HCC1143 INV subpopulation expressed many of the traditional markers of EMT, while the HCC1143 NON subpopulation expression profile did not, suggesting that the HCC1143 INV population had undergone EMT and the NON subpopulation had not (Figure 4-1 A). Interestingly, both the SUM159 INV and NON subpopulations expressed markers of EMT, suggesting that traditional markers of EMT are not sufficient to distinguish INV and NON subpopulations (Figure 4-1 A). The EMT profiles of the SUM159 and HCC1143 INV and NON populations were consistent with western blot data we generated previously (Figure 2-5), validating multiple targets within the signature.

To further characterize and determine a molecular program that distinguished invasive from noninvasive subpopulations, we performed genome-wide mRNA gene expression profiling on the SUM159 and HCC1143 INV and NON populations. The isogenic INV and NON populations clustered closer together than to their phenotypic correlate, suggesting that sister INV and NON expression profiles are more closely related to each other than to other cell lines (Figure 4-1 B).

Supervised clustering analysis was performed using probe sets with four fold expression differences and a false discovery rate of less than five percent (Figure 4-1 C). Indeed, using the supervised clustering significance analysis, we were able to distinguish similarities between the SUM159 and HCC1143 INV and NON populations (Figure 4-1 D). We identified known regulators of invasion such as platelet derived growth factor α (PDGFRα) and some that have not been implicated in breast cancer invasion such as dedicator of cytokinesis 10 (DOCK10), a guanine exchange factor (GEF) which activates small G proteins (Figure 4-1 D) (Eckert et al., 2011; Nishikimi et al., 2005).

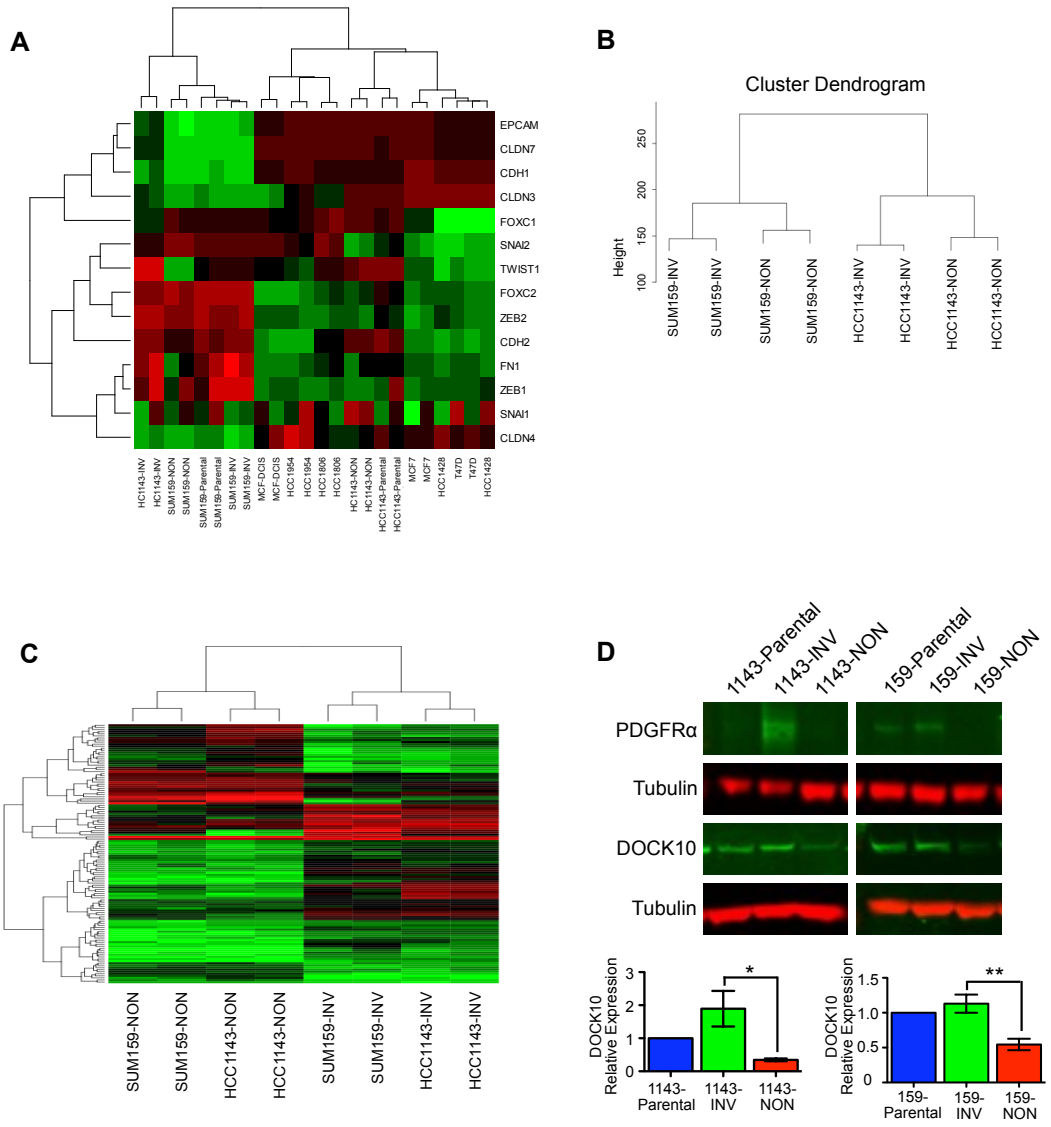



**Figure 4-1. Molecular definition of the INV and NON daughter subpopulations.** (**A**) Heatmap showing the mRNA expression of EMT-related genes in the indicated HCC1143 and SUM159 subpopulations and NON breast cancer cell lines. Mesenchymal markers (FOXC1, SNAI2, TWIST1, FOXC2, ZEB2, CDH2, FN1, ZEB1, SNAI1) and epithelial markers (EPCAM, CLDN7, CDH1, CLDN3, CLDN4) are shown. (**B**) Dendogram depicting unsupervised hierarchical clustering of the HCC1143 and SUM159 daughter subpopulations with complete linkage and Euclidean distance. (**C**) Heatmap showing differentially expressed genes in the INV and NON SUM159 and

HCC1143 subpopulations of cells. Median values of replicate probe sets for the same genes were used to summarize expression values for each gene are shown. (**D**) HCC1143 and SUM159 parental and daughter subpopulation lysates were imunoblotted with anti-PDGFRα, anti-DOCK10 and anti-tubulin antibodies. The mean and standard error mean (S.E.M) of at least 3 independent experiments are shown. HCC1143 p=0.0458 and SUM159 p=0.0089 (Student's t-test).

**Determining genes necessary for invasion**

We performed a small functional screen using siRNA pools against genes that were upregulated four fold in the INV populations as a method to uncover differentially expressed proteins that were necessary for invasion. We chose to work with genes that were upregulated in the INV population to identify regulators of invasion. Using a single cell assay, we transfected SUM159-INV cells, plated them in organotypic culture, and assessed the phenotype 24 hours later (Figure 4-2 A and B). Twenty six potential regulators of invasion were tested using this assay over three rounds of testing (Figure 4-2 A and B). The non-targeting control 159 INV cells were elongated and displayed filopodial projections, while six gene candidates displayed short cell phenotypes, lacking filopodial projections, reminiscent of the 159 NON cell phenotype (Figure 4-2 C and D). Candidate regulators of invasion included PPAP2B, LPAR1, FOXD1, ITGA11, DAB2, and TWIST (Figure 4-2 C and D). Candidate gene knockdown was validated by QPCR, however future experiments include validating protein knockdown by western blot (Figure 4-2 E).

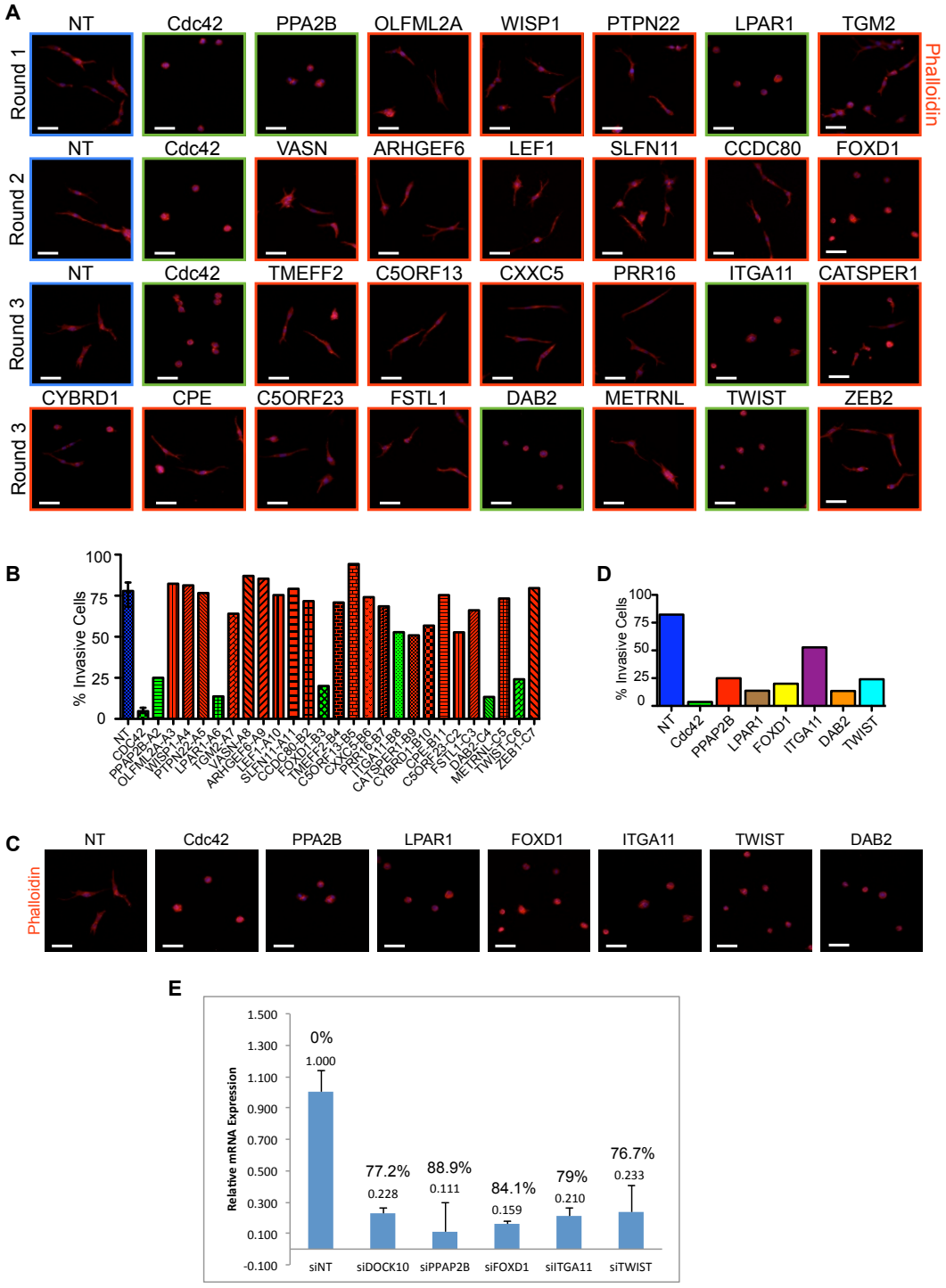

A
NT
Cdc42
PPA2B
OLFML2A
WISP1
PTPN22
LPAR1
TGM2
Round 1
Phalloidin
NT
Cdc42
VASN
ARHGEF6
LEF1
SLFN11
CCDC80
FOXD1
Round 2
NT
Cdc42
TMEFF2
C5ORF13
CXXC5
PRR16
ITGA11
CATSPER1
Round 3
CYBRD1
CPE
C5ORF23
FSTL1
DAB2
METRNL
TWIST
ZEB2
Round 3
B
% Invasive Cells
100
75
50
25
0
D
% Invasive Cells
100
75
50
25
0
NT
Cdc42
PPAP2B
LPAR1
FOXD1
ITGA11
DAB2
TWIST
C
Phalloidin
NT
Cdc42
PPA2B
LPAR1
FOXD1
ITGA11
TWIST
DAB2
E
Relative mRNA Expression
1.500
1.300
1.100
0.900
0.700
0.500
0.300
0.100
-0.100
0%
1.000
77.2%
0.228
88.9%
0.111
84.1%
0.159
79%
0.210
76.7%
0.233
siNT
siDOCK10
siPPAP2B
siFOXD1
siITGA11
siTWIST

**Figure 4-2. Candidate genes required for INV cell elongation and cooperative invasion.** (**A**) SUM159-INV cells were transfected with the indicated siRNAs for 48 h and then plated in organotypic culture for 24 h. Transfected cultures were stained with phalloidin (red, F-actin) and Hoechst (blue, nuclei). Testing of the candidates was done in three rounds of transfection and re-plating with siNon-targeting and siCdc42 controls included in each round. (**B**) Quantification of invasive cells is shown below, a cell was considered invasive if the cell displayed membrane projections. Data are the mean of 50 cells analyzed per condition. Scale bar, 50 μm. (**C**) Collection of candidate genes that are necessary for cell elongation in organotypic culture. (**D**) Quantification of invasive cells is shown below. Scale bar, 50 μm. (**E**) QPCR validation of siRNA knockdown. SUM159-INV cells were transfected with indicated siRNAs for 48 hours, harvested, and QPCR was performed to validate knockdown of mRNA transcripts. Values are normalized to the Non-targeting control. Percentages located on top of each bar represents percent knockdown. The mean and standard error mean (S.E.M) of three replicates from one experiment is shown. QPCR was performed by Erin Maine.

**DOCK10 is necessary for cooperative invasion**

Higher expression of DOCK10 was observed in the INV compared to the NON population of both HCC1143 and SUM159 cells (Figure 4-1 C and D), thus to determine if DOCK10 expression was necessary for invasion, we used the single cell assay to test the requirement of DOCK10 expression in SUM159 INV cell invasion (Figure 4-3 A). When SUM159 INV cells were transfected with siRNAs targeting DOCK10, cell length and invasion was reduced, suggesting that DOCK10 expression is necessary for invasion (Figure 4-3 A). Knockdown of DOCK10 expression was confirmed by western blot (Figure 4-3 B).

To determine if DOCK10 expression was required for invasion in additional invasive cell lines, we tested the highly invasive human breast cancer cell line, HS.578T. Transient siRNA mediated knockdown of DOCK10 expression did suppress elongation and invasiveness in 578T cells similar to the SUM159 INV results (Figure 4-3 C). This suggests a new function for DOCK10 in regulating filopodia formation in breast cancer

cells. Earlier we demonstrated that Cdc42 and N-WASP, are also required for invasion (Figure 3-4) and DOCK10 is a known activator of Cdc42, suggesting that DOCK10 is signaling through Cdc42 and N-WASP to promote actin polymerization and possibly filopodia formation (Nishikimi et al., 2005). Cdc42 activation by DOCK10 is necessary for melanoma cell invasion, supporting our hypothesis that DOCK10 may also regulate invasion of breast cancer cells through Cdc42 (Gadea et al., 2008).

To determine if DOCK10 expression was necessary for cooperative invasion, DOCK10 expression was knocked down in the INV cell population using siRNA, then clustered with untransfected NON cells and plated in organoytpic culture. While the control non-targeting heterogeneous clusters collectively invaded, the heterogeneous clusters with knockdown of DOCK10 in the INV cells did not form invasive projections, thus preventing cooperative invasion from occurring (Figure 4-3 D). These data suggest that DOCK10 expression is necessary for cooperative NON cell invasion. DOCK10 is an example of a candidate gene that is necessary for invasion, which was identified using gene expression profiling of the INV and NON populations.

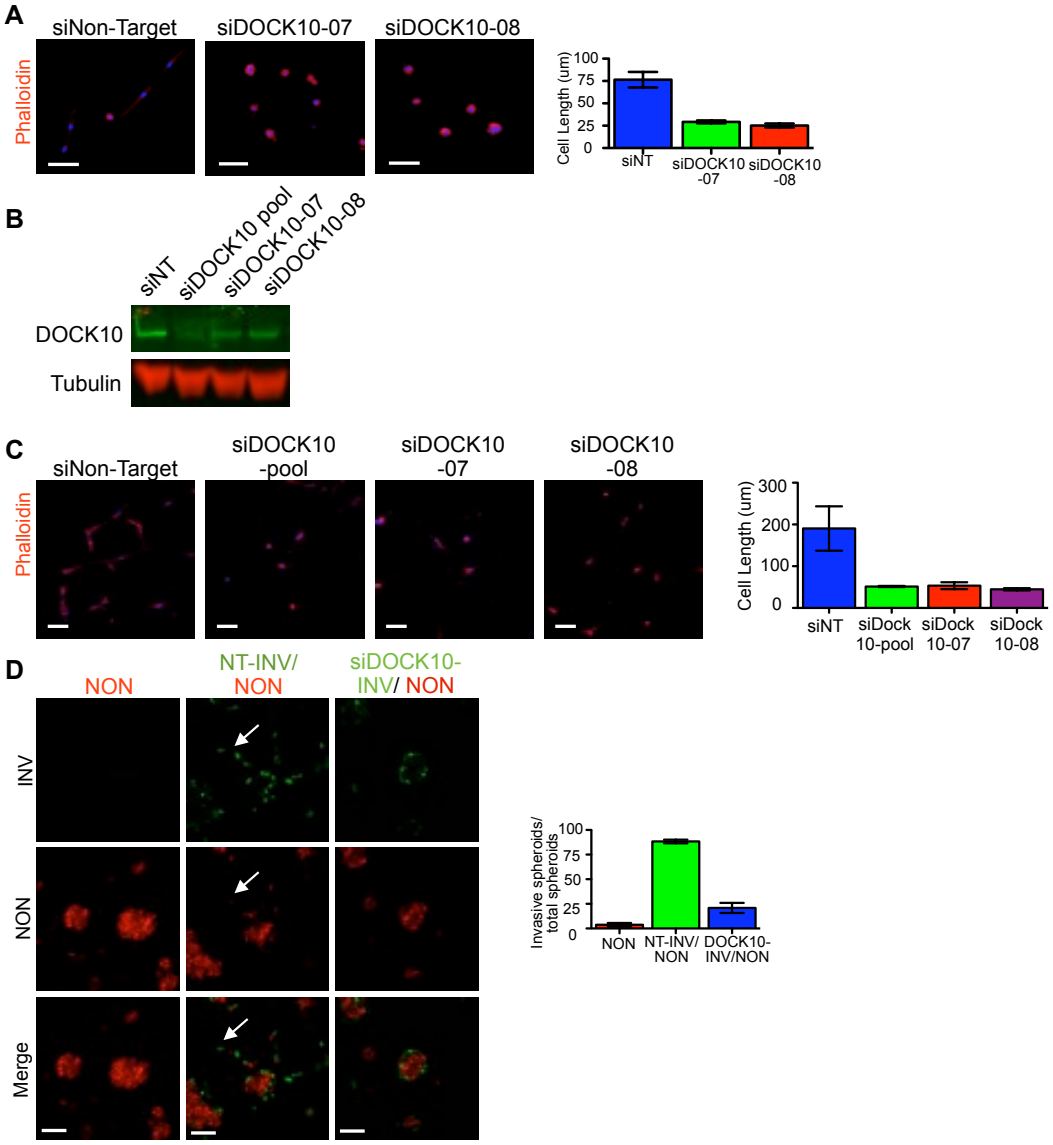



**Figure 4-3. DOCK-10 is required for INV cell elongation and cooperative invasion.** (**A**) SUM159-INV cells were transfected with the indicated siRNAs for 48 h and then plated in organotypic culture for 24 h. Transfected cultures were stained with phalloidin (green, F-actin) and Hoechst (blue, nuclei). Quantification of the length of the longest edge of individual cells is shown. Data are the mean +/- range of 50 cells analyzed per condition. Statistical significance was determined by Student's t-test. Scale bar, 50 µm. (**B**) Western blot validation of siRNA mediated knockdown. Lysates of SUM159-INV cells transfected for 48 h with the indicated siRNAs were immunoblotted with the indicated antibodies. (**C**) 578T cells were transfected with the indicated individual or pooled siRNAs for 48 h. The transfected cells were then plated in

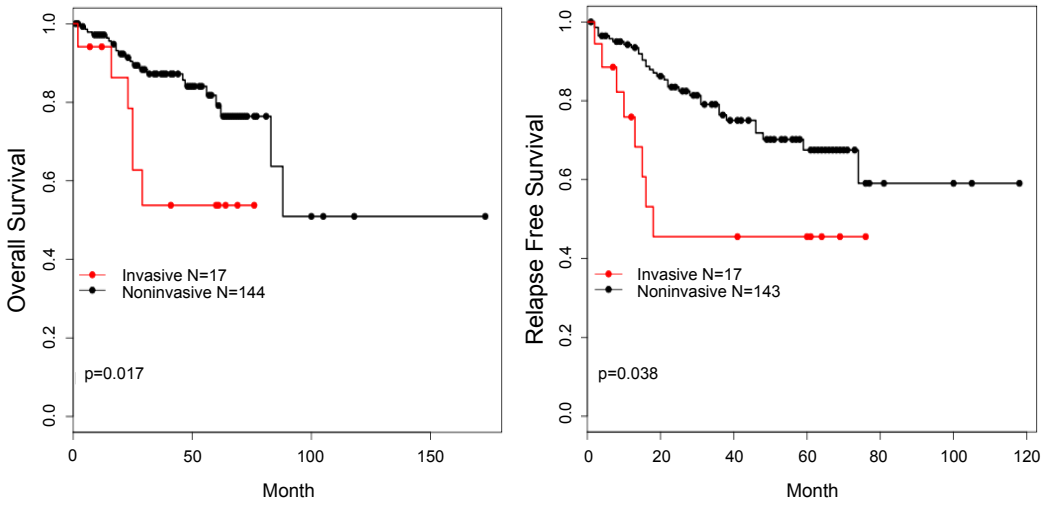
organotypic culture for 24 h stained with phalloidin (red, F-actin) and Hoechst (blue, nuclei). Quantification of average cell length is shown in the bar graph on the right. Data are the mean +/- range of 30 spheroids analyzed per condition in two independent experiments. Experiment performed by Mary Topolovski. Scale bar, 50 μm. (**D**) SUM159-INV (H2B:GFP, green, nuclei) cells were transfected for 24 h with either the noni-targeting (NT) control or DOCK-10 pooled siRNAs before clustering with untransfected SUM159-NON (H2B:mCherry, red, nuclei) cells in spheroids at a ratio of 25% SUM159-INV:75% 159-NON cells. Spheroid clusters were then embedded in organotypic culture for 48 h. Solid arrows identify invasive projections. The quantification of invasion is shown in the bar graphs to the right. Spheroids composed of at least 50 cells and heterogeneous clusters that contained 10% or more SUM159-INV cells were quantified. Spheroids containing at least one projection of three or more cells invading away from the primary mass were classified as invasive. Data are the mean +/- S.D. of 50 spheroids analyzed per condition in three independent experiments. Statistical significance was determined by Student's t-test. Scale bar, 50 μm.

**An invasive cell signature predicts poor patient outcome**

Using the gene expression profiles from the invasive and noninvasive cell lines, we generated an invasive cell expression signature that predicted poor patient outcome (Figure 4-4 A). To generate the invasive cell signature, we focused our analysis on genes that were overexpressed four fold in the INV population, restricting our list to a set of 49 genes (Appendix 1). Platform disparities and unavailable data from the patient cohort reduced our gene list to 29, which was used for predictive analysis on the two INV and NON subpopulations (SUM159 and HCC1143 INV and NON), and the eight noninvasive cell lines (Appendix 2). Using these data, a Random Forest approach was used to produce a prediction model, which was applied to the patient cohort and classified patients into either an "invasive" or "noninvasive" group. The patients within the "invasive" group had a shorter relapse free time and poorer overall survival when compared to the "noninvasive" group, suggesting that the presence of an invasive subpopulation within a tumor may promote a worse clinical outcome (Figure 4-4 A). The invasive signature was present across ER/PR positive, HER2 positive, and triple negative

breast cancer subtypes, suggesting that it was not merely recapitulating a breast cancer subtype specific signature (Figure 4-4 B).

**A**




**B**

| ER,HER2,and PGR (all negative) | non-invasive | Invasive |
|---|---|---|
| NO | 91 | 17 |
| Yes | 25 | 6 |

Fisher's exact test p-value = 0.5948

**Figure 4-4. The presence of the invasive subpopulations mRNA signature in primary tumors correlates with poor patient outcome.** (**A**) The expression of 29 genes in the invasive HCC1143 and SUM159 subpopulations and nine non-invasive cell lines was used to develop a prediction model using the Random Forest approach. The model was then used to classify each primary tumor as "invasive" or "noninvasive". Kaplan Meier survival curves were drawn for both the "invasive" and "noninvasive" groups and the survival differences between the groups were compared using the log-rank test. (**B**) All three subtypes are represented in the invasive cell signature.

## DISSCUSSION

The progression to an invasive phenotype is usually associated with the development of mesenchymal properties, thus it is not surprising that the INV population displays markers of EMT (Tomaskovic-Crook et al., 2009). However, traditional EMT markers are not sufficient to predict patient outcome (Chikaishi et al., 2011). We have identified an invasive gene signature that predicts poor patient outcome and shorter relapse free survival in breast cancer patients. This signature may help determine which patients are at risk for invasion and metastasis, and prevent patients who are not likely to relapse from being over-treated.

The 29-gene invasive cell signature may not identify patients with tumors that contain an invasive population that is too small to be detected by microarray. Microarrays are population averages, thus a small population of INV cells may not be detected using this technique (Altare et al., 2001). The invasive cell signature will identify only those patients with tumors that contain enough of an invasive cell population to display an invasive signature (Figure 4-5). Additional techniques such as laser capture microdissection may be necessary to isolate small subpopulations of invasive cells that exist within some patient tumors.

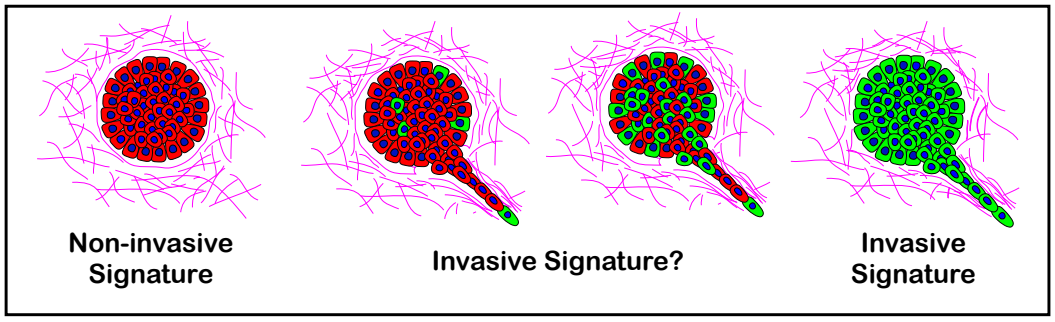



**Figure 4-5. Tumors with small populations of INV cells may not be detected using the 29-gene invasive cell signature.** The 29-gene invasive cell signature will likely identify tumors containing large populations of invasive cells, however microarrays survey the population average of a sample, thus small populations of invasive cells may not be detected.

# CHAPTER FIVE

## Conclusions and Future Directions

### Overview

A major challenge in breast cancer research has been determining how tumor cells transition from a noninvasive to invasive, malignant phenotype, as it is the metastases, not the primary tumor that causes death in breast cancer patients (Fidler, 1999). It appears that invasiveness does not simply result from the acquisition of cell motility because some noninvasive breast cancer cell lines and subpopulations are capable of motility (Pearson and Hunter, 2007). However, components of the microenvironment can provide an avenue of invasion for autonomously noninvasive yet motile breast cancer cells via secreted factors or remodeling of the microenvironment (Dang et al., 2011). In this study, we investigate the molecular requirements and cooperative elements necessary for breast cancer cell invasion.

### Comparison of invasive and noninvasive subpopulations of cells

Laser capture microdissection (LCM) has been used to identify genes associated with invasive breast cancer by comparing gene expression profiles of matched-pair ductal carcinoma *in situ* (DCIS) and invasive breast cancer (IBC) tissue isolated from patient tumors (Ma et al., 2003; Schuetz et al., 2006). However, distinct signatures for various pathological stages of breast cancer were not identified. In fact expression profiles of DCIS and IBC lesions were remarkably similar in comparison with normal breast tissue, supporting a clonal theory of tumorigenesis (Ma et al., 2003; McSherry et al., 2007; Schuetz et al., 2006). The similarity in expression profiles between DCIS and IBC

lesions perhaps highlight the heterogeneity observed in tumors because it is possible that the bulk of the IBC tumors were composed of noninvasive DCIS-like cells, containing only a small subpopulation of invasive cells which may have been undetectable by expression profiling (McSherry et al., 2007).

Using a functional approach, cells invading into matrigel filled needles toward epidermal growth factor (EGF) *in vivo* were isolated to compare against the bulk tumor using gene expression profiling (Wang et al., 2004). An invasive cell signature was derived (Wang et al., 2004) and as might be expected due to the difference in assays, there was limited overlap in the data acquired from previous gene expression profiles derived by LCM and functional analysis of patient tumors (Ma et al., 2003; Schuetz et al., 2006). While the functional study succeeded in collecting a potentially pure population of invasive cells, a limitation of this technique is that the comparison was to the bulk tumor, which likely contains a spectrum of invasive and noninvasive populations of cells, perhaps causing a decrease in gene expression variation. We identified and isolated invasive and noninvasive subpopulations from breast cancer cell lines by phenotype, thus allowing for the comparison of highly enriched invasive and noninvasive subpopulations of cancer cells.

We found that invasive (INV) and noninvasive (NON) populations existed in multiple human and murine breast cancer cell lines. The INV populations were enriched for markers of mesenchymal cells, while the NON populations retained epithelial markers such as the cell-cell adhesion molecule, EpCAM. Recently, others in the lab have isolated HCC1143 EpCAM-low and EpCAM-high cells, which are enriched for INV and NON cells by fluorescent activated cell sorting (FACS). In addition, they were able to

isolate EpCAM-high and EpCAM-low subpopulations from additional human breast cancer cell lines SUM149, SUM229, and HCC38 that previously were not attainable by separation by phenotype in organotypic culture because the invasive structures did not retain cell-cell contacts. Sorting for EpCAM-high and low cells by FACS yielded stable HCC1143, SUM149, SUM229, and HCC38 EpCAM-high (NON) and EpCAM-low (INV) populations (data not shown). Isolation of subpopulations using a cell surface marker such as EpCAM is a simple procedure that others can quickly and easily perform to repeat the experiments carried out in this manuscript.

It is possible that INV and NON-like subpopulations may exist in multiple types of cancer. Thus in the future, attempts to isolate invasive and noninvasive subpopulations from other cancer cell lines should be performed. Cell lines from lung, pancreatic, and colorectal cancers may contain invasive and noninvasive subpopulations. Lung, pancreatic, and colorectal cancer would benefit from identifying invasive and noninvasive populations because all are leading causes of cancer related death in which metastases are a major concern and are reported to display heterogeneity (Calbo et al., 2011; Froeling et al., 2010; Losi et al., 2005; Samuel and Hudson, 2012). The ability to distinguish invasive from noninvasive populations in cancer may provide insight into invasive cell targeting, potentially allowing for the prevention of deadly metastases.

**Commensalism**

Commensalism is a term in ecology used to define a type of cooperation between species that can be used to describe the interaction between tumor cell subpopulations (Saetre et al., 2012). In a commensal relationship, one species benefits from a relationship while the other remains unaffected. We propose that breast tumors contain

subpopulations of cells that display commensal behavior during invasion, allowing autonomously non-invasive cells to invade.

An example of commensalism is the relationship between lions and hyenas. Lions catch and eat their prey then scavenger hyenas consume the left over carcass. Hyenas benefit from their relationship with lions because they gain nutrition but lions neither experience benefit nor loss due to this relationship. In a similar way, INV and NON cells display a commensal relationship during invasion. NON cells benefit by gaining the ability to invade in the presence of INV cells, but INV cells seem to neither gain nor lose from the interaction with NON cells.

**Remodeling of the microenvironment is necessary for cooperative invasion**

Our results indicate that remodeling of the microenvironment by INV cells is required to allow cooperative invasion of NON cells by knocking down Cdc42 and N-WASP expression in INV cells and clustering them with NON cells. The inability of NON cells to invade in the presence of transfected INV cells suggests that secreted factors are not sufficient to induce cooperative invasion. Cdc42 plays a role in vesicle trafficking and cell polarity, so it is possible that secretion of invasion inducing factors or cell polarity in the INV cells may have been decreased upon Cdc42 knockdown (Cerione, 2004). However, the inability of NON cells to invade during N-WASP knockdown in INV cells suggests that secreted factors are not sufficient to drive cooperative NON cell invasion, as N-WASP is not known to regulate vesicle trafficking.

Additional experiments that would demonstrate the importance of remodeling of the microenvironment as opposed to secreted factors in cooperative invasion would be

embedding the INV cells in organotypic culture and allowing them to form tracks and remodel the ECM. The INV cells will be treated with cytotoxic drugs, causing the INVcells to undergo apoptosis and die, and drug resistant NON cells will be plated on top of the remodeled ECM. If the NON cells invade into the remodeled ECM in the absence of INV cells, it would suggest that remodeling of the microenvironment is sufficient for NON cell invasion, if not it may suggest that a secreted factor may be necessary for cooperative NON cell invasion. However, it is possible that secreted factors may be left behind by the INV cells after they have been eliminated, thus additional studies will be needed to support the conclusion that secreted factors are not necessary for cooperative invasion.

**Molecular regulation of invasion**

The identification of regulators of invasion is necessary to better understand and target malignant cells. Here, we identify candidate genes that are differentially regulated in INV and NON populations that may induce invasion in breast cancer cell lines. It is likely that multiple proteins work together to induce invasion in cells, thus most likely multiple candidate genes will be required to induce invasion in NON cells. Candidate genes PPAP2B, LPAR1, ITGA11, and DAB2 likely have a direct role in inducing invasion, while transcription factors FOXD1 and Twist1 likely regulate multiple proteins and pathways contributing to invasion.

*PPAP2B*

Phosphatidic acid phosphatase type 2B (PPAP2B), also known as lipid phosphate phosphatase 3 (LPP3) is a membrane glycoprotein which regulates embryonic patterning during development and vasculogenesis (Escalante-Alcalde et al., 2003). PPAP2B

activates the Wnt pathway and has been implicated in inducing endothelial cell migration and regulating tumor progression (Chatterjee et al., 2011; Humtsoe et al., 2010). The Wnt pathway is crucial in embryonic development and is upregulated in many human cancers including breast cancer, stimulating tumor proliferation and progression (Howe and Brown, 2004; Klaus and Birchmeier, 2008). The Wnt pathway has been implicated in breast cancer invasion, thus PPAP2B could be mediating invasion via Wnt signaling (Nguyen et al., 2005).

*LPAR1*

Lysophosphatidic acid receptor-1 (LPAR1), also known as EDG2, is a G-protein coupled receptor that binds lysophosphatidic acid (LPA) and induces multiple cellular responses including cell migration, proliferation and cytoskeletal dynamics (Hayashi et al., 2012; Van Leeuwen et al., 2003). LPAR1 has been shown to induce breast cancer cell motility and metastasis (Horak et al., 2007a; Horak et al., 2007b). This regulation of motility may explain why knockdown of LPAR1 reduces invasion in SUM159 INV cells, perhaps through the regulation filopodial extension. LPAR1 has also been shown to enhance the production of matrix metalloproteinase-2 (MMP-2), which has been shown to promote invasiveness in multiple cancers including breast cancer (Kato et al., 2012). Thus, LPAR1 associated invasion may be linked to an increase in cellular projections and protease production in breast cancer cells.

*ITGA11*

Integrin alpha-11 (ITGA11) is a heterodimeric membrane adhesion protein which binds components of the extracellular matrix, specifically collagen (Hynes, 2002). This intigrin may be regulating invasion due to its role in adhesion to the ECM. Cells that

overexpress ITGA11 may acquire the ability to bind components of the ECM through structures such as filopodia and thus develop invasive capabilities. ITGA11 has known functions in tumorigenesis as it is overexpressed in non-small cell lung cancer (NSCLC) and promotes tumor growth *in vivo* (Wang et al., 2002; Zhu et al., 2007).

*DAB2*

Disabled-2 (DAB2) is a mitogen-responsive phosphoprotein, which interacts with Grb2 and SOS, members of the ERK pathway (Xu et al., 1998). It is possible that DAB2 may work indirectly through Erk activation of invasion or directly through the regulation of integrins that form focal adhesions and are involved in motility (Teckchandani et al., 2009). Additionally, DAB2 has been reported to be involved during EMT and angiogenesis, suggesting that DAB2 may regulate invasion linked to EMT and the recruitment of vasculature (Cheong et al., 2012; Prunier and Howe, 2005). However, DAB2 expression is frequently lost in ovarian and breast cancer indicating that DAB2 may be a tumor suppressor (Bagadi et al., 2007). Further studies on this protein will be needed to clarify its role in invasion.

*Transcription factors*

Forkhead box protein D1 (FOXD1) and Twist-related protein 1 (Twist1) are transcription factors that likely play an indirect role in invasion, perhaps through the regulation of proteins that may be required for invasion.

*FOXD1*

In mammals, FOXD1 is required for correct patterning during retinal development and kidney morphogenesis (Carreres et al., 2011; Levinson et al., 2005). Little is known about the role of FOXD1 in cancer. There are a limited number of studies implicating a

role for FOXD1 and other members of the Forkhead box (FOX) proteins in cancer (Myatt and Lam, 2007; Zhang et al., 2003). However, FOXD1 is a transcription factor and thus may regulate a number of pathways that as of yet have not been linked to tumorigenesis or invasion.

*Twist*

There is a large body of evidence demonstrating that the transcription factor Twist1 promotes EMT and metastasis in a wide range of carcinomas (Kwok et al., 2005; Lee et al., 2006; Yang et al., 2004). Twist1 is required for the metastasis of mouse mammary carcinoma, 4T1 cell line, which we found to contain stable INV and NON subpopulations (Yang et al., 2004). Twist1 has been shown to induce Platelet derived growth factor receptor α (PDGFRα) expression, thus promoting invadopodia formation (Eckert et al., 2011).

PDGFRα is a receptor tyrosine kinase that plays a role in angiogenesis and is associated with promoting tumorigenesis (Dong et al., 2004; Hermanson et al., 1992; Jechlinger et al., 2006). We identified PDGFRα as a candidate gene from our 29 gene signature which regulates SUM159 INV cell elongation and invasion, however we were unable to confirm protein knockdown of cells transfected with siRNA targeting PDGFRα transcripts. However, we have since used PDGFRα siRNA sequences from a different company and obtained similar results, in which we were able to confirm knockdown of PDGFRα using quantitative polymerase chain reaction (QPCR), suggesting PDGFRα is necessary for SUM159 INV cell elongation. Twist1 may be acting on multiple pathways to promote EMT induced invasion and metastasis, but one way may be the Twist1 regulation of PDGFRα to induce filopodia or invadopodia formation (Eckert et al., 2011).

*In vivo*, PDGFRα may also function to recruit vasculature that facilitates cancer cell metastasis. Thus Twist1 may regulate multiple aspects of metastasis by inducing both local invasion and the recruitment of vasculature that allows the dissemination of cancer cells.

It is possible that FOXD1 and Twist1 regulate the expression of signaling proteins such as PDGFRα, dedicator of cytokinesis 10 (DOCK10), PPAP2B, LPAR1, and DAB2 which in turn may activate direct regulators of invasion such as ITGA11 and ARP2/3 (Figure 5-1). The candidate genes are likely not acting in a linear pathway, rather proteins that have a direct interaction with the cytoskeleton or ECM to induce invasion are likely regulated upstream by signaling proteins which expression is modulated by transcription factors.

**Future directions**

We have shown that knockdown of specific gene products can reduce the invasiveness of INV subopopulations. Testing across additional breast cancer cell lines as well as other types of cancer cell lines, such as lung and prostate cancer, will support these results. Testing on other cancer types will indicate that the candidate genes are necessary for invasion across a wide variety of breast cancer subtypes and other cancers, thus making them more robust targets for cancer treatment. Future experiments will include testing of the candidate genes to determine if their overexpression in noninvasive breast cancer cell lines is sufficient to promote invasion, thus identifying which genes are sufficient for invasion and potentially targets for treatment. To determine if the candidate genes are present in human breast cancer patient samples IHC will be performed to identify levels of candidate proteins in tumor samples. If the candidate proteins are

present in patient samples, it may be possible to use them as diagnostic tools to identify which patients are more likely to require intensive treatment and which can be spared intense treatment regiments.

To determine if the candidate genes are necessary for metastasis, the genes will be constitutively knocked down using short hairpin ribonucleic acid (shRNA) in a metastatic cell line, such as the SUM159 INV line. Orthotropic injections in the mouse mammary fat pads of the SUM159 INV cell line produce micrometastatses in the lungs about 4-6 weeks after injection (data not shown). Constitutive knockdown of the candidate genes may reduce the number of metastatic cells found on the lung suggesting that the gene is necessary for metastasis.

The identification of the candidate genes as important factors in invasion may lead to treatments and therapies that target these genes to prevent tumor cells from invading and metastasizing in breast cancer patients. Metastasis is the primary cause of mortality in breast cancer patients, which if suppressed, will result in the prevention of breast cancer related death. If targeted therapies towards these candidate genes and diagnostic tools are found to be effective in breast cancer, these methods should be applied to other types of cancer as well. The diagnostic tools and therapies that could result from these studies could serve to impede cancer cell metastasis and prevent the majority of cancer related deaths.

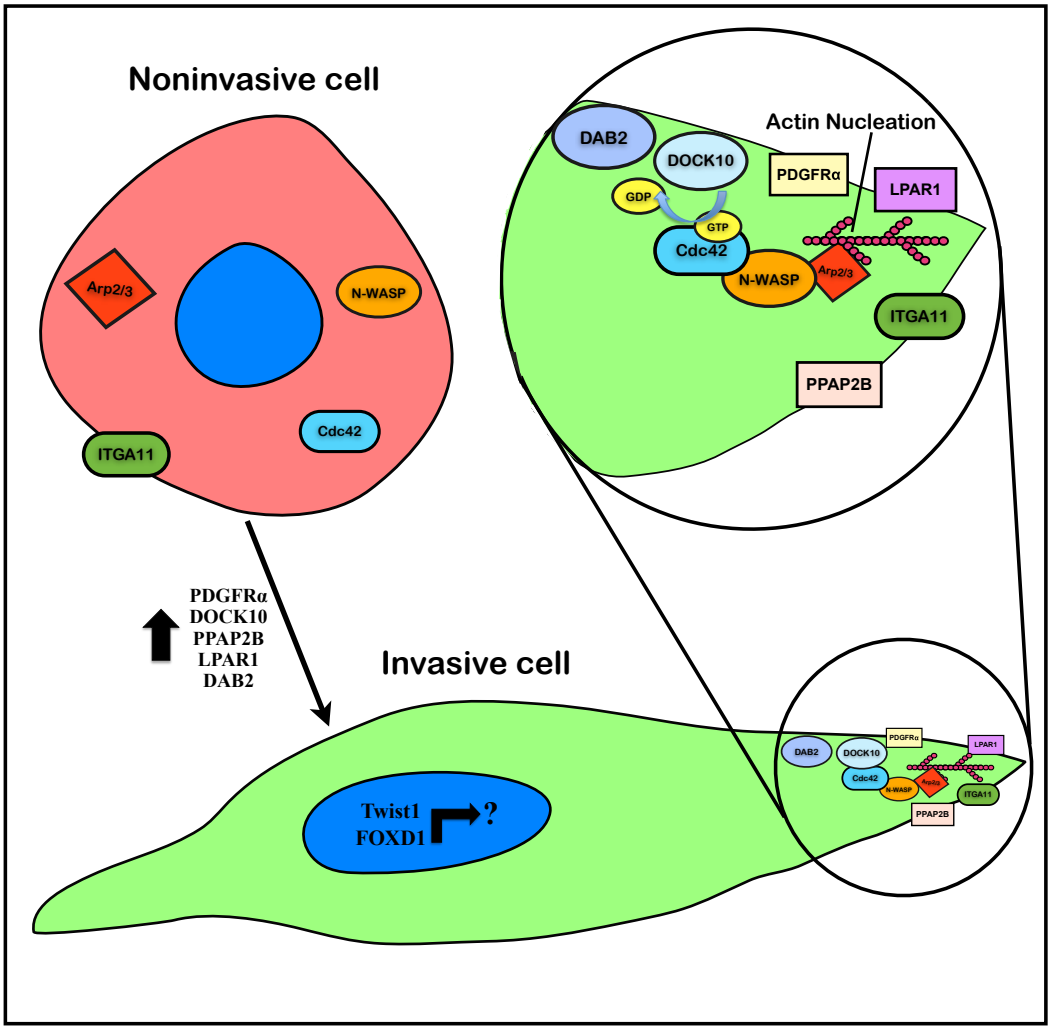



**Figure 5-1. Molecular interaction of candidate genes to promote invasion.** It is likely that the upregulation of multiple candidate genes are necessary to promote the invasion of a noninvasive tumor cell. Transcription factors, Twist1 and FOXD1 may increase the expression of signaling molecules, PPAP2B, LPAR1, DAB2, DOCK10 or PDGFRα to induce downstream signaling through direct regulators of adhesion and the cytoskeleton, ITGA11 and Arp2/3, thus causing invasion. Thus, to determine which genes are sufficient for NON cell invasion, overexpression of multiple candidate gene proteins may be necessary to induce NON cell invasion.

## APPENDIX A
## 49 Gene Expression Candidate List

| Gene ID | Gene Name | Fold Change |
|---|---|---|
| ILMN_2388800 | PPAP2B | 17.66755452 |
| ILMN_1761425 | OLFML2A | 14.00261328 |
| ILMN_1713499 | WISP1 | 21.74472479 |
| ILMN_2408683 | PPAP2B | 15.19570738 |
| ILMN_2246328 | PTPN22 | 10.86500449 |
| ILMN_1739496 | PRRX1 | 16.38682086 |
| ILMN_1701441 | LPAR1 | 8.769618795 |
| ILMN_1776842 | DKFZP451A211 | 10.1198135 |
| ILMN_2086470 | PDGFRA | 37.21545756 |
| ILMN_1679267 | TGM2 | 5.561287037 |
| ILMN_1702301 | DOCK10 | 4.328901072 |
| ILMN_1679060 | LOC642559 | 4.412202347 |
| ILMN_3191393 | LOC100128892 | 7.999666711 |
| ILMN_1667295 | VASN | 7.807246618 |
| ILMN_1775268 | HECW2 | 4.545215385 |
| ILMN_1658917 | SLC1A1 | 5.133860411 |
| ILMN_1803423 | ARHGEF6 | 6.359743062 |
| ILMN_2213136 | LEF1 | 5.919048036 |
| ILMN_1681949 | PDGFRA | 18.23925707 |
| ILMN_1752520 | SLFN11 | 5.669328303 |

| | | |
|---|---|---|
| ILMN_1715662 | CCDC80 | 5.53603794 |
| ILMN_1704418 | FOXD1 | 4.628827669 |
| ILMN_1730645 | TMEFF2 | 19.41349784 |
| ILMN_1742866 | F2R | 7.055647333 |
| ILMN_2065773 | SCG5 | 27.49268014 |
| ILMN_2121408 | HBEGF | 7.877542307 |
| ILMN_1680738 | C5ORF13 | 11.45523732 |
| ILMN_1745256 | CXXC5 | 5.947463767 |
| ILMN_3232894 | CNRIP1 | 17.22463604 |
| ILMN_3307729 | CXXC5 | 5.79209357 |
| ILMN_1709153 | PRR16 | 7.311946417 |
| ILMN_1746618 | PAQR7 | 4.336490652 |
| ILMN_1801516 | GPC1 | 4.790553221 |
| ILMN_1651343 | ITGA11 | 4.579752047 |
| ILMN_2162860 | SLFN11 | 4.906947764 |
| ILMN_1751276 | BDNF | 10.40566195 |
| ILMN_2068499 | POU5F1P1 | 5.007135841 |
| ILMN_1689431 | APCDD1L | 4.33526196 |
| ILMN_1789394 | CATSPER1 | 5.600855861 |
| ILMN_1712305 | CYBRD1 | 10.01674537 |
| ILMN_1731374 | CPE | 4.053985604 |
| ILMN_1769556 | C5ORF23 | 4.543801553 |

| | | |
|---|---|---|
| ILMN_1676361 | ARHGAP22 | 9.313720389 |
| ILMN_1717934 | SYT11 | 7.442010158 |
| ILMN_2201678 | FSTL1 | 15.64961731 |
| ILMN_1764228 | DAB2 | 5.56233706 |
| ILMN_1801610 | METRNL | 4.179714007 |
| ILMN_1819854 | HS.36053 | 7.207528077 |
| ILMN_1803338 | CCDC80 | 4.153756424 |

## APPENDIX B
## 29 Gene Expression Candidate List

Gene Name

ARHGEF6

C5ORF13

C5ORF23

CATSPER1

CCDC80

CPE

CXXC5

CYBRD1

DAB2

DOCK10

F2R

FOXD1

FSTL1

HBEGF

ITGA11

LEF1

LPAR1

METRNL

OLFML2A

PDGFRA

PPAP2B

PRR16

PTPN22

SCG5

SLFN11

TGM2

TMEFF2

VASN

WISP1

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