UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Twist1-Mediated Induction of FAP-alpha in Invadopodia and Breast Cancer Metastasis

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

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2014

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Chair

University of California, San Diego

2014

DEDICATION

This dissertation is dedicated to my family, without whom this work and everything leading up to it, would not be possible.

EPIGRAPH

It always seems impossible until it’s done.

Nelson Mandela

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LIST OF ABBREVIATIONS

ADAM12: A Disintegrin And Metalloprotease 12

BM: Basement Membrane

ChIP: Chromatin ImmunoPrecipitation

CTC: Circulating Tumor Cell

DAPI: 4',6-diamidino-2-phenylindole

DCIS: Ductal Carcinoma In Situ

DMEM: Dulbecco's Modified Eagle Medium

DPP4: Dipeptidyl Peptidase 4

ECM: Extracellular Matrix

EGR1: Early Growth Response protein 1

EMSA: Electrophoretic Mobility Shift Assay

EMT: Epithelial-Mesenchymal Transition

ER: Estrogen Receptor

FAPα: Fibroblast Activation Protein α

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green Fluorescent Protein

HER2: Human Epidermal growth factor Receptor 2

HMLE: Human Mammary Epithelial cells immortalized with Large T antigen

IF: Immunofluorescence

IHC: Immunohistochemistry

MARE: MAF Recognition Element

MET: Mesenchymal-Epithelial Transition

MMP: Matrix Metalloprotease

MMTV: Mouse Mammary Tumor Virus

MT1-MMP: Membrane-Tethered Matrix Metalloprotease

N-WASP: Neuronal Wiskott-Aldrich Syndrome Protein

NCBI: National Center for Biotechnology Information

NFκB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

PDGFRα: Platlet-Derived Growth Factor Receptor α

PFA: Paraformaldehyde

PyMT: Polyoma virus Middle T antigen

RNAi: RNA interference

SDS: Sodium Dodecyl Sulfate

shRNA: small hairpin RNA

siRNA: small interfering RNA

TGFβ: Transforming Growth Factor β

Tks5: Tyrosine Kinase Substrate 5

LIST OF SUPPLEMENTAL FILES

Control HS578T cells labeled with mCherry LifeAct
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ABSTRACT OF THE DISSERTATION

The Role of Twist1-Mediated Induction of FAP-alpha in Invadopodia and Breast Cancer Metastasis

by

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Professor Jing Yang, Chair

Breast cancer metastasis, the dissemination of primary tumor cells to distant organs, is the leading cause of death among breast cancer patients. During metastasis, tumor cells must invade through the basement membrane and local tissues, intravasate into the circulatory system, travel to a distant

organ, extravasate into the new tissue, and establish micrometastases, which eventually become secondary tumors. The basic helix-loop-helix transcription factor Twist1 is expressed in a variety of aggressive cancers, and is essential for metastasis to take place. Twist1 activates a developmental program known as the Epithelial-Mesenchymal Transition (EMT), during which tumor cells lose their cell-cell junctions and become more migratory and invasive. Twist1 endows tumor cells with an increased invasive capacity by inducing formation of subcellular structures known as invadopodia. Located on the ventral surface of invasive cells, invadopodia are actin-rich protrusions of the cell membrane that recruit proteases to points of cell-matrix contact, and thus degrade the surrounding extracellular matrix (ECM). It was found that Twist1-mediated formation of invadopodia is required for metastasis to take place, but the precise role that Twist1 plays in invadopodia assembly was not completely understood. This dissertation presents data demonstrating that Twist1 is involved in regulation of a serine protease known as Fibroblast Activation Protein alpha (FAPα). FAPα is located at invadopodia, and while not required for EMT or cellular migration, is essential for metastasis to take place. Further investigation found that FAPα expression is required for ECM degradation, but its protease activity is not. Although ECM degradation is impaired when FAPα expression is suppressed, early stages of invadopodia formation still take place, suggesting that FAPα may be involved in the later steps of invadopodia assembly, when proteases are recruited. Indeed, results show that the matrix metalloprotease MT1-MMP is mislocalized in the absence of FAPα expression,

providing additional evidence that the role of FAPα at invadopodia is more structural than proteolytic. Further studies must be undertaken to examine how FAPα is trafficked to invadopodia, and which domain of the protein is important for its function.

CHAPTER 1

Introduction

1.1 Cancer

People have been investigating how and why tumors form for the past 4000 years. Hippocrates coined the term “cancer”, which refers to the cut surface of a tumor resembling a crab (Hajdu, 2011). During the 19th century, Rudolph Virchow made the landmark discovery that tumors consisted of cells, and that epithelial tumors arose from pre-existing epithelial cells (Hajdu, 2012). Our knowledge of cancer biology has grown substantially since then, and we now know that tumors are more than simply masses of aberrantly proliferating cells.

Hundreds of distinct types of cancer have been described to date, and some exhibit various tumor subtypes. This complexity prompted a number of questions: how many regulatory signaling pathways need to be disrupted for cancer to arise? Are these pathways the same in all cancer types? Which signaling pathways are cell-autonomous, and which are triggered by cues from the surrounding tumor microenvironment? In 2000, Douglas Hanahan and Robert Weinberg published a seminal article postulating that there are 6 “hallmarks of cancer” – 6 essential capabilities that cells must possess to undergo malignant growth (Hanahan and Weinberg, 2000). These are: self-sufficiency in growth signals, ability to evade apoptosis, insensitivity to growth-inhibitory signals, sustained angiogenesis, limitless replicative potential, and

the ability to metastasize and invade surrounding tissues (Hanahan and Weinberg, 2000). It was proposed that these 6 key alterations in cells were shared in most, if not all, human cancer types. In light of the advances in cancer biology research that took place over the course of the following decade, Hanahan and Weinberg updated these original 6 hallmarks in 2011 to include two key enabling characteristics that allow cells to acquire the other 6 core hallmarks: genomic instability and mutation, and tumor-promoting inflammation (Hanahan and Weinberg, 2011). The authors also added two other hallmarks involved in the pathogenesis of the majority of cancers: reprogramming cellular metabolism to support neoplastic proliferation, and the ability to avoid destruction by the immune system. Because these latter two hallmarks are still undergoing validation and are not yet fully generalized, they are referred to as “emerging hallmarks” (Hanahan and Weinberg, 2011).

The characterization of the key hallmarks of cancer has sparked a fundamental shift in approaching cancer treatment. A decade ago, cancer therapy largely consisted of blanket cyotoxic chemotherapy, in which almost every patient received a standard drug cocktail. Today, treatments that target specific hallmarks, or combinations of hallmarks, are widely used (Figure 1).

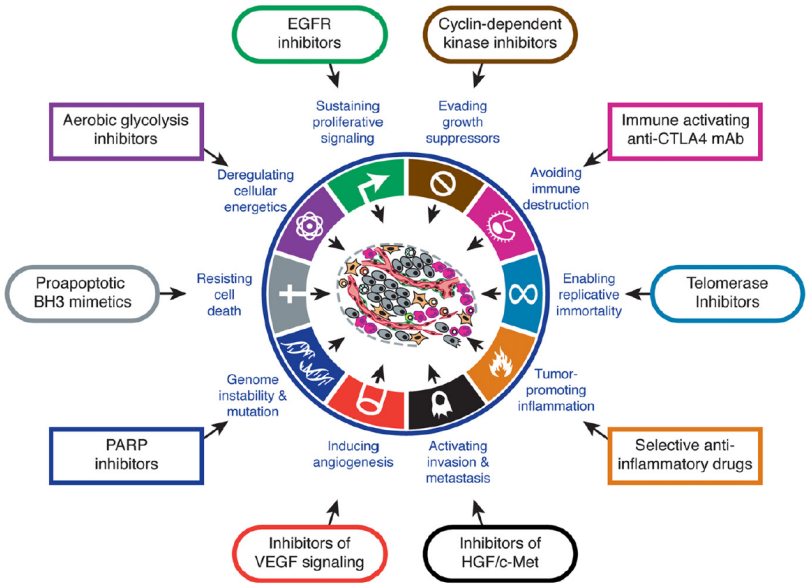

Figure 1-1: **Therapeutic Targeting of the Hallmarks of Cancer.** Examples of the types of drugs that interfere with each of the hallmarks of cancer necessary for tumor growth and progression. These drug categories are either being developed and are in clinical trials, or have been approved for use in the clinic. Figure is from Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646-

For example, the monoclonal antibody Bevacizumab (Avastin), blocks the pro-angiogenic factor VEGF-A. Finally, large-scale genomic, proteomic, transcriptomic, and metabolomic profiling of a patient's tumor cells provides an ever more accurate prediction of how tumors will respond in the short- and long-term to various therapies, thus paving the way to more personalized cancer treatment.

1.2 Breast Cancer

Breast cancer is the most common cancer affecting women in the United States today. Approximately 1 in 8 American women will develop breast cancer over the course of her lifetime, 232,340 new cases of breast cancer are expected to be diagnosed in 2014, and 39,620 breast cancer deaths are expected to occur (DeSantis et al., 2014). While the average 5-year survival rate for localized breast cancer is above 95%, the survival rate of metastatic breast cancer is only 25%, indicating that metastasis is the main cause of mortality in breast cancer patients (Howlader et al., 2014).

There are two main types of breast cancer: ductal, arising from the milk ducts, and lobular, arising from the milk-producing glands. Both of these can be found in in situ and invasive forms. Ductal carcinoma in situ, or DCIS, is characterized by the growth of non-invasive neoplastic cells that have not yet invaded the surrounding tissue. Invasive ductal carcinoma, the most commonly diagnosed type of breast cancer, involves neoplastic cells that have the ability to invade the surrounding tissue and potentially metastasize.

The molecular characteristics of breast tumors were described in the early 2000s, through gene expression profiling (Sorlie et al., 2001; van 't Veer et al., 2002). Breast tumors typically fall under 3 main subtypes: estrogen receptor (ER) positive, HER2-positive, and triple negative. Approximately 70% of breast tumors are ER-positive; the tumor cells have enriched expression of estrogen receptors, which allows for enhanced binding of estrogen, prompting cell survival and proliferation. ER-positive tumors typically respond well to

endocrine therapy, which consists of treatment with either tamoxifen, which blocks the estrogen receptors and thus blunts the downstream signaling pathways, or aromatase inhibitors, which inhibit the production of estrogen altogether (Lumachi et al., 2013). In HER2-positive tumors, expression of the human epidermal growth factor receptor 2 is amplified, leading to aberrant signaling that triggers the PI3K/Akt/mTOR pathway, which in turn drives angiogenesis, cell proliferation, survival, and migration (Krishnamurti and Silverman, 2014). Although these tumors tend to be more fast-growing and aggressive, treatment in the form of the monoclonal antibody Trastuzumab (Herceptin), which binds to the extracellular domain of HER2 and blocks downstream signaling, is effective in some patients. However, some patients exhibit disease progression in spite of initially responding to Trastuzumab. This is because HER2 can both homo- and heterodimerize to initiate downstream signaling, and thus treatment with other monoclonal antibodies, such as Pertuzumab, that bind the dimerization domain of HER2, are necessary (Baselga, 2010). Finally, triple negative tumors, that is, those that are ER-negative, progesterone receptor-negative, and HER2-negative, are the most aggressive types of tumors with the worst patient prognosis, since many tumors undergo metastasis. Patients with triple negative tumors often have a higher prevalence of *BRCA* gene mutations (Greenup et al., 2013). Triple negative breast cancer is difficult to treat, because tumors do not respond to endocrine therapy or most other targeted therapies, leaving chemotherapy as the only viable treatment option (Hudis and Gianni, 2011).

1.3 Breast Cancer Metastasis

Surgical intervention and adjuvant therapy can treat primary tumors that remain local. It is when the tumor becomes more aggressive and metastasizes, and the resulting secondary tumors become resistant to various forms of therapy, that breast cancer becomes essentially incurable. Metastasis is the main cause of death in the majority of breast cancer patients, and yet it is quite possibly the least understood facet of cancer pathogenesis. For a tumor cell to metastasize, it has to accomplish several tasks (Figure 2): 1) gain the ability to migrate and invade, 2) intravasate into the circulatory system, 3) travel through the circulatory system to a distant organ, 4) extravasate out of the circulatory system into organ tissue, and 5) establish micrometastases in the new organ (Nguyen et al., 2009).

Carcinomas are tumors that arise from epithelial tissues, and, similar to their tissue of origin, are also constrained by the basement membrane (BM), a specialized extracellular matrix (ECM) that functions to protect, organize, and polarize epithelial cells to form epithelial tissues and acts as a barrier against cell invasion (Glentis et al., 2014). In order for tumors cells to metastasize, the BM must first be breached. This is accomplished by cells expressing matrix metalloproteases (MMPs), which degrade and remodel the basement membrane, allowing cells to invade through (Kessenbrock et al., 2010). A number of ECM-degrading MMPs, such as MMP1, 2, 13, and 14 are involved in this process (Kessenbrock et al., 2010).

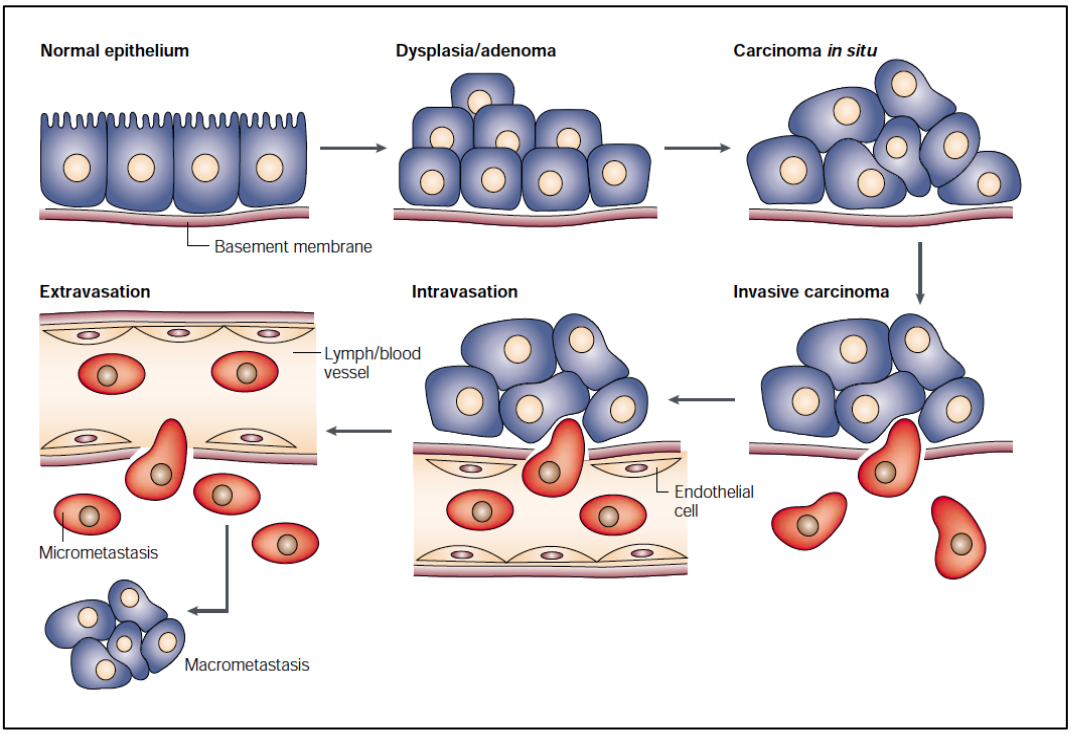

Figure 1-2: The multiple steps of tumor cell metastasis. Figure adapted from Thiery, J.P. 2002. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2:442-454.

MMP14 in particular, also known as MT1-MMP, has been shown to be important in collagen turnover; by first cleaving collagen fibers, MT1-MMP allows cells to re-orient the collagen fragments into parallel bundles, thereby forming a track that facilitates cellular migration and invasion (Friedl and Wolf, 2008). Once the BM is breached, tumor cells then enter the tumor microenvironment, also known as the stroma. The stroma becomes more "reactive" over the course of tumor progression; tumor cells that breach the BM come in contact with cancer-associated fibroblasts and tumor-associated macrophages, both of which secrete growth factors and cytokines that further

increase the aggressive behavior of the BM-breaching carcinoma cells (Bissell and Hines, 2011; Kalluri and Zeisberg, 2006).

Once the carcinoma cells have successfully intravasated into the circulatory system, they can spread widely all over the body. Carcinoma cells that are traveling through the circulatory system are known as circulating tumor cells (CTCs), and it is these cells that experience the main bottleneck in metastasis: surviving the journey through the blood/lymphatic vessels. CTCs must be able to survive without any attachment to the ECM. Epithelial cells that are not attached to any kind of matrix undergo anoikis, a type of apoptosis that arises through lack of anchorage to the ECM. CTCs use a variety of strategies to overcome anoikis, such as modifying cell surface integrin expression, bypassing integrin signaling through hyperactivation of tyrosine kinases, and bypassing metabolic stress (Guadamillas et al., 2011). CTCs must also survive damage caused by hemodynamic shear forces exerted upon them while in the blood vessels, and predation by immune cells, especially natural killer cells.

Upon extravasating out of the circulatory system, the carcinoma cells must then colonize new tissue to ultimately form secondary tumors. Establishing secondary tumors in a distant organ is fraught with difficulties, thus representing another metastatic bottleneck. After undergoing the stress of extravasation, cells must attempt to colonize and proliferate in a potentially hostile environment with a lack of both pro-survival signals and a supportive stroma, and exposure to immune cells (Vanharanta and Massague, 2013). It is

most likely for this reason that metastatic latency – that is, the time between the diagnosis of the primary tumor and the emergence of detectable metastatic lesions (Vanharanta and Massague, 2013) – takes place. While the cause of metastatic latency is still currently under investigation, there are a few possible reasons as to why carcinoma cells can take several years to form secondary tumors, including slow attrition over a long period of time, growth arrest (also known as dormancy), and cycles of cell proliferation and apoptosis, resulting in no net gain of cell number (Valastyan and Weinberg, 2011). Tsai et al. also suggest that the inability of disseminated cells to revert the epithelial-mesenchymal transition, discussed below, could also contribute to tumor dormancy (Tsai et al., 2012). The fact that carcinoma cells have difficulty establishing metastatic colonies in distant organs is not a new idea. Over 100 years ago, Stephen Paget published the “seed and soil” hypothesis, in which he explained that certain tumor cells (the “seeds”) have a specific affinity for only a few organs (the “soil”). Metastasis, Paget reasoned, could only take place if the seed and the soil were compatible (Fidler, 2003). Indeed, breast carcinomas metastasize most often to the lungs, bone, and brain (Chaffer and Weinberg, 2011; Valastyan and Weinberg, 2011). In a different study, melanoma cells metastasized to subcutaneous grafts of lung tissue, but did not metastasize to subcutaneous grafts of renal tissue, despite being similarly vascularized (Hart and Fidler, 1980), thereby recapitulating the propensity of melanomas to metastasize to the lungs.

1.4 Epithelial-Mesenchymal Transition (EMT)

To detach from the primary tumor and breach the BM, carcinoma cells first need to break down cell-cell junctions, become more motile, remodel cell-matrix adhesion sites, and invade through the ECM. A developmental program termed Epithelial-Mesenchymal Transition (EMT) enables tumor cells to obtain these properties (Thiery, 2002).

EMT was first described in chick embryos by Elizabeth Hay in the 1960s (Hay, 1968). Using the primitive streak, a structure that is indicative of the early signs of gastrulation in reptilian, avian, and mammalian development (Acloque et al., 2009), as a model, Hay found that epithelial cells were able to undergo phenotypic changes that effectively “transformed” them into mesenchymal cells (Hay, 1968). Because it is now known that this transformation is reversible – mesenchymal cells that have undergone EMT can revert back to an epithelial state through a process called mesenchymal-epithelial transition (MET) – the term “transition” is preferred (Acloque et al., 2009). While EMT was first discovered during development, it also plays a role in other processes, such as wound healing, tissue regeneration, and metastatic cancer. To reflect the fact that EMT occurs in different settings with different functional consequences, a proposal was made to classify EMT into three distinct subtypes (Kalluri, 2009). Type 1 EMTs take place during development, with the ultimate purpose of generating new tissues with a variety of functions. EMT is an integral part of embryogenesis: without the formation of mesenchymal cells that have the ability to move and differentiate

into different tissues, organogenesis would fail to take place (Thiery and Sleeman, 2006). In mammalian development, EMT first takes place during implantation, when trophoblasts located at the tip of the chorionic vili undergo EMT and invade into the endometrium, anchoring the placenta (Lim and Thiery, 2012). The next important instance of developmental EMT takes place during gastrulation, when, in the case of mammalian development, epiblast cells (primitive epithelial cells) move to the midline of the embryo, forming the primitive streak. These epiblast cells then undergo EMT: they detach from the epithelial layer and become internalized, thereby forming the mesoderm and endoderm of the embryo, and the epiblasts left behind become the ectoderm (Acloque et al., 2009). EMT also takes place during vertebrate neural development when epithelial cells located near the dorsal midline of the neural tube undergo EMT and become migratory neural crest cells, which then migrate over long distances and differentiate to eventually form a variety of different tissue subtypes, such as pigment cells, bone and cartilage of the jaws, and ganglia of the peripheral nervous system (Acloque et al., 2009). Formation of these derivatives often requires an MET event (Nieto, 2013), further underscoring the fact that EMT is reversible.

Type 2 EMTs arise during wound healing, tissue regeneration, and organ fibrosis. EMTs in this category occur initially as part of a healing process involving the generation of fibroblasts that secrete ECM molecules in response to inflammation. During wound healing, EMT ceases once inflammation is abrogated, but in the case of organ fibrosis, EMT can continue in response to

the ongoing inflammatory response, ultimately resulting in organ degeneration and failure (Nieto, 2013). This is most often observed in fibrosis of the kidney, liver, lung, and intestine (Kalluri and Weinberg, 2009)

Finally, Type 3 EMT takes place in cancer cells of epithelial origin that have undergone genetic and epigenetic changes that favor tumor formation, thereby making them fundamentally different from untransformed epithelial cells. These alterations, affecting mainly oncogenes and tumor suppressor genes, conspire with the EMT regulatory network to produce outcomes that are vastly different from the other two types of EMT; the main result is that carcinoma cells that undergo EMT gain the ability to invade and metastasize (Kalluri and Weinberg, 2009). The reversibility of EMT is especially necessary during metastasis so that cells are able to proliferate, re-epithelialize, and form secondary metastases (Tsai et al., 2012).

1.5 Molecular Mechanisms of EMT

Epithelial cells maintain specialized cell-cell junctions, including tight junctions, adherens junctions, and desmosomes, that are important for epithelial integrity. During EMT, these junctions are broken and the proteins that form them are degraded. Dissolution of tight junctions is characterized by a decrease in the occludin and claudin proteins, along with the loss of the protein zonula occludens 1 (ZO-1) (Lamouille et al., 2014). The prototypical epithelial cell marker, E-cadherin, is also downregulated during EMT, ultimately resulting in the loss of adherens junctions. E-cadherin transcriptional

repression, promoter methylation, protein phosphorylation and degradation (Tsai and Yang, 2013), all contribute towards stabilizing the loss of epithelial junctions. Disassembly of the epithelial junctions also results in a loss of apical-basal polarity through destruction of the PAR complex (Huang et al., 2012).

In addition to the loss of cell-cell junctions, cells undergoing EMT also experience cytoskeletal changes, allowing for greater cell motility. Alterations to the cytoskeleton include activation of RhoGTPases, which are responsible for controlling actin dynamics. RhoA promotes actin stress fiber formation and regulates cytoskeletal changes involved in cell-cell and cell-matrix adhesion, Rac1 is responsible for lamellipodia and membrane ruffle formation, and Cdc42 is involved in filopodia formation (Yilmaz and Christofori, 2009). Another noteworthy cytoskeletal change that takes place during EMT is the shift in intermediate filament from cytokeratin to vimentin, which has a positive correlation with increased invasiveness and metastasis and is thus a consistent marker of Type 3 EMT. A number of other EMT effector molecules that are important for cell migration, such as N-cadherin, CD44, and integrin β6 are induced during EMT, and are also commonly used to define the onset of EMT in carcinoma cells (Tsai and Yang, 2013).

Triggering EMT requires transcriptional modification of several genes involved in cell-cell adhesion, cell migration, and invasion. These changes are orchestrated by three groups of core transcription factors that regulate EMT: the Snail zinc finger family, the ZEB (zinc finger E-box-binding) family, and the

basic helix-loop-helix family (Lamouille et al., 2014; Tsai and Yang, 2013). The Snail family of transcription factors, namely Snail1 and Snail2 (also known as Slug), function by directly binding to E-boxes on the E-cadherin promoter, thereby repressing its transcription. The Snail1 in particular can cooperate with the transcription factor ETS1 to stimulate MMP expression (Lamouille et al., 2014). The ZEB family of transcription factors, Zeb1 and Zeb2, also bind to E-boxes in the E-cadherin promoter (Tsai and Yang, 2013), but can also initiate expression of the serine protease TMPRSS4, as well as repress expression of various cell polarity genes (Thiery et al., 2009). Finally, the basic helix-loop-helix transcription factors Twist1, Twist2 and E12/E47 also downregulate epithelial genes, and can also induce expression of mesenchymal genes. For example, Twist1 can act independently of Snail1 to repress E-cadherin and upregulate expression of N-cadherin and fibronectin (Zeisberg and Neilson, 2009). Twist1 can also activate programs involved in tumor cell invasion (Eckert et al., 2011), demonstrating coordination of multiple facets of EMT.

EMT can also be initiated by factors in the ECM, such as collagen and halyuronic acid, along with soluble growth factors including members of the TGFβ and fibroblast growth factor families, and epidermal growth factor (Thiery and Sleeman, 2006). TGFβ in particular can activate the core EMT regulators Snail1/2, Zeb1/2, and Twist1 (Thiery et al., 2009), and induce expression of microRNAs that repress expression of epithelial proteins (Lamouille et al., 2013). Other factors present in the tumor microenvironment, such as pro-inflammatory cytokines and exposure to hypoxia can also induce

EMT through stabilization of Snail1 and upregulation of Twist1, respectively (Tsai and Yang, 2013).

1.6 The Transcription Factor Twist1

The basic helix-loop-helix transcription factor Twist1 was originally identified in *Drosophila melanogaster* as required for dorso-ventral patterning in the early embryo, ultimately giving rise to mesoderm formation (Thisse et al., 1987). During mammalian development, Twist1 is also involved in neural tube closure. Unlike in *Drosophila*, *Twist1*$^{-/-}$ mice are able to survive gastrulation, but then the embryos exhibit lethal defects in neural tube closure at E9.5 (Chen and Behringer, 1995). Further studies in mice revealed that Twist1 is essential for the patterning of the forebrain and differentiation of the craniofacial mesenchyme (Soo et al., 2002). In humans, mutations in the *TWIST1* gene result in Saethre-Chotzen syndrome, a congenital disorder characterized by abnormal craniofacial growth caused by premature closure of one or more of the sutures between the bones of the skull (Woods et al., 2009).

A class II basic helix-loop-helix transcription factor (Teng and Li, 2014), Twist1 contains within its structure a basic domain that can interact with the core E-box sequence “CANNTG”, a helix-loop-helix domain that mediates homodimerization or dimerization with E12/E47 (E proteins make up the group of Class I basic helix-loop-helix transcription factors (Teng and Li, 2014), and a highly conserved C-terminal domain, also known as the WR domain or Twist

box (Castanon and Baylies, 2002). While the function of the WR domain is currently unclear, it has been demonstrated that it is required for the transactivation activity of Twist1 (Laursen et al., 2007), and that phosphorylation by GSK3β at key residues within the WR domain is required for proper Twist1 function (Lander et al., 2013).

It has been shown that the stability of the Twist1 protein can be regulated in a couple of different ways. First, phosphorylation of S68 by several kinases, but most notably mitogen-activated protein kinase (MAPK), prevents ubiquitination and degradation of Twist1 (Hong et al., 2011). Twist1 protein stability can also be determined by its binding partners: Twist1 is stable when bound to E47, but targeted for ubiquitination when bound to Id1 (Hayashi et al., 2007). In carcinoma cells, Twist1 expression is upregulated by a variety of factors, including SRC-1, STAT3, MSX2, HIF-1α, integrin-linked kinase, and NFκB (Qin et al., 2012).

As mentioned in the previous section, Twist1 plays a role in triggering the onset of EMT through downregulation of epithelial cell markers, and upregulation of mesenchymal markers. A seminal study by Yang et al. demonstrated that Twist1 is in fact required for EMT to take place, and is thus also essential for tumor cell metastasis (Yang et al., 2004). Indeed, subsequent investigations revealed that Twist1 is in fact expressed in a variety of aggressive cancers, including breast cancer (Vesuna et al., 2008), hepatocellular carcinoma (Lee et al., 2006), head and neck cancer (Yuen et al., 2007), cervical, ovarian, bladder, and gastic cancers (Kajiyama et al.,

2006; Shibata et al., 2008; Vecchi et al., 2007; Zhang et al., 2007). Further studies found that metastasis depends on a reversible induction of Twist1, in which EMT is activated, and then turned off (Tsai et al., 2012).

One of the consequences of Twist1-mediated EMT induction is that carcinoma cells become more invasive (Yang et al., 2004). An elegant study carried out by Eckert et al demonstrated that Twist1 is a key mediator of formation of invasive subcellular structures known as invadopodia through inducing expression of the growth factor PDGFRα (Eckert et al., 2011). Twist1-mediated induction of PDGFRα promotes Src kinase activation and invadopodia formation. This study also showed that PDGFRα expression, along with invadopodia assembly, were both required for carcinoma cell metastasis to take place (Eckert et al., 2011).

1.7 Invadopodia

Several key stages of metastasis, including invasion, intravasation, and extravasation, are thought to involve ECM degradation and remodeling. In recent years, actin-rich subcellular protrusions known as invadopodia have been shown to be critical for ECM degradation (Linder, 2007). Invadopodia consist of an actin-rich core surrounded by a number of important protein components, including cytoskeletal modulators, adhesion proteins, scaffolding proteins, and signaling molecules (Murphy and Courtneidge, 2011). The central function of invadopodia is to recruit various matrix proteases to cell-ECM focal contacts for matrix degradation. Unlike other actin-based

protrusions such as lamellipodia and filopodia that are present in normal cells, invadopodia are uniquely present in invasive cancer cells and are considered the transformed version of podosomes, which are present in highly invasive normal cells such as macrophages, osteoclasts, and dendritic cells. Figure 3 provides an outline of components that are unique to invadopodia, and that are specifically modulated in human cancers.

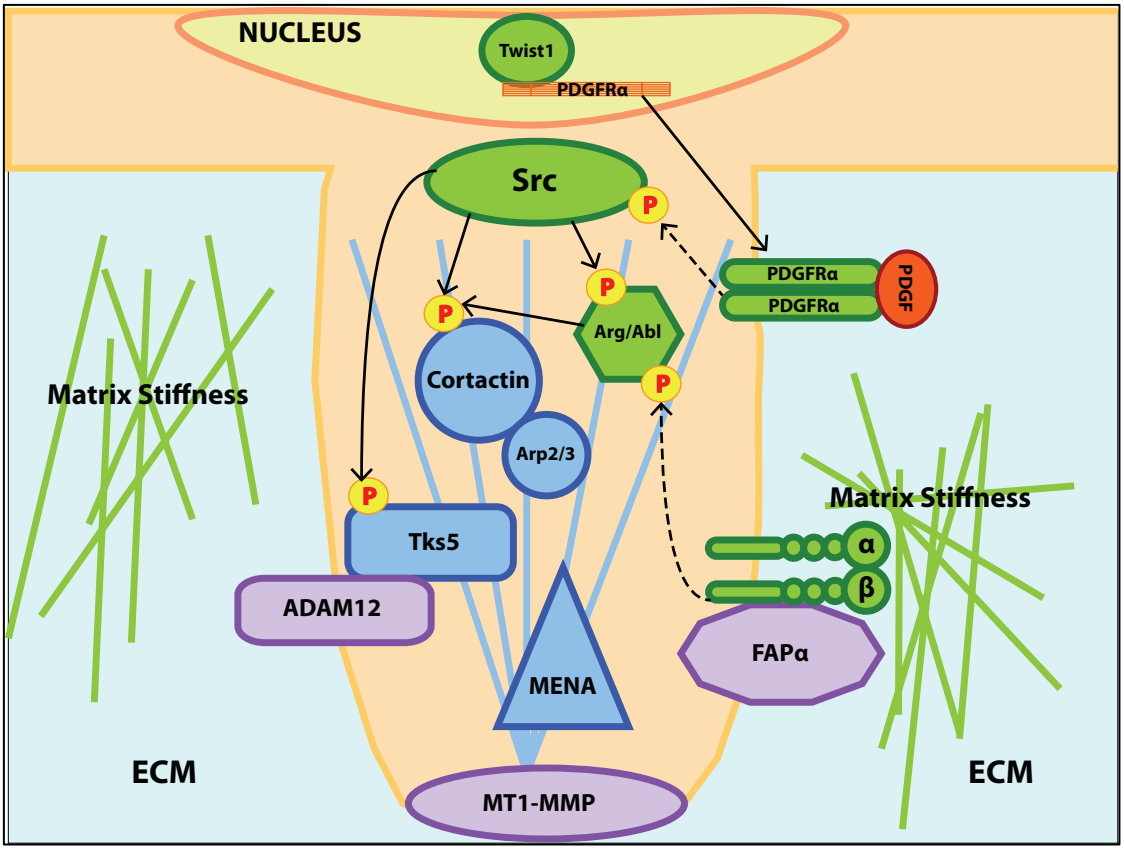

Figure 1-3: Overview of the invadopodia components and regulators. Twist1-induced expression of PDGFRα leads to increased Src kinase activity, which serves as a trigger for invadopodia formation. Src-mediated phosphorylation of the structural components cortactin and Tks5 and the Arg/Abl tyrosine kinase promotes invadopodia assembly. Integrin β1 serves as an adhesion mediator between invadopodia and ECM, an activator of Abl/Arg at invadopodia, a sensor of matrix stiffness to regulate invadopodia assembly, and a potential docking site for FAPα. Structural components of invadopodia, which include the actin core, are labeled in blue, proteases are labeled in purple, and regulatory components are labeled in green.

1.7-A Structural Components of Invadopodia

As an actin-based structure, invadopodia engage a large number of structural and regulatory proteins that control actin dynamics, such as Arp2/3, Ena/Vasp, and various small GTPases. Cortactin and MENA are both key players of actin polymerization and dynamics, therefore their roles in tumor invasion and metastasis go beyond invadopodia to general cell migration and other actin-based cellular processes. In contrast, Tks proteins are known to be more specifically involved in invadopodia formation, therefore its impact on tumor invasion and metastasis is thought to be largely due to its functions at invadopodia.

Cortactin

Cortactin is a cytoskeletal protein that when phosphorylated can recruit the Arp2/3 complex to promote invadopodia formation. Cortactin was originally identified as a Src phosphorylation target in Src-transformed chicken embryo fibroblasts (Weed and Parsons, 2001). Src binds to cortactin through direct interaction with the SH2 domain of Src (Okamura and Resh, 1995) and phosphorylates cortactin in v-Src transformed 3T3 fibroblasts (Huang et al., 1998; Weed and Parsons, 2001).

Since Src kinase plays an essential role in invadopodia regulation (discussed in detail in a later section), cortactin has been shown to be a key regulator of actin polymerization at invadopodia in response to Src activation. The association of cortactin with invadopodia was first described in MDA-MB-231 cells, in which microinjection of anti-cortactin antibodies reduced their

ability to degrade extracellular matrix (Bowden et al., 1999). Furthermore, immunoprecipitation of cortactin revealed its presence in invadopodia-enriched membrane fractions (Bowden et al., 1999). Finally, immunofluorescence indicated cortactin at actively degrading invadopodia (Artym et al., 2006; Bowden et al., 1999). Knockdown of cortactin in MDA-MB-231 cells resulted in inhibition of actin/cortactin positive puncta and matrix degradation, suggesting that cortactin is required for invadopodia formation and function (Artym et al., 2006). Cortactin localization at invadopodia coincided with phosphotyrosine puncta, which is consistent with Src regulation of cortactin phosphorylation (Bowden et al., 2006). Src activation of cortactin resulted in the localization of Nck1 and N-WASP at invadopodia, in addition to the disengagement of cofilin, all of which are required for Arp2/3-mediated actin polymerization to promote invadopodia formation (Oser et al., 2009; Yamaguchi et al., 2005a).

Since cortactin regulates various actin-based cellular programs, including invadopodia and lamellipodia formation and dynamics, its role in tumor cell invasion and metastasis is well documented. Overexpression of cortactin in NIH3T3 cells resulted in increased invasion in a Matrigel Boyden chamber assay (Patel et al., 1998). Similarly, overexpression of cortactin in MDA-MB-231 cells resulted in increased invasion, which correlated with increased metastatic bone lesions (Li et al., 2001). In contrast, overexpression of a phosphorylation deficient cortactin inhibited invasion and resulted in minimal bone metastatic lesions (Li et al., 2001). Similar results were obtained

in a hepatocellular carcinoma (HCC) model, where overexpression of wild-type cortactin in the non-metastatic HCC cell line KIM1 increased metastatic incidence without affecting primary tumor growth (Chuma et al., 2004).

Cortactin was first implicated in the progression of human cancers through gene amplification at Chromosome 11q in breast and squamous cell carcinomas (Li et al., 2001; Schuuring, 1995; Weaver, 2008). Additionally, high levels of cortactin expression are also observed in human ovarian, bladder, and lung cancer (Bowden et al., 1999; Li et al., 2001; Schuuring, 1995; Weed and Parsons, 2001). Importantly, overexpression of cortactin is associated with poor patient prognosis in breast and head and neck squamous carcinomas, further highlighting a critical role of cortactin in human tumor progression (Li et al., 2001; Weaver, 2008).

MENA

Mena is a member of the Enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) family of proteins, which are involved in the regulation of actin polymerization. Mena protein is upregulated in breast, pancreatic, colon, gastric, cervical cancers, and melanoma, with the expression of specific isoforms regulating the invasive properties of breast cancer cells (Gertler and Condeelis, 2011; Goswami et al., 2009; Philippar et al., 2008). Due to its role in actin polymerization, Mena is a logical regulator of invadopodia formation. Mena was found to co-localize with cortactin and F-actin at invadopodia (Philippar et al., 2008). Additionally, Mena-null MMTV-PyMT mammary tumors exhibited reduced invasion into the surrounding stroma (Roussos et

al., 2010). In conjunction, Mena-null mice had significantly fewer circulating tumor cells and lung metastases, when compared to control mice, suggesting that Mena expression is necessary for tumor cell intravasation and invasion (Roussos et al., 2010).

Interestingly, gene profiling in rat MTLn3 and mouse PyMT breast tumors identified an invasion specific isoform of Mena, called Mena^{INV}, whose expression was correlated with invasive ability (Goswami et al., 2009). Mena^{INV} increased invasion of MTLn3 cells into collagen gels, indicating a unique role for Mena^{INV} in carcinoma cell invasion (Philippar et al., 2008). Additionally, MtLn3 cells expressing Mena^{INV} exhibited increased membrane protrusions compared to parental MTLn3 cells (Philippar et al., 2008). In terms of metastatic progression, Mena^{INV} expression was associated with highly metastatic carcinomas in the PyMT mouse mammary tumor model (Roussos et al., 2011). Philippar et al. also observed that overexpression of Mena^{INV} in MTLn3 cells resulted in increased micrometastatic lung formation, despite no effect on primary tumor growth (Philippar et al., 2008), again indicating the importance of regulating Mena mRNA splicing in tumor invasion and metastasis (Roussos et al., 2010).

Tks Adaptor Proteins

The Tks adaptor proteins Tks4 and Tks5 are named after Tyrosine Kinase Substrate with 4 or 5 SH3 Domains, respectively. Tks proteins only contain SH3 and PH domains for protein-protein and protein-lipid interactions;

therefore they are thought to serve as adaptor proteins that recruit other proteins and lipids(Courtneidge et al., 2005) for invadopodia assembly.

The role of Tks5 in invadopodia was first discovered using a Src substrate screening assay, and further characterized based on its localization to invadopodia in Src-transformed fibroblasts (Seals et al., 2005). Tks5 is required for both invadopodia formation and invasion activity in a variety of human cancer cell lines, as knocking down Tks5 reduced matrix degradation activity and invasion (Eckert et al., 2011). Likewise, introduction of Tks5 into the human breast epithelial cell line T47D, which lacks endogenous Tks5 expression, promoted invadopodia formation (Seals et al., 2005). Tks5 was shown to bind to ADAM12 (Abram et al., 2003), a metalloproteinase associated with invadopodia. Furthermore, Tks5 is also associated with the actin regulatory protein N-WASP (Oikawa et al., 2008) and involved in the recruitment of AFAPA-110, p190RhoGAP, and cortactin to invadopodia (Crimaldi et al., 2009). Finally, Tks5 binds to $p22^{phox}$, a part of the NADPH oxidase complex that generates reactive oxygen species (ROS), which facilitates invadopodia assembly and function (Diaz et al., 2009).

The family member Tks4 also localizes to invadopodia in Src-transformed cells and is required for invadopodia assembly (Buschman et al., 2009). However, Tks4 and Tks5 seem to play non-redundant roles in invadopodia function. Cells lacking Tks4 formed actin puncta resembling invadopodia, but these cells failed to degrade ECM components, even in the presence of high levels of Tks5 (Buschman et al., 2009). This is thought to be

due to a crucial role of Tks4 in recruiting MT1-MMP to invadopodia since no MT1-MMP was detected in the rudimentary invadopodia present in Tks4 knockdown cells (Buschman et al., 2009).

To test the role of Tks5 in tumor metastasis, Eckert et al. showed that knocking down Tks5 in Ras-transformed human mammary epithelial cells that overexpress Twist1 inhibited both local invasion and the ability of these cells to form lung metastases, while primary tumor formation rates were not altered (Eckert et al., 2011). These data strongly indicate that Tks5, and likely its role in invadopodia assembly, are required for the early steps of metastasis. To test whether Tks5 functions during extravasation and metastatic outgrowth, Blouw et al. injected Src-transformed 3T3 cells with Tks5 knockdown into immunocompromised mice via tail vein. While Tks5 knockdown did not significantly affect the number of lung colonies, the metastases derived from the cells with Tks5 knockdown were significantly smaller (Blouw et al., 2008). These data suggest that Tks5 could be further required for the expansion of secondary tumors in distant sites.

1.7-B Regulatory Components of Invadopodia

Since extracellular matrix is essential for cell survival and proliferation, invadopodia-mediated matrix degradation is a highly regulated process. Understanding the upstream inducing signals of invadopodia formation and function is still in its infancy. A significant numbers of signaling regulators, including EGFR, PDGFR, PI3 kinases, c-Met have been implicated in

invadopodia regulation in various cancer cell lines. Since many of them play critical roles in multiple cellular processes in cancer, including cell proliferation and apoptosis, it is difficult to attribute their functional impact on tumor progression specifically to invadopodia. The following key upstream signaling pathways have been more uniquely implicated in invadopodia function during tumor progression and metastasis.

Phosphorylation via Src and Arg tyrosine kinases

Tyrosine phosphorylation of many core components is critical to trigger invadopodia assembly and function. Especially, two tyrosine kinases, Src and Arg, stand out as essential activators of invadopodia.

Src kinase

The Src kinase is the founding member of the Src family of non-receptor tyrosine kinases (Summy and Gallick, 2003), of which, Src is the only family member that is uniquely linked to invadopodia. The role of Src in invadopodia formation was first described by Chen et al., where Rous sarcoma viral (RSV) transformation of chicken embryonic fibroblasts resulted in actin rosette, or podosome formation. Additionally, RSV mediated cellular transformation correlated with the appearance of $pp60^{Src}$ accumulation at these rosettes (Chen et al., 1985). Similarly, invadopodia formation was enhanced with constitutive expression of active c-Src, as evidenced by co-localization of F-actin and cortactin staining (Balzer et al., 2010). In contrast, overexpression of a kinase inactive c-Src and knockdown of c-Src by RNAi showed decreased invadopodia formation and degradation activities (Bowden

et al., 2006; Kelley et al., 2010; Mader et al., 2011). Closer examination of protrusion formation in MDA-MB-231 cells revealed that overexpressing wild-type Src or constitutively active Src exhibited invadopodia extension into the collagen gel (Bowden et al., 2006).

Src kinase has been shown to play a major role in the invasive process. Expression of c-Src in SYF (src-/-, yes-/-, fyn-/-) murine embryonic fibroblasts (MEF) promoted invasion, while Ras^{V-12} failed to do so in a Boyden chamber assay (Chan and Chen, 2012). Similarly, MDA-MB-231 breast cancer cells treated with Src siRNAs exhibited reduced matrix degradation and invasion through matrix-coated chambers (Mader et al., 2011). Treatment with Src inhibitors, Dasatinib, PP2, or SU6656, in MDA-MB-231 cells reduced invasion through Matrigel, indicating the importance of Src activity for tumor cell invasion into the extracellular matrix (ECM) (Sanchez-Bailon et al., 2012).

Although Src was originally isolated as an oncogene, recent studies suggest a more critical role for Src in tumor metastasis (Chan and Chen, 2012; Ishizawar and Parsons, 2004; Yeatman, 2004). Specifically, c-Src activity is correlated with increased bone metastases, poor clinical prognosis, and reduced survival for breast and colon carcinoma patients (Aligayer et al., 2002; Sanchez-Bailon et al., 2012; Zhang et al., 2009). Src gene deletion in MMTV-polyoma middle T antigen (PyMT) mammary tumor models resulted in a reduction in circulating tumor cells, despite no defect in primary mammary tumor initiation and proliferation (Wang et al., 2009). Similarly, Src siRNA treated L3.6pl pancreatic cancer cells exhibited a reduction in lung and liver

metastasis (Trevino et al., 2006). c-Src was required for formation of metastatic lung colony formation by H-Ras^{V-12} expressing SYF (src-/-, yes-/-, fyn-/-) MEF cells, supporting the role of Src in tumor progression (Chan and Chen, 2012). Likewise, BoM-1833, a bone metastatic derivative of MDA-MB-231 cells, which were injected into recipient mice, showed increased survival and reduced bone metastases upon treatment with Src siRNA (Zhang et al., 2009).

Since Src activity plays a prominent role in cancer progression, it becomes an ideal therapeutic target. The Src selective inhibitor, SU6656, has been found to inhibit Src kinase activity, as evidenced by reduced levels of phospho-Y418-Src. SU6656 was also found to significantly reduce invadopodia formation, as well as migration and invasion of the human breast cancer line MDA-MB-231 (Balzer et al., 2010). KX2-391 is a first-in-class Src selective inhibitor on clinical trial that targets the unique Src substrate binding site (Puls et al., 2010). KX2-391 has shown promising preclinical data, with KX2-391 treatment, in combination with paclitaxel, resulting the regression of pre-established MDA-MB-231 xenograft tumors (Anbalagan et al., 2012). Additionally, KX2-391 treatment led to reduced metastasis formation of MDA-MB-231 tumors the lung and liver (Anbalagan et al., 2012). Phase I trials in patients with solid tumors showed that KX2-391 is well tolerated and demonstrated preliminary antitumor activity, with several patients displayed halted disease progression (Naing et al., 2013).

Abl/Arg

Similar to Src kinase, the Abl family of non-receptor tyrosine kinases, which includes c-Abl and the Abl-related gene (Arg/Abl2), plays an important role in tumor progression in human leukemia, non-small cell lung cancer, breast cancer, melanoma, and pancreatic cancer (Lin and Arlinghaus, 2008). Specifically, c-Abl and Arg kinases activities have been shown to correlate with poorly differentiated and highly invasive breast cancer lines (Srinivasan and Plattner, 2006). The role of Abl/Arg kinase was initially hypothesized in invadopodia formation due to previously known activation of Abl by Src kinases. More specifically, PDGF and EGF stimulation of fibroblast and breast cancer cells resulted in c-Abl activity through Src and Fyn phosphorylation (Plattner et al., 1999; Srinivasan and Plattner, 2006). Treatment of various highly invasive breast cancer cell lines with Src inhibitor SU6656 reduced c-Abl and Arg activity (Srinivasan and Plattner, 2006). Src activation of Arg is required for cortactin phosphorylation and actin polymerization at invadopodia, as knockdown of Arg in MDA-MB-231 cells resulted in no cortactin phosphorylation and reduced F-actin barbed end generation (Mader et al., 2011).

The Abl family of non-receptor tyrosine kinases has also been localized directly to the invadopodia structure. YFP-tagged wildtype and constitutively active Arg co-localized with cortactin positive invadopodia, in Src expressing NIH3T3 cells, while kinase-inactive Arg expression disrupted invadopodia formation (Smith-Pearson et al., 2010). Similarly, immunofluorescence staining

of Arg in MDA-MB-231 cells showed co-localization with Tks5 positive invadopodia (Mader et al., 2011).

Abl and Arg kinases also play a significant role in tumor invasion and metastasis. Knockdown of Abl and Arg reduced the ability of MDA-MB-231, and its metastatic derivatives, to degrade extracellular matrix and invade (Smith-Pearson et al., 2010; Srinivasan and Plattner, 2006). Inhibition of Abl kinase activity with STI571, an Abl/Arg inhibitor, reduced invasion of MDA-MB-435S breast cancer cells in a Matrigel invasion assay (Srinivasan and Plattner, 2006). MDA-MB-231 cells, expressing Arg and Abl shRNA constructs, showed fewer circulating tumor cells *in vivo* compared to control tumor-bearing mice (Gil-Henn et al., 2013). Similarly, Gil-Henn et al. showed that STI571-treated mice showed fewer circulating tumor cells than control mice bearing MDA-MB-231 tumors (Gil-Henn et al., 2013). These results indicate that Abl kinase activity is required for tumor cell intravasation.

A number of tyrosine kinase inhibitors with dual specificities toward Src and Abl family tyrosine kinases, including Dasatinib, Saracatinib, and Bosutinib, have been developed and showed promising activities against tumor invasion and metastasis in several solid tumors preclinical studies. Specifically, these inhibitors were found to significantly reduce invadopodia formation, as well as migration and invasion of the human breast cancer line MDA-MB-231 (Jallal et al., 2007; Mayer and Krop, 2010; Sanchez-Bailon et al., 2012). Additionally, treatment of mice with Dasatinib led to reduced formation of bone metastases by BoM-1833 cells (Zhang et al., 2009). Similar

results have been observed in pancreatic tumors, with Dasatinib treatment leading to a reduction in primary tumor growth and metastasis formation by L3.6pl cells (Trevino et al., 2006). However, these inhibitors have shown limited activity in monotherapy trials (Puls et al., 2010). It is important to note, however, that completed clinical trials studying the efficacy of Src/Abl inhibitors have been conducted in unselected cancer patients. Many ongoing trials using biomarkers (such as cortactin phosphorylation) to pre-select patients who are more likely to benefit from Src/Abl inhibition, hold promise for the future success of Src/Abl inhibitors in cancer treatment (Puls et al., 2010).

Integrin-mediated signaling

Given that integrins are the key connection between cell protrusions and the surrounding extracellular matrix, it is not surprising that integrins play important roles in invadopodia regulation. Integrin clustering at invadopodia was first described in Rous sarcoma viral transformation of chicken embryonic fibroblasts (RSVCEF). RSVCEFs cultured with fibronectin coated beads exhibited invadopodia formation, which was associated with β1 staining (Mueller and Chen, 1991). Interestingly, fibronectin positive vesicles were found to co-stain with β1 integrin; further validating the functional role of invadopodia in matrix degradation (Mueller and Chen, 1991). In contrast, murine embryonic fibroblasts overexpressing Src and depleted for β1 integrin exhibit reduced rosette formation (Destaing et al., 2010). In addition, treatment of SCC61 squamous cell carcinoma cells with an integrin blocking peptide or a β1 integrin blocking antibody led to a reduction in actively degrading

invadopodia per cell (Branch et al., 2012). Lastly, knocking down β1 integrin in MDA-MB-231 and MTLn3 mammary adenomacarcinoma cells led to a reduction in mature invadopodia formation (Beaty et al., 2013).

How integrins regulate invadopodia function has not been clearly elucidated. Various studies indicate that integrins promote invadopodia maturation by serving as a docking station for various proteases and/or activating Arg kinase for actin stabilization. Laminin peptide activation of β1 integrin in LOX human melanoma cells led to increased invadopodia-mediated degradation (Nakahara et al., 1996) due to increased seprase/FAPα recruitment to invadopodia via binding to β1 integrin (Bowden et al., 1999; Nakahara et al., 1996). More recently, β1 integrin has been found to activate Arg kinase. FRET-based experiments point to direct interaction between β1 integrin and Arg at Tks5 positive invadopodia (Beaty et al., 2013).

Changes in integrin-mediated adhesion signaling complexes are known to play an important role in tumor cell proliferation, migration and survival (Desgrosellier and Cheresh, 2010). Specifically, loss of β1 integrin in MMTV-driven Erb2 breast tumor mice reduced Y416 c-Src phosphorylation and metastatic lesion formation in the lungs (Huck et al., 2010). Similarly, tail vein injection of MDA-MB-435 breast cancer cells, expressing a constitutively active mutant αvβ3 exhibited enhanced metastatic lung colonization (Huck et al., 2010). To date, it is unclear which integrin subunits are the predominant forms required for invadopodia function. Since the focus of current integrin-targeted therapies has been on their anti-angiogenic properties, their potential

as an anti-metastatic treatment via invadopodia inhibition requires identifying and targeting invadopodia-specific integrins in the near future.

Matrix stiffness and mechano-regulation

Recent evidence has implicated matrix stiffness in tumor progression and increased incidence in metastasis, through increased collagen deposition (Levental et al., 2009; Parekh and Weaver, 2009; Ramaswamy et al., 2003). Several elegant studies show that increasing matrix stiffness without altering biochemical components of extracellular matrix (ECM) can induce a malignant phenotype, suggesting that mechanical force exerted by stiff ECM could play a critical role in tumor invasion and metastasis (Paszek et al., 2005). In mice, Lysyl oxidase-mediated collagen crosslinking stiffens tumor ECM and promotes breast tumor progression (Erler et al., 2006). Furthermore, inhibition of lysyl oxidase (LOX) blocks tumor invasion and eliminates metastasis formation from orthotopically grown breast tumors (Yana and Weiss, 2000).

Indeed, ECM rigidity is indicated in invadopodia regulation. Specifically, Alexander et al. noted that CA1d breast cancer cells plated on increasing concentrations of gelatin showed increased invadopodia formation and ECM degradation (Alexander et al., 2008). Interestingly, two studies showed that both CA1d and 804G bladder cancer cells exhibited increased invadopodia formation when placed on matrix substrates with increasing mechanical rigidity without changing their biochemical components (Parekh et al., 2011). Furthermore, these studies showed that invadopodia-mediated ECM degradation only increased when placed on surfaces within the kPa range,

which corresponds to the stiffness in tumors (Parekh et al., 2011). While it is not well understood how matrix rigidity regulates invadopodia formation, this study suggests that activation of p130Cas and FAK via myosin II, which acts as mechanosensors that transmit mechanical signals from ECM, could play a prominent role (Alexander et al., 2008). Given the effects of increasing matrix stiffness on invadopodia functions, it is possible that mechanical forces generated by rigid tumor matrix stiffness could modulate invadopodia function to impact tumor progression and metastasis.

1.7-C Proteases Associated with Invadopodia

Given their central function to recruit proteases to cell-matrix contacts for matrix remodeling, invadopodia are shown to contain a large numbers of proteases. The proteases found at invadopodia include metalloproteases (both secreted and membrane-tethered matrix metalloproteinases [MMPs]), the ADAM (A Disintegrin And Metalloproteinase) family members, and membrane-bound serine proteases, all of which have been implicated in cancer progression and metastasis. Past research has focused on developing metalloproteinase inhibitors to suppress ECM degradation and tumor metastasis. Although these inhibitors show promising results in cell culture and tumor xenograft models, numerous metalloproteinase inhibitors have failed in clinical trial (Overall and Kleifeld, 2006). Further studies indicate that some metalloproteinases could have anti-tumorigenic effects (Freije et al., 2003). Therefore, the strategy of broadly blocking metalloproteinases to

abrogate metastasis might not be a viable approach to prevent tumor metastasis. Some proteases are unique to invadopodia and might be promising new targets in inhibiting tumor invasion and metastasis.

Metalloproteinases

MT1-MMP

MT1-MMP (also known as MMP14), a membrane-anchored metalloproteinase, is considered a central player of invadopodia-mediated ECM degradation. MT1-MMP cleaves (Yana and Weiss, 2000) several substrates in vitro, including ECM components such as fibronectin, type I, II, and III collagen, laminins, vitronectin, and aggrecans (d'Ortho et al., 1997; Fosang et al., 1998; Koshikawa et al., 2000; Ohuchi et al., 1997). Additionally, MT1-MMP is capable of activating other MMP zymogens: MT1-MMP activates MMP2 by cleaving the N-terminal prodomain of pro-MMP2 (Deryugina et al., 2001), and MMP9 is activated through an activation cascade involving MT1-MMP, MMP2, and MMP3 (Toth et al., 2003). MT1-MMP is shown to be required for the matrix degradation activity of invadopodia. Artym et al. found that cortactin aggregation initiated accumulation of MT1-MMP at invadopodia (Artym et al., 2006). This study also found that while MT1-MMP knockdown moderately impacted the initial stages of invadopodia formation, matrix degradation was strongly suppressed (Artym et al., 2006), indicating that MT1-MMP is essential for functional invadopodia.

MT1-MMP is delivered to invadopodia via multiple routes. Studies from Yu et al. show that N-WASP, which promotes actin nucleation, promotes the

delivery of MT1-MMP from late endosomes to invadopodia (Steffen et al., 2008; Yu et al., 2012). MT1-MMP can also be mobilized by the Rab8-dependent secretory pathway and delivered to collagen-contact sites (Bravo-Cordero et al., 2007). Finally, MT1-MMP can also be internalized by both clathrin- and caveolae-mediated endocytosis (Murphy and Courtneidge, 2011; Poincloux et al., 2009) and this internalization serves to recycle MT1-MMP back to invadopodia when needed.

A key inducer of invadopodia, Src kinase, has also been shown to directly regulate the delivery of MT1-MMP to invadopodia. Src-mediated phosophorylation of MT1-MMP in its AP2 clathrin adaptor binding domain slows endocytosis of MT1-MMP and increases matrix degradation activity (Poincloux et al., 2009). In addition, phosphorylation of MT1-MMP by Src at Tyr573 has been shown to be required for tumor cell proliferation, invasion of 3D collagen matrices, and tumor growth in nude mice (Nyalendo et al., 2008; Nyalendo et al., 2007). Finally, a recent study found that this phosphorylation was required for mono-ubiquitination of Lys581, which is involved in MT1-MMP trafficking to the cell surface and cellular invasion through collagen matrices (Eisenach et al., 2012).

An increase in MT1-MMP expression is generally associated with poor prognosis in a wide variety of human cancers, including breast, lung, melanoma, colorectal, and squamous cell carcinomas (Egeblad and Werb, 2002). MT1-MMP expression has also been directly linked to metastasis in mouse tumor models. MT1-MMP-deficient mice were bred with MMTV-PyMT

mice, and then PyMT-positive mammary glands lacking MT1-MMP were orthotopically transplanted into wild-type mice. While palpable tumors developed faster with MT1-MMP-deficient mammary glands, metastatic spread was reduced by 50% (Szabova et al., 2008). Consistent with this study, Perentes et al. injected MDA-MB-231 cells with MT1-MMP knockdown into the mammary fad pad of SCID mice (Perentes et al., 2011) and found that MT1-MMP knockdown resulted in a significant decrease in lung metastasis without affecting primary tumor growth (Perentes et al., 2011). These results suggest that MT1-MMP is required for metastatic development in vivo.

Since blocking MMP activity has failed in clinical trials as an anti-metastasis therapy, possibly due to the broad spectrum of inhibition and severe toxicities (Devy and Dransfield, 2011; Gialeli et al., 2011), new therapeutic strategies aim to target the specific MMPs that contribute to disease progression (Devy and Dransfield, 2011). A fully humanized monoclonal antibody (DX-2400, Dyax Corporation) that targets MT1-MMP at its catalytic domain showed great promise in pre-clinical studies. DX-2400 abrogated MMP2 cleavage on tumor and endothelial cells, blocked angiogenesis, and reduced tumor formation and metastasis (Devy and Dransfield, 2011; Devy et al., 2009). Another humanized antibody targeting the non-catalytic hemopexin domain of MT1-MMP has recently shown promise in inhibiting invasion and angiogenesis in pre-clinical studies (Basu et al., 2012). The therapeutic potential of targeting MT1-MMP to inhibit invadopodia-mediated tumor invasion and metastasis holds great future promise.

ADAM Proteases

The ADAMs are a family of disintegrin and metalloproteinases that are involved in a variety of biological processes, including cell adhesion, migration, proteolysis, myoblast fusion, and fertilization (Seals and Courtneidge, 2003). Here, we focus on ADAM12 due to its more established presence at invadopodia. ADAM12 has two alternatively spliced variants: ADAM12-L, which consists of pro-, metalloprotease, disintegrin, cysteine-rich, transmembrane, and cytoplasmic domains, and ADAM12-S, which lacks the transmembrane and cytoplasmic domains(Kveiborg et al., 2008).

ADAM12 contributes to invadopodia function at multiple levels, including degrading the ECM, modulating integrin function, and functioning as a sheddase to activate growth factors (Moss and Lambert, 2002). ADAM12 is localized to invadopodia; it binds to the scaffold protein Tks5 (Abram et al., 2003), and has been found to trigger invadopodia assembly(Albrechtsen et al., 2011). The sheddase activity of ADAM12 may contribute to the overall degradation activity of invadopodia. A recent study by Días et al. demonstrated that ADAM12 expression was elevated in a Notch-dependent manner under hypoxic conditions (Diaz et al., 2013). ADAM12 promoted the ectodomain shedding of heparin-binding EGF-like growth factor, which in turn induced invadopodia formation and the invasive activity of cancer cells (Diaz et al., 2013).

ADAM12 is implicated in a variety of cancers, including breast, prostate, lung, liver, brain, and bone cancers, as well as aggressive fibromatosis. In

human breast cancer patients, Roy et al. showed that ADAM12 is a prognostic marker: urinary levels of ADAM12 increased along with disease stage (Roy et al., 2004). Transgenic mice expressing the ADAM12-S isoform driven by the MMTV-LTR promoter were bred with mice carrying the polyoma middle T (PyMT) oncogene in the mammary gland; PyMT expression in the mammary gland led to rapid formation of mammary carcinomas. Tumors in mice expressing ADAM12-S developed faster than in littermates expressing PyMT alone (Kveiborg et al., 2005). Similarly, ADAM12-S isoform significantly increased the ability of MCF-7 cells to migrate and invade, which led to a higher incidence of local and distant metastases in vivo (Roy et al., 2011). Interestingly, cells expressing a catalytically dead mutant of ADAM12-S failed to promote tumor development, indicating that the proteolytic activity of ADAM12-S is required to promote formation of distant metastases (Roy et al., 2011).

Serine Proteases

Two transmembrane type II serine proteases of the Dipeptidyl-Peptidase (DPP) family, Fibroblast Activation Protein (FAP, FAPα, also known as seprase), and DPP4, have also been associated with invadopodia. Formerly known as F19 Cell Surface Antigen, FAPα was originally identified in 1986 in cultured fibroblasts using the monoclonal antibody F19 (O'Brien and O'Connor, 2008). DPP4 and FAPα both have very similar structures, consisting of a very short cytoplasmic domain, a single-pass transmembrane domain, a β-propeller domain that is glycosylated and rich in cysteine

residues, and an α/β hydrolase domain, which contains the catalytic domain (Chen and Kelly, 2003). The two proteases are similar to one another, but not identical: DPP4 and FAPα share 68% homology at their catalytic domains (Chen and Kelly, 2003). Both DPP4 and FAPα contain exopeptidase activity and FAPα also exhibits endopeptidase activity, owing to a key alanine residue that shapes the active site (Meadows et al., 2007; O'Brien and O'Connor, 2008). Previous studies have shown that FAPα is localized at invadopodia as a complex with DPP4 (Ghersi et al., 2006), or alternatively, associated with α3β1 integrin, with the integrin serving as a docking site for FAPα (Mueller et al., 1999). The role of FAPα in invadopodia is currently unclear, but some studies suggest that its gelatinase activity may contribute to the overall degradation activity of invadopodia. Christiansen et al. found that FAPα digests collagen I into smaller fragments following initial cleavage by MMP-1, suggesting that FAPα works together with other proteases to cleave partially degraded ECM components (Christiansen et al., 2007).

A key difference between FAPα and DPP4 is that expression of DPP4 is ubiquitous throughout all tissues, whereas that of FAPα is restricted to tissues undergoing wound healing and epithelial cancers (Busek et al., 2004; O'Brien and O'Connor, 2008), thus making FAPα a unique player in tumor progression. Indeed, FAPα has been shown to be expressed in a variety of aggressive cancers, including breast, colon, and ovarian cancers, and malignant melanoma (Chen et al., 2006). Additionally, genetic deletion of FAPα inhibited tumor growth in a K-ras-driven model of endogenous lung

cancer and in a mouse model of colon cancer. Pharmacological inhibition of FAPα also attenuated tumor growth in these mouse models, indicating that FAPα is a promising target for therapeutic intervention (Santos et al., 2009). In human and mouse tumors, FAPα has been shown to be expressed in stromal fibroblasts, carcinoma cells, and immune cells (Chen et al., 2006; Henry et al., 2007; Kraman et al., 2010; Pure, 2009). What remains to be answered is whether and how FAPα in individual cell types contributes to tumor progress and whether the role of FAPα at invadopodia is critical for tumor invasion and metastasis.

Previous attempts to target FAPα for therapeutic intervention have proved to be challenging. In 2003, Phase I/II clinical trials for the humanized FAPα monoclonal antibody Sibrotuzumab failed to demonstrate measurable therapeutic activity in patients with metastatic colorectal cancer, (Hofheinz et al., 2003) with only 2 out of 17 patients having stable disease during the Phase II trial (Hofheinz et al., 2003). However, this antibody has not been shown to block any cellular or protease function of FAPα, which might explain the lack of therapeutic effects. In 2007, a small molecule inhibitor of FAPα, Talabostat, was developed to inhibit the protease activity of FAPα. Again, minimal clinical activity was observed in patients with metastatic colorectal cancer receiving Talabostat alone (Narra et al., 2007), or in metastatic melanoma patients receiving Talabostat in conjunction with cisplatin treatment (Eager et al., 2009). However, the stability of this inhibitor in vivo is thought be extremely poor, thus limiting its effectiveness.

Given these recent setbacks in targeting FAPα, efforts have turned to FAPα –mediated immunotherapy. One approach is to develop DNA vaccines to target FAPα, thus eliminating all FAPα-positive cell types in a tumor. Several groups reported that through CD8+ T cell-mediated killing, such therapy successfully suppressed primary tumor cell growth and metastasis of implanted breast and colon tumors without obvious toxicity (Lee et al., 2005; Loeffler et al., 2006). Another approach is to deliver radioisotopes specifically to the tumor site using FAPα antibodies as cargoes. Pre-clinical studies involving two humanized FAPα monoclonal antibodies (ESC11 and ESC14) labeled with the radiolanthanide ^{177}Lu have yielded promising results: both antibodies accumulated in human FAPα-positive xenografts and delayed tumor growth (Fischer et al., 2012). Given that FAPα is expressed in various cell types in a tumor, it is important to recognize that the effect of targeting FAPα on tumor progression cannot solely be explained by inhibition of invadopodia. However, these results suggest a unique approach to targeting components of invadopodia in human cancers.

1.7-D The role of Invadopodia in Tumor Invasion and Metastasis

The critical role of invadopodia in ECM degradation explains why the ability to form invadopodia largely correlates with the invasive and metastatic potential of tumor cells (Yamaguchi, 2012). Suppressing invadopodia formation by inhibiting Src, Twist1, or Tks5 have been convincingly shown to inhibit tumor metastasis in various tumor models. Invadopodia could play

critical roles during three steps of the metastatic process: invasion into the surrounding stroma, intravasation into the vasculature, and extravasation. Direct in vivo visualization and assessment of invadopodia formation during the metastatic process has proven to be challenging due to the limited numbers of invadopodia-specific markers in a 3D microenvironment. Furthermore, it remains unclear whether actin-based protrusions observed in 2D culture, including filopodia and invadopodia, share similar components or merge into one structure in 3D; thus confusing the issue of defining invadopodia in vivo.

New imaging techniques have made it possible to begin to identify invadopodia-like protrusions in vivo. Using 3D time-lapse imaging, Gligorijevic et al. (2012) observed protrusion formation by MTLn3 rat mammary adenocarcinoma xenograft tumors growing in the mammary fat pad of SCID mice. These protrusions were positive for cortactin and proteolytic activity, as evidenced by cleaved collagen 3/4 staining, indicating the presence of invadopodia in vivo (Gligorijevic et al., 2012). Looking specifically at the intravasation, Gligorijevic et al. used the photoconvertible Dendra2 protein to trace tumor cell intravasation in vivo. Control MTLn3 primary tumor cells were shown to disappear from the imaging area due to dissemination of cells into the blood stream. In contrast, knocking down N-WASP resulted in no visible dissemination (Gligorijevic et al., 2012). Yamaguchi, H. et al (2005) observed similar invadopodia-like protrusions during tumor cell intravasation using intravital imaging. GFP expressing MTLn3 cells revealed invadopodia-like

protrusions extending into the blood vessel wall (Yamaguchi et al., 2005b). These invadopodia-like protrusions were shown to help tumor cells to penetrate the ECM surrounding the blood vessel walls and to squeeze through the endothelial barrier (Yamaguchi et al., 2005b). Together, these data strongly support the notion that invadopodia are required for tumor cell intravasation.

Assessment of invadopodia formation during the extravasation process is more limited; however, protrusive structures have been identified by actively extravasating tumor cells (Ito et al., 2001; Stoletov et al., 2010). Specifically, time course observations of intra-meseneric vein injection of GFP-positive rat tongue carcinoma cells reveal clusters of tumor cells in the sinusoids (Ito et al., 2001). Over time, the authors noted focal loss of basement membrane at sinusoidal areas where extravasation was taking place (Ito et al., 2001). The loss of basement membrane indicates potential sites of active ECM degradation by invadopodia. Protrusive structures have also been identified by actively extravasating MDA-MB-435 cells expressing Twist1 upon injection into the vasculature of zebrafish (Stoletov et al., 2010). Direct imaging of tumor cells in the vasculature revealed that Twist1-overexpressing cells display large rounded protrusions (Stoletov et al., 2010), suggesting that invadopodia-like protrusions by extravasating tumor cells contribute to ECM degradation and breaking through the endothelium barrier.

1.7-E Perspectives and therapeutic implications

As discussed, recent progresses suggest an essential role of invadopodia in tumor invasion and metastasis. The specific presence of invadopodia in invasive tumor cells and their unique ability to precisely coordinate localized ECM degradation with cell movement make them ideal targets for anti-metastasis therapies.

A number of critical issues need to be resolved to put invadopodia at the forefront of tumor metastasis research and treatment. First, although actin assembly and elongation during invadopodia initiation have been extensively studied, it remains unclear what and how matrix degrading enzymes are recruited to invadopodia to perform their functions. Since broad inhibition of MMPs has not been successful in blocking metastasis, the strategy to inhibit protease recruitment could be a promising new route to specifically targeting ECM degradation and tumor invasion. Second, it remains unclear whether and how the molecular components and regulation of invadopodia and other actin-based membrane protrusions are different. Since many invadopodia components play critical roles in various cellular processes, such as proliferation, apoptosis, and migration, it is difficult to attribute all their observed activities on tumor progression to invadopodia function. Understanding such differences would further solidify the unique role of invadopodia in tumor metastasis in vivo, and lead to more specific targeting therapies against invadopodia in tumors without affecting normal cellular functions.

To move invadopodia inhibitors into therapeutic applications against tumor metastasis, we first need to determine how to apply invadopodia inhibitors for cancer treatment. As discussed, the main function of invadopodia in tumors is to promote matrix degradation and tumor invasion, but not to regulate cell proliferation or survival. Therefore, the main utility of invadopodia inhibitors should be to prevent primary and secondary metastasis occurrences, instead of inhibiting the growth of established primary tumors and metastases. Invadopodia inhibitors could be beneficial in preventing new metastasis development in a number of metastasis-prone cancer patient groups. Using breast cancer as an example, a group of cancer patients who have already developed limited metastatic diseases, such as a single brain metastasis, may use invadopodia inhibitors to prevent secondary metastasis lesions in the brain. Also patients that have presented lymph node positivity could benefit from invadopodia inhibitors to prevent distant metastasis development. Furthermore, recent gene expression profiling and biomarker studies make it possible to predict long-term metastasis occurrence and survival outcome in early-stage breast cancer patients. Invadopodia inhibitors, in combination with traditional chemotherapies, could potentially reduce metastasis development in the selected high-risk patient population based on such molecular profiling.

Another pressing issue that faces the entire metastasis field, including invadopodia research, is how to develop proper clinical trials to test anti-metastatic agents, such as invadopodia inhibitors. As discussed above,

invadopodia mainly function to promote matrix degradation, thus perturbation of their functions in tumors have little or no effect on tumor proliferation. Unfortunately, the current clinical trial system requires all anti-cancer agents to show efficacy in phase II by shrinking established primary tumors and/or distant metastases in patients before moving to phase III trials and regulatory approval. Only after approval can these agents be tested in metastasis prevention trials for more early-stage cancer patients. Since invadopodia-specific inhibitors are unlikely to shrink existing tumors and metastases, these inhibitors would fail in current phase II clinical trials even though they might be potent to prevent new metastasis occurrence. A stimulating article by Dr. Patricia Steeg (Nature 2012) has proposed that the rate of new metastasis occurrence in metastasis high-risk patients as a more appropriate end point for metastasis prevention trials (Steeg, 2012). Given the unique role of invadopodia in tumor invasion and metastasis, invadopodia inhibitors need to be tested in better-designed metastasis prevention trials to explore their full potentials in combating tumor metastasis.

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CHAPTER 2

Fibroblast Activation Protein alpha (FAPα) regulates invadopodia activity and promotes tumor metastasis

2.1 Introduction

Metastasis is associated with a poor prognostic outcome, and is the main cause of mortality in breast cancer patients. During metastasis, carcinoma cells must acquire the ability to migrate and invade through surrounding tissues to gain access to systemic circulation. Our previous studies found that expression of the basic helix-loop-helix transcription factor Twist1 is essential for the ability of tumor cells to metastasize from the mammary gland to the lungs in a mouse breast tumor model, and that Twist1 expression is highly elevated in invasive human lobular breast cancer (Yang et al., 2004). Twist1 triggers a developmental program known as the epithelial to mesenchymal transition (EMT), which involves the dissolution of epithelial cell-cell adhesion and loss of epithelial cell polarity, and the acquisition of mesenchymal characteristics, along with an increased migratory capacity (Hay, 1968). EMT, orchestrated in part by Twist1, confers the ability to migrate and invade upon tumor cells, thereby giving them the ability to metastasize.

In our prior investigations, we sought to determine how Twist1 controls EMT. We found that Twist1 plays a key role in regulating extracellular matrix (ECM) degradation to promote tumor cell metastasis, through inducing formation of subcellular invasive structures known as invadopodia.

Invadopodia are actin-rich membrane protrusions found in carcinoma cells that degrade the ECM through localization of proteases (Chen, 1989). In addition to the actin core, invadopodia are enriched with components necessary for regulating actin machinery, such as cortactin, N-WASP, and the Arp2/3 complex (Stylli et al., 2008). The Tks adaptor proteins Tks4 and Tks5 are thought to recruit other proteins and lipids for invadopodia assembly (Courtneidge et al., 2005), and the ECM degradation activity of invadopodia is mediated by a variety of proteases, chief among them being the membrane-associated matrix metalloprotease MT1-MMP (Artym et al., 2006). Invadopodia assembly and activity requires phosphorylation of different components, including cortactin (Bowden et al., 2006), Tks5 (Seals et al., 2005), and MT1-MMP (Poincloux et al., 2009) by Src kinase. Our prior study showed that Twist1 promotes invadopodia assembly through inducing the expression and activation of PDGFRα, which directly activates Src family kinases (Eckert et al., 2011). We also demonstrated that invadopodia formation is required for Twist1-mediated metastasis in vivo, as knocking down components of invadopodia significantly reduces the number of disseminated tumor cells (Eckert et al., 2011).

The molecular machinery behind the regulation of invadopodia assembly is still not completely understood (Murphy and Courtneidge, 2011), and we thus sought to determine other ways in which Twist1 is involved in invadopodia formation. Here, we describe the role of Twist1-induced serine protease FAPα in invadopodia.

2.2 Fibroblast Activation Protein Alpha (FAPα) is induced by Twist1

Our previous studies found that Twist1 is required for EMT and subsequent metastasis (Yang et al., 2004), and that Twist1 is required for invadopodia assembly (Eckert et al., 2011). We carried out DNA microarray analysis using an inducible Twist1 (Twist-ER) construct (Mani et al., 2008) to examine induction of genes downstream of Twist1 whose products are involved in invadopodia. We found that expression of Fibroblast Activation Protein Alpha (FAPα) mRNA begins increasing at 36 hours post-Twist1 induction, and reached 8-fold induction after 12 days (Figure 2-1A). FAPα protein expression also increased 3 days post-Twist1 induction (Figure 2-1B).

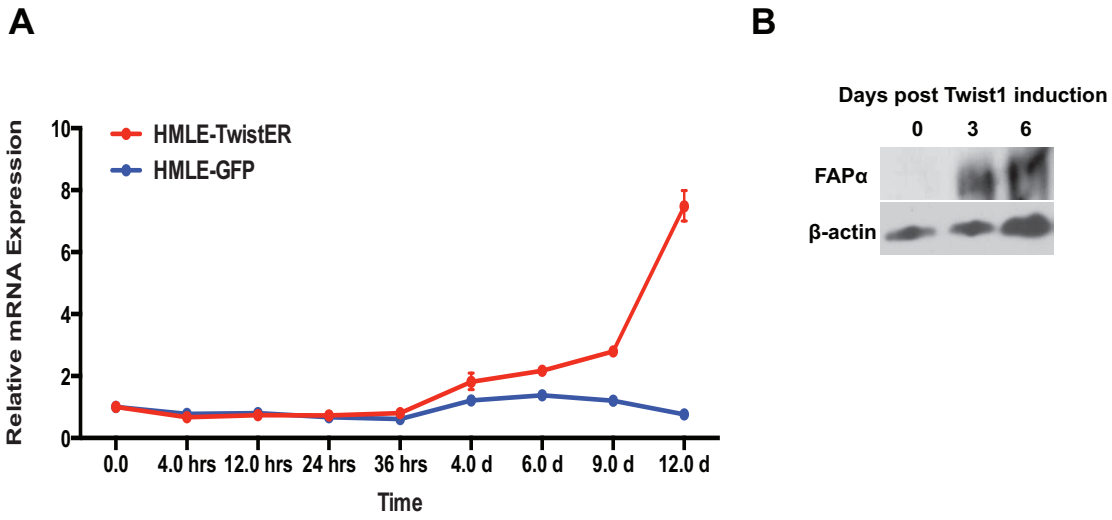

Figure 2-1: FAPα is induced by Twist1. A: FAPα mRNA levels were measured in HMLE-TwistER cells undergoing Twist1 induction with continuous administration of tamoxifen and compared to those in uninduced control cells. B: Cell lysates from induced HMLE-TwistER cells were analyzed by SDS-PAGE and probed for FAPα and β-actin.

FAPα, also known as seprase, is a type II integral serine protease best known for its expression in activated tumor-associated fibroblasts (O'Brien and

O'Connor, 2008), but it has also been shown to be expressed in tumors of epithelial origin (Chen et al., 2006). Consistent with this, we found that knocking down Twist1 using two independent shRNAs also reduces FAPα mRNA levels in the HS578T human breast cancer cell line (Figure 2-2A), and that FAPα expression is higher in human mammary epithelial cells overexpressing Twist (HMLE-Twist cells), HS578T cells, and in the human breast cancer cell line SUM1315 relative to normal human mammary epithelial cells (Figure 2-2B).

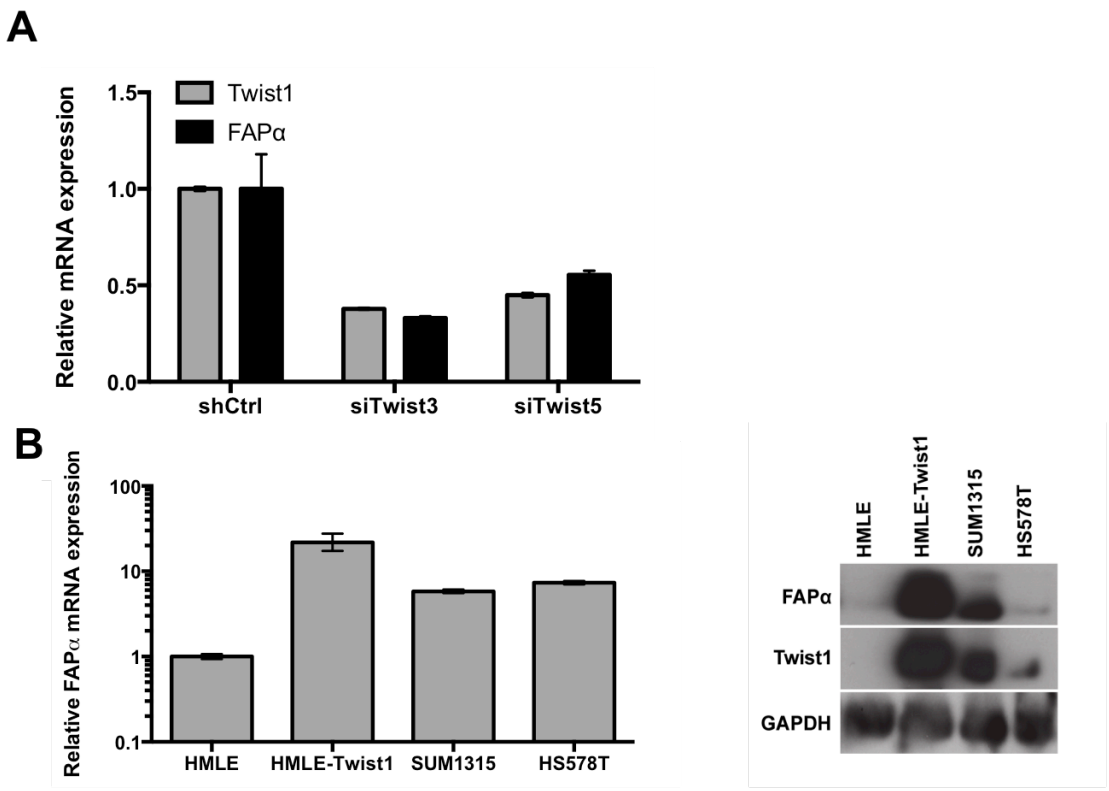

Figure 2-2: FAPα mRNA expression in human cell lines. A: Twist1 was knocked down in HS578T cells using two independent siRNAs, and then FAPα mRNA expression was measured using qRT-PCR. B: FAPα mRNA and protein levels in various human breast cancer cell lines.

2.3 FAPα expression is required for Twist1-mediated tumor metastasis

Twist1 is required for cells to metastasize from the mammary gland to the lungs; given that Twist1 induces FAPα expression, we sought to determine whether FAPα plays a role in metastasis in vivo. HMLE-Twist cells expressing either a control shRNA, or one of two independent shRNAs targeting FAPα (Figure 2-3), were labeled with GFP, transformed with oncogenic Ras, and then injected subcutaneously into nude mice.

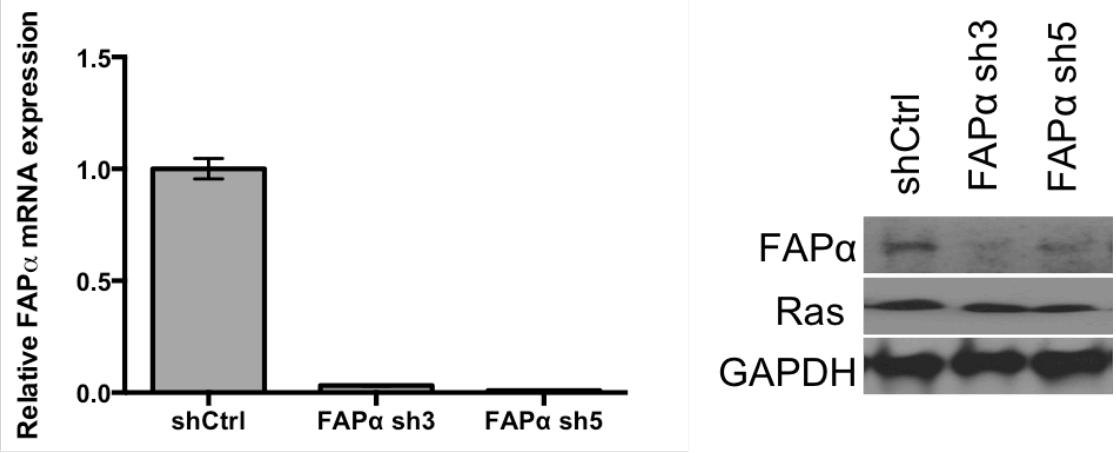

Figure 2-3: FAPα expression in HMLE-Twist cells. A: FAPα mRNA expression in HMLE-Twist cells expressing two independent shRNAs targeting FAPα. B: FAPα and Ras protein expression in HMLE-Twist cells.

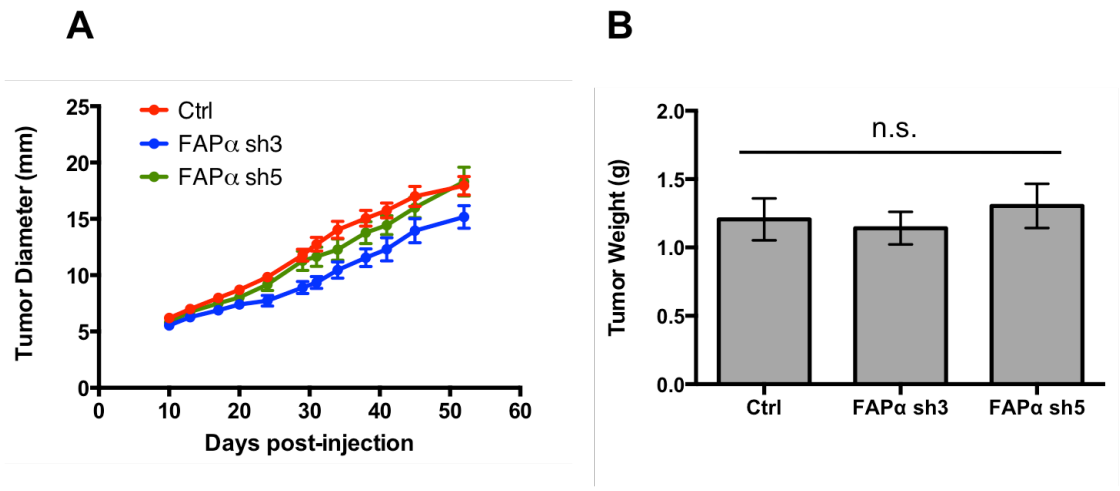

Figure 2-4: Tumor growth rate (A) and tumor weight (B) are not affected by FAPα knockdown. n.s.: not significant ($p > 0.60$, Student's T-test).

FAPα suppression did not significantly affect primary tumor growth rate or tumor size (Figure 2-4A, 2-4B). Strikingly, FAPα knockdown greatly affected the ability of tumor cells to metastasize to the lungs. While HMLE-Twist cells expressing a control shRNA generated hundreds of small GFP-positive micrometastases throughout the lungs (Figure 2-5A), the FAPα knockdown cells failed to metastasize to the lungs by an average of 400-fold, even though the primary tumors were of similar weights (Figure 2-5B). An initial primary tumor is necessary for cells to metastasize; mice in which injected cells did not form tumors (i.e., the cells did not "take") also did not have GFP-positive cells in their lungs (data not shown), indicating that the cells were not released upon injection straight into the vasculature, but rather are required to extravasate away from a primary tumor. These data demonstrate that FAPα expression is critical for the ability of tumor cells to metastasize to the lung.

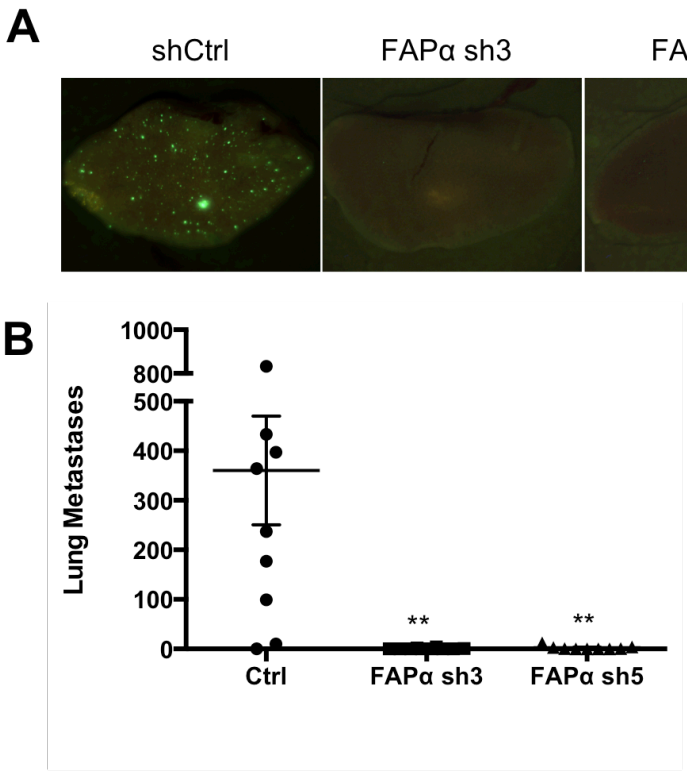

Figure 2-5: FAPα expression is required for metastasis in vivo. A: Representative images of lungs of nude mice injected with HMLE-Twist cells expressing indicated shRNAs. Dissemination of GFP-positive cells decreases upon FAPα suppression. B: Quantification of GFP-positive cells in each individual lung. N = 10 mice per group. **p < 0.01.

2.4 FAPα is expressed in invadopodia

Our previous studies have shown that, in addition to Twist1 expression, proper invadopodia formation is required for tumor cell metastasis (Eckert et al., 2011). Previous data also show that FAPα is located at invadopodia of endothelial cells (Ghersi et al., 2006). We set out to verify that FAPα was also located at invadopodia in the epithelial HS578T cell line. Co-localization of F-actin and the actin-binding protein cortactin is used to properly identify invadopodia (Bowden et al., 2006). We found that F-actin, cortactin, and FAPα

co-localized together in the same discrete punctate structures (Figure 2-6), indicating that FAPα is indeed expressed in the invadopodia of epithelial cells.

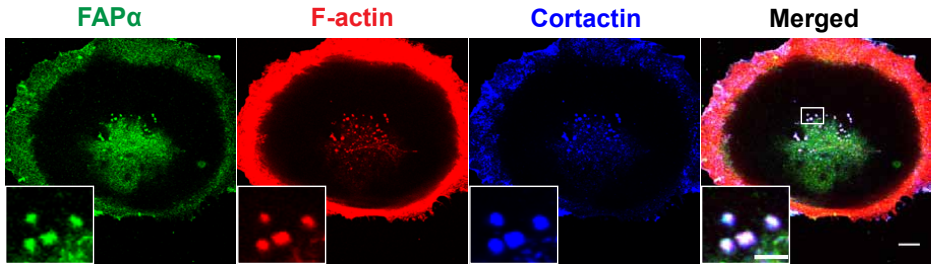

Figure 2-6: FAPα is expressed in invadopodia. HS578T cells were cultured on collagen-coated coverslips and then stained for FAPα (green), F-actin (red), and cortactin (blue). Scale bar: 10 μm for main picture and 3 μm for inset.

We further examined these invadopodia by Z-sectioning, and showed that F-actin, cortactin, and FAPα all co-localize at structures that protrude out from the basal surface of the cell (Figure 2-7), further emphasizing that FAPα is expressed in invadopodia.

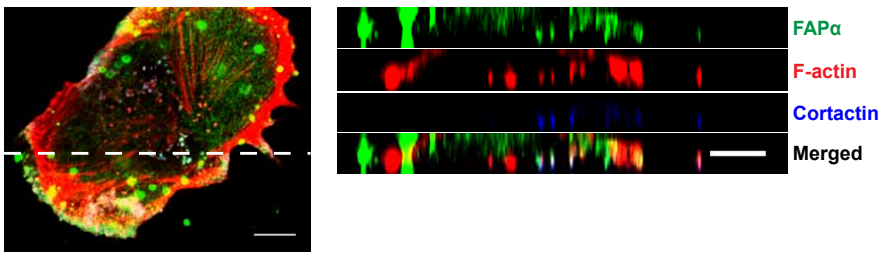

Figure 2-7: Confocal image of an HS578T cell stained for FAPα, F-actin, and cortactin. XZ image was taken along indicated dotted line. Scale bars: 10 μm.

To determine whether FAPα is present in functioning invadopodia, i.e., invadopodia that are actively degrading the ECM, HS578T cells were seeded

onto an Oregon Green-conjugated gelatin matrix, upon which areas of localized invadopodia-mediated ECM degradation show up as dark patches. As indicated by the arrowheads in Figure 2-8, F-actin and FAPα co-localize at several invadopodia that also overlap with the dark areas of degradation. Taken together, these data demonstrate that FAPα is expressed at active invadopodia in epithelial cells.

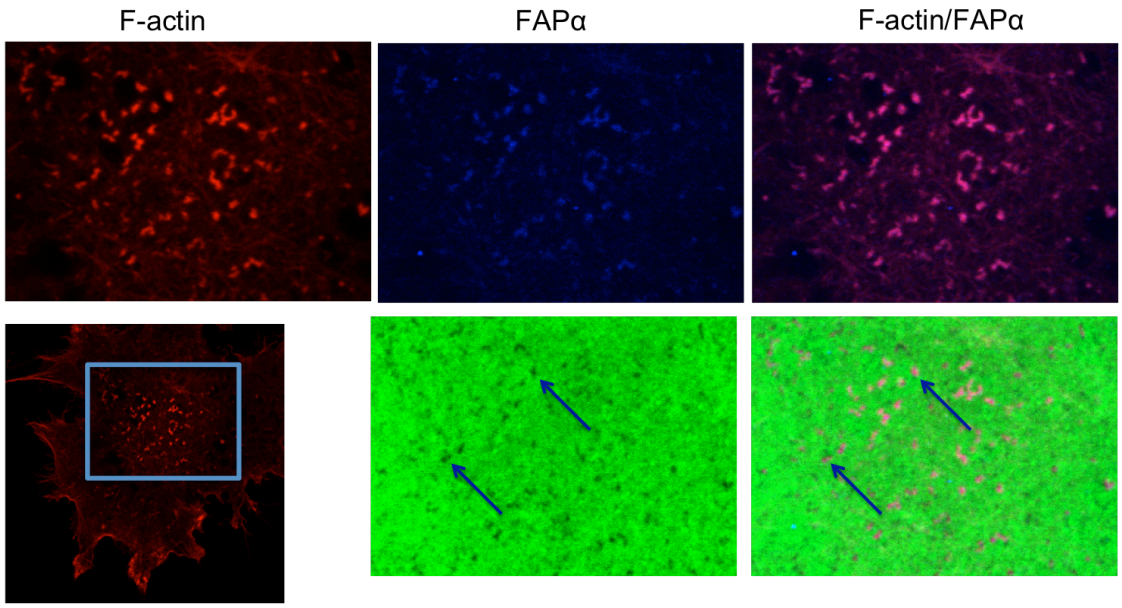

Figure 2-8: FAPα is expressed at invadopodia that are actively degrading the ECM. HS578T cells are stained for cortactin (blue) and F-actin (red). Arrows indicate actively degrading invadopodia.

2.5 FAPα knockdown reduces ECM degradation

Suppression of FAPα did not affect the cells' ability to undergo EMT: normal human mammary epithelial cells (HMEC) that were expressing either control or shRNAs targeting FAPα prior to ectopic expression of Twist1 still exhibited a scattered, mesenchymal phenotype (Figure 2-9). Further

characterization of these cells through Western blotting of epithelial vs. mesenchymal proteins to ensure that EMT has indeed taken place is currently ongoing.

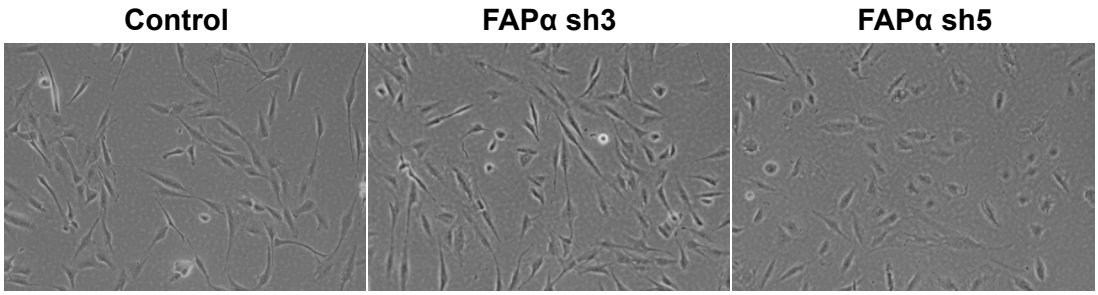

Figure 2-9: FAPα expression is not required for EMT. HMLE cells were stably expressing the indicated shRNAs prior to ectopic Twist1 expression.

FAPα knockdown also did not affect the ability of cells to migrate; both control and FAPα knockdown HS578T cells were plated to confluency and then underwent an in vitro wound healing assay. Both control and FAPα knockdown cells migrated into the “wounded” area and were able to successfully close the wound by 48 hours (Figure 2-10).

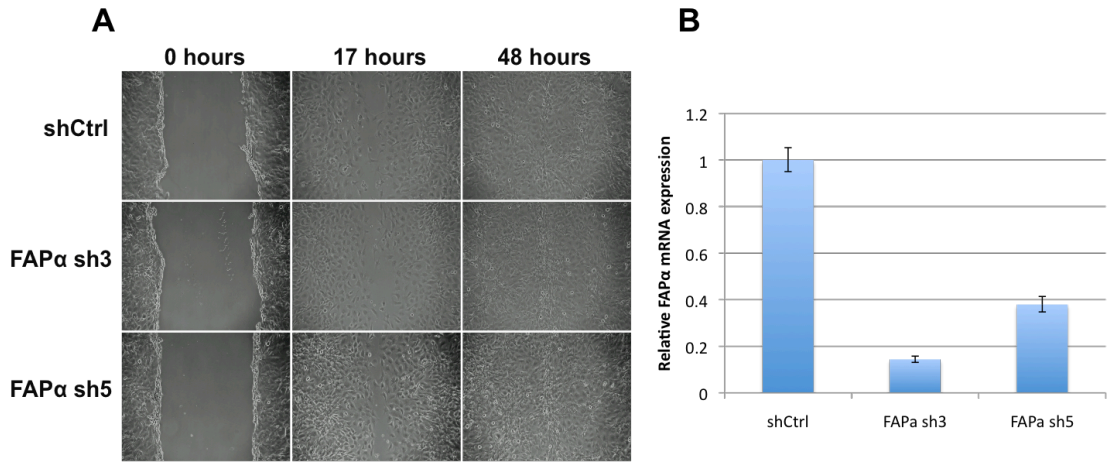

Figure 2-10: Suppression of FAPα expression does not affect cell migration. A: HS578T cells were seeded at 90% confluency, and a p-1000 tip was scratched through the cells, creating a wound. Cells were monitored for 48 hours after wounding, and images were captured at the indicated timepoints. B: Relative mRNA expression of FAPa in HS578T cells expressing the indicated shRNAs.

We therefore hypothesized that the inability of the FAPα knockdown cells to metastasize to the lung was likely due to a defect in invadopodia-mediated ECM degradation activity. To test this hypothesis, we seeded HMLE-Twist control and FAPα knockdown cells onto the Oregon Green-conjugated gelatin matrix and then measured their ability to degrade the matrix. Control cells degraded the matrix over the course of 16 hours (Figure 2-11A and 2-11B), whereas ECM degradation activity was significantly reduced in the FAPα knockdown cells.

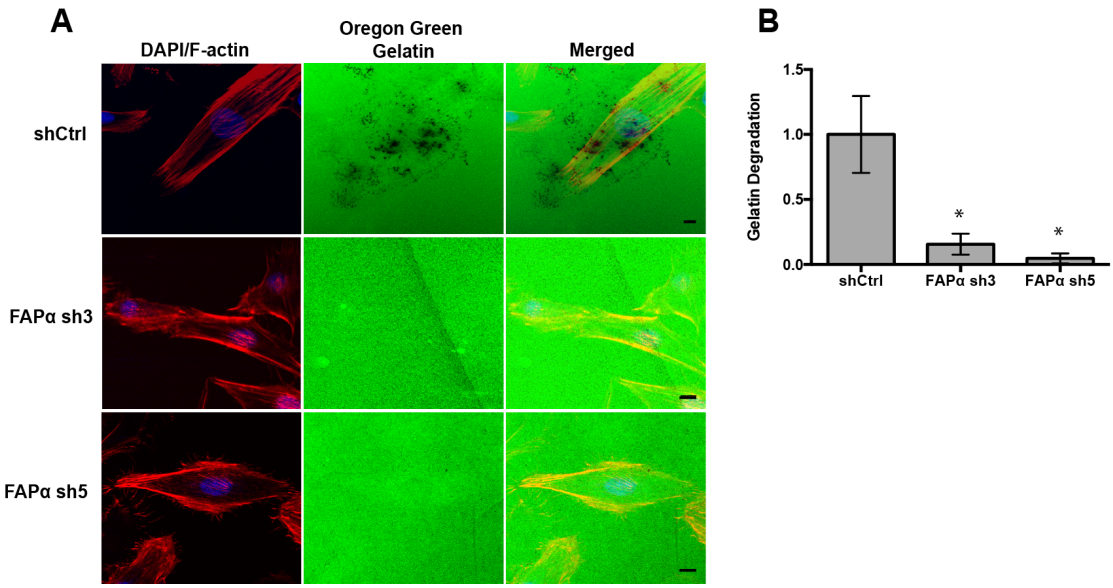

Figure 2-11: FAPα knockdown reduces ECM degradation in HMLE-Twist cells. A: HMLE-Twist cells were seeded on gelatin labeled with Oregon Green for 16 hours. Nuclei are stained with DAPI (blue) and F-actin with phalloidin (red). Scale bars: 10 μm. B: Quantification of Oregon Green gelatin degradation. *p < 0.02

We carried out the same assay in the SUM1315 breast cancer cell line, in which Twist1 is not exogenously expressed, but rather contains endogenously high levels of Twist1. We found that, similar to the HMLE-Twist cells, the SUM1315 cells expressing a control shRNA were able to degrade the matrix, but those expressing shRNAs against FAPα showed a reduced degradation capacity (Figure 2-12).

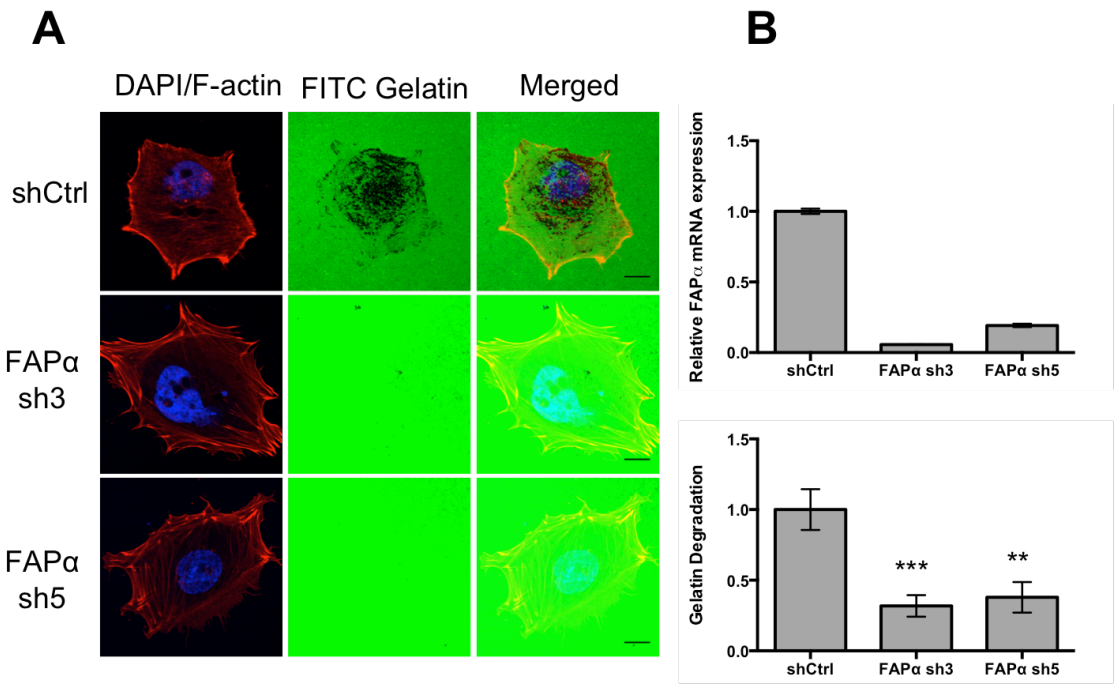

Figure 2-12: FAPα knockdown reduces ECM degradation in SUM1315 cells. A: SUM1315 cells were seeded onto Oregon Green-labeled gelatin for 6 hours. Nuclei are stained with DAPI (blue), and F-actin with phalloidin (red). Scale bars: 10 μm. B: (Top) Relative mRNA levels of FAPa in SUM1315 cells expressing the indicated shRNAs. (Bottom) Quantification of Oregon Green gelatin degradation. **p < 0.003; ***p < 0.002.

A prior study showed that a homolog of FAPα, DPP4, is also present at invadopodia, and forms a heterodimer complex with FAPα (Ghersi et al., 2006). Our results suggest that DPP4, which shares over 68% homology with FAPα (Chen and Kelly, 2003), is not able to compensate for the absence of FAPα. Knocking down DPP4 in SUM1315 cells has no significant effect on ECM degradation (Figure 2-13). These results show that FAPα expression is required for proper invadopodia function.

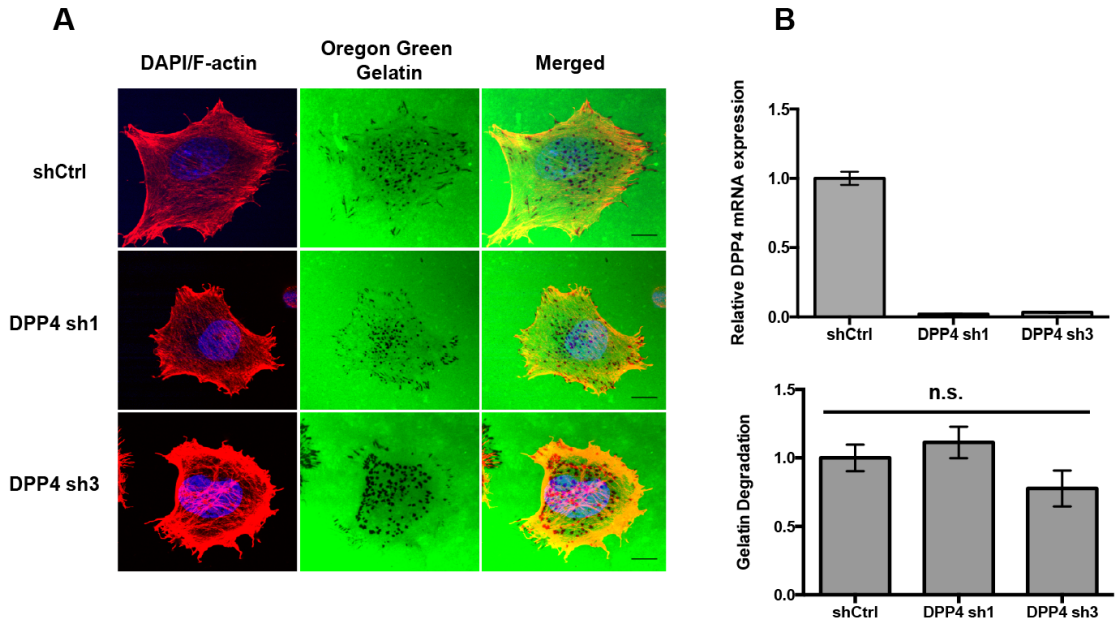

Figure 2-13: DPP4 knockdown does not significantly reduce ECM degradation. A: SUM1315 cells were seeded onto Oregon Green-labeled gelatin for 6 hours. Scale bars: 10 μm. B: Top – DPP4 mRNA expression in cells expressing a control shRNA and two different shRNAs targeting DPP4. Bottom – Quantification of Oregon Green gelatin degradation. n.s.: not significant.

2.6 The proteolytic activity of FAPα is not required for ECM degradation

We next set out to determine whether the proteolytic activity of FAPα is required for ECM degradation, thereby explaining the lack of ECM degradation in FAPα knockdown cells. We obtained an FAPα-specific inhibitor, *Ac*-Arg-*peg*-D-Ala-L-BoroPro (also known as M83), which is 1000-fold more specific to FAPα over DPP4 (Lee et al., 2011). We used the synthetic substrate Tic-Pro-*AFC* (also known as C95), a prolyl-specific fluorescent substrate that emits fluorescence upon cleavage, to verify that M83 abrogates FAPα catalytic activity in SUM1315 cells, and found that fluorescence did not increase in a

linear fashion over time in the presence of the FAPα inhibitor, as expected (Figure 2-14).

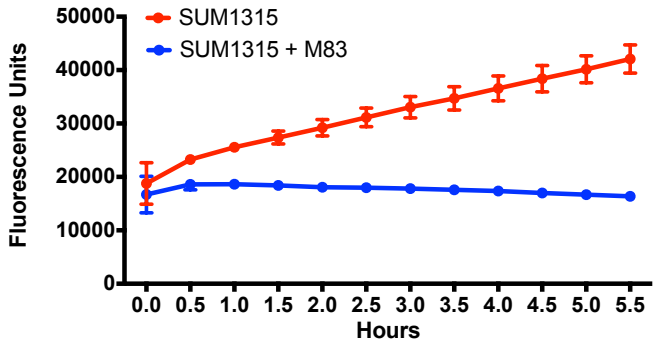

Figure 2-14: The FAPα inhibitor M83 abrogates FAPα catalytic activity.

We then used M83 to inhibit FAPα protease activity in SUM1315 cells seeded on top of Oregon Green-labeled gelatin matrix. Surprisingly, cells treated with M83 were able to degrade the matrix to levels similar to cells treated with PBS alone (Figure 2-15B), suggesting that the protease activity of FAPα may not be required for invadopodia-mediated ECM degradation. To ensure that the cells underwent proper M83 treatment, we treated a separate group of SUM1315 cells with the pan-matrixmetalloprotease inhibitor GM6001, which is known to dramatically reduce ECM degradation (Clark et al., 2007).

The pattern of degradation varies substantially between the HMLE-Twist and SUM1315 cells, with the latter exhibiting less punctate degradation. One possible reason for this difference could be that SUM1315 have more degradation activity at focal adhesions than HMLE-Twist cells. Further studies involving visualizing the matrix metalloprotease MT1-MMP at the edge of SUM1315 cells would confirm this. 3-D studies of ECM degradation would

most likely show that the pattern of invadopodia-mediated degradation is similar between cell lines. We are currently carrying out studies to confirm this.

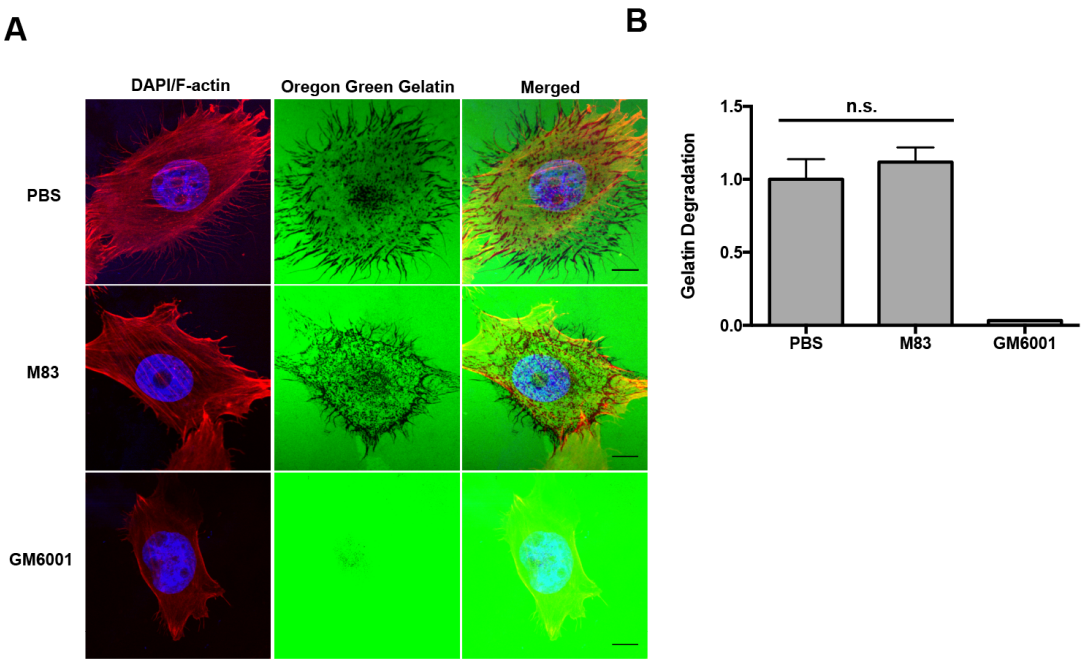

Figure 2-15: Chemical inhibition of FAPα does not affect ECM degradation. A: SUM1315 cells were seeded onto Oregon Green gelatin for 6 hours, then fixed and stained for DAPI and F-actin. B: Quantification of ECM degradation. Scale bars: 10 μm. n.s.: not significant.

Most proteins in the DPP family of proteases, such as DPP4, can only carry out exopeptidase activity. FAPα is unique because it has the capacity to carry out both exopeptidase and endopeptidase activity (Wolf et al., 2008). We sought to determine whether FAPα's endopeptidase activity is necessary to drive ECM degradation. We mutated two key residues in FAPα's catalytic domain: S624, which is part of the catalytic triad of FAPα and vital for both exo- and endopeptidase activity, and A657, which controls the geometry of the active site (Meadows et al., 2007; O'Brien and O'Connor, 2008), and is only necessary for endopeptidase activity. We verified that the mutants carried out

the appropriate protease activities using the C95 substrate described above (Figure 2-16), and found that the S624A mutant displayed little activity, while the A657S mutant had intermediate protease activity, which confirms previous data from other studies using this mutant (Huang et al., 2011; Meadows et al., 2007).

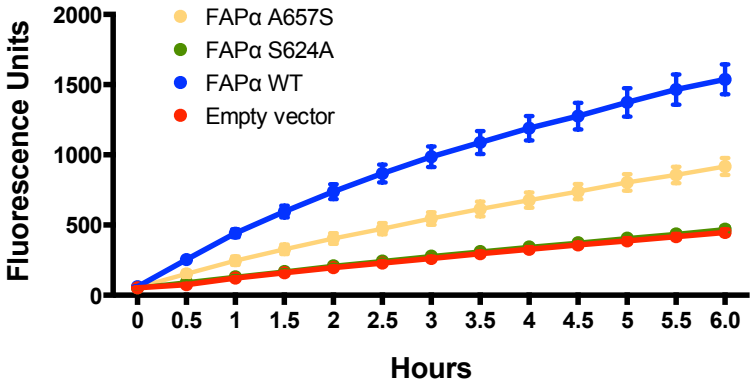

Figure 2-16: Point mutations of the FAPα catalytic site result in varying catalytic activity. 293T cells were transfected with the indicated mutant constructs prior to catalytic activity measurement.

We then re-expressed either a control vector, wild-type FAPα, the FAPα S624A mutant, or the FAPα A657S mutant back into FAPα knockdown SUM1315 cells to find out whether ECM degradation activity would be restored (Figure 2-17).

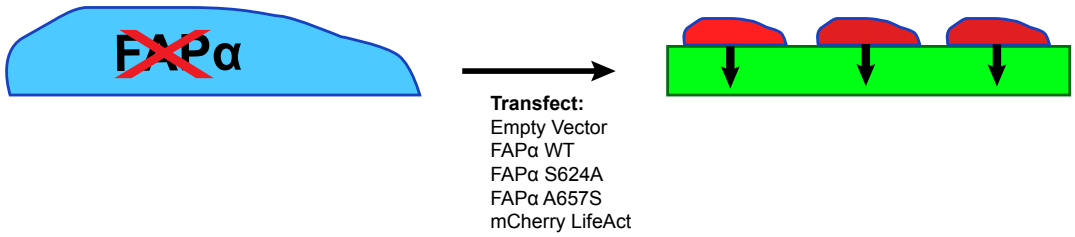

Figure 2-17: Overview of FAPα rescue experiment. SUM1315 cells that have FAPα knocked down are transiently transfected with the indicated constructs, and then ECM degradation is measured and compared to SUM1315 cells expressing a control vector.

We found that overexpression of wild-type FAPα, the S624A protease-dead mutant, and the A657S endopeptidase-dead mutant were able to restore ECM degradation activity in both lines of FAPα knockdown cells (Figure 2-18 and 2-19). Taken together, these data show that the protease activity of FAPα is not required for invadopodia-mediated ECM degradation, but FAPα expression – even devoid of protease activity – can restore the loss of ECM degradation activity in FAPα knockdown cells.

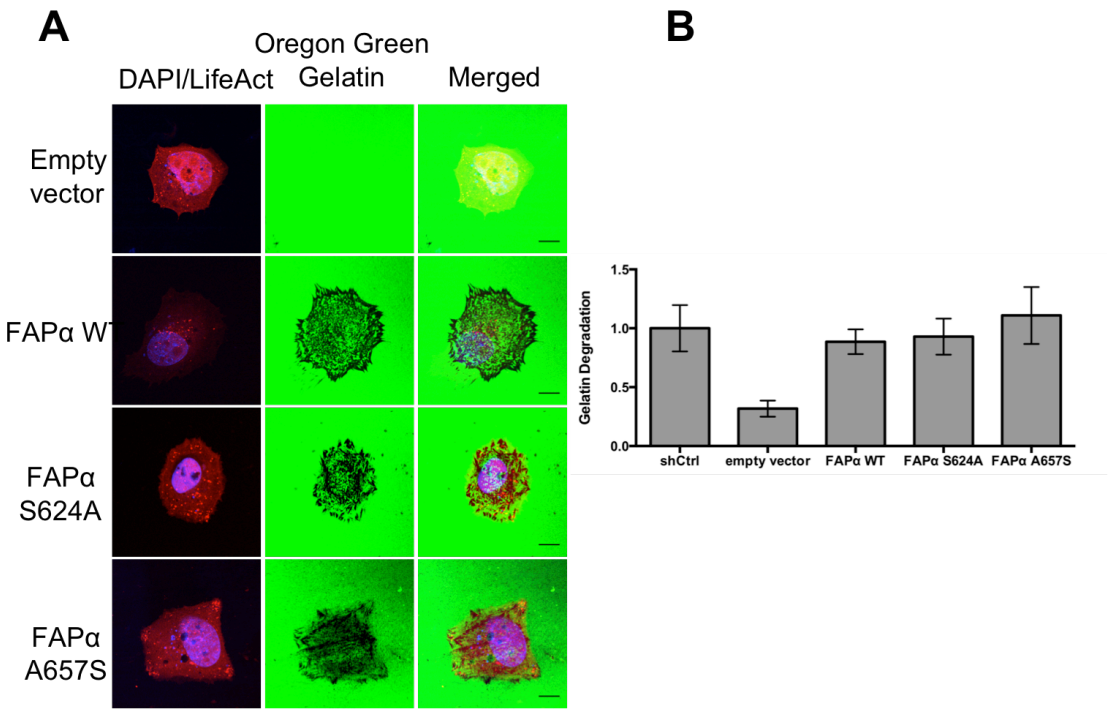

Figure 2-18: Expression of FAPα mutants with compromised catalytic activity in FAPα sh3 knockdown cells restores ECM degradation. A: SUM1315 FAPα sh3 cells were transfected with the indicated constructs and mCherry LifeAct. 48 hours after transfection, cells were seeded onto Oregon Green-labeled gelatin for 6 hours. Nuclei are stained with DAPI (blue). Scale bars: 10 μm. B: Quantification of Oregon Green-labeled gelatin degradation, relative to control cell degradation activity.

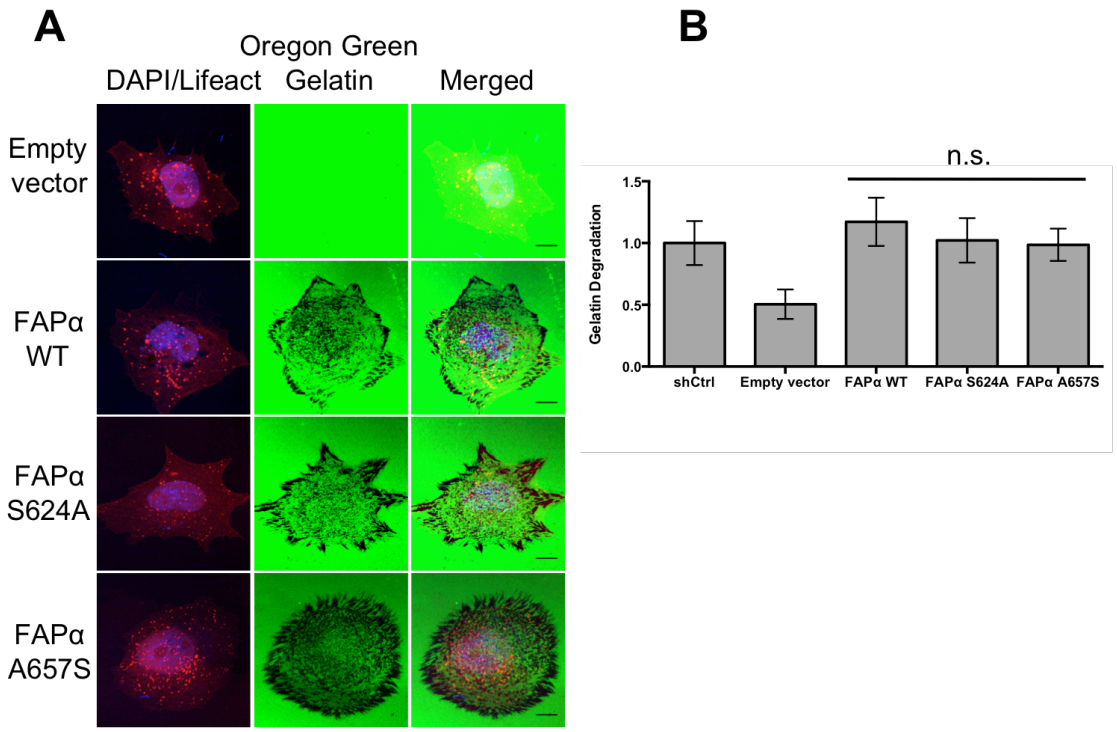

Figure 2-19: Expression of FAPα mutants with compromised catalytic activity in FAPα sh5 knockdown cells restores ECM degradation. A: SUM1315 FAPα sh3 cells were transfected with the indicated constructs and mCherry LifeAct. 48 hours after transfection, cells were seeded onto Oregon Green-labeled gelatin for 6 hours. Nuclei are stained with DAPI (blue). Scale bars: 10 μm. B: Quantification of Oregon Green-labeled gelatin degradation, relative to control cell degradation activity.

2.7 Late-stage invadopodia assembly is disrupted in FAPα knockdown cells

Invadopodia are assembled in a series of discrete steps (Artym et al., 2006; Murphy and Courtneidge, 2011). First, the structural components such as actin and cortactin converge onto the site of invadopodia formation, and after the subcellular protrusion has formed, proteases such as the membrane-tethered matrixmetalloprotease MT1-MMP localize to the maturing invadopodium, which is able to degrade the ECM. Given that FAPα expression

is required for invadopodia-mediated ECM degradation, but its catalytic activity is not required, we hypothesized that FAPα is playing a key structural role in the formation of invadopodia that is critical for proper invadopodia function. We first examined invadopodia in both control and FAPα knockdown HMLE-Twist cells, and found that F-actin and cortactin co-localization at puncta, which are indicative of invadopodia formation, in all cells was intact (Figure 2-20), demonstrating that the initial steps of invadopodia assembly were proceeding normally.

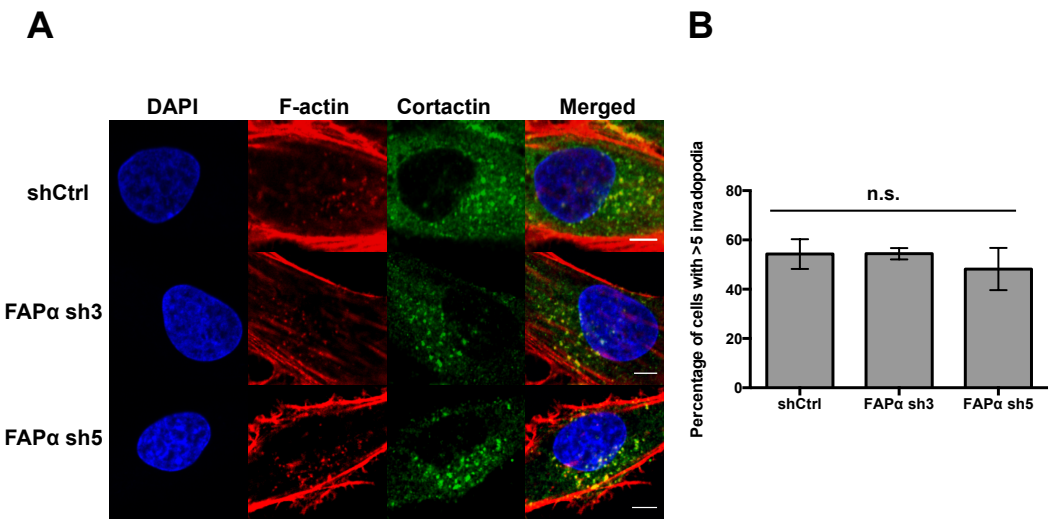

Figure 2-20: The initial stages of invadopodia assembly proceed normally in FAPα knockdown cells. A: HMLE-Twist cells expressing the indicated shRNAs underwent IF staining for F-actin (red), cortactin (green), and nuclei were stained with DAPI (blue). Scale bar: 10 μm. B: Quantification of cells that contain more than 5 invadopodia per cell.

We then set out to determine whether protease secretion and/or localization to invadopodia were affected by FAPα suppression. Gelatin zymography showed that MMP2 was secreted into the conditioned media from

control and FAPα knockdown SUM1315 cells (Figure 2-21), but the ratio of active to pro-MMP2 was lower in the conditioned media from FAPα knockdown cells versus that of the control cells, indicating that there may be a defect in MMP2 processing.

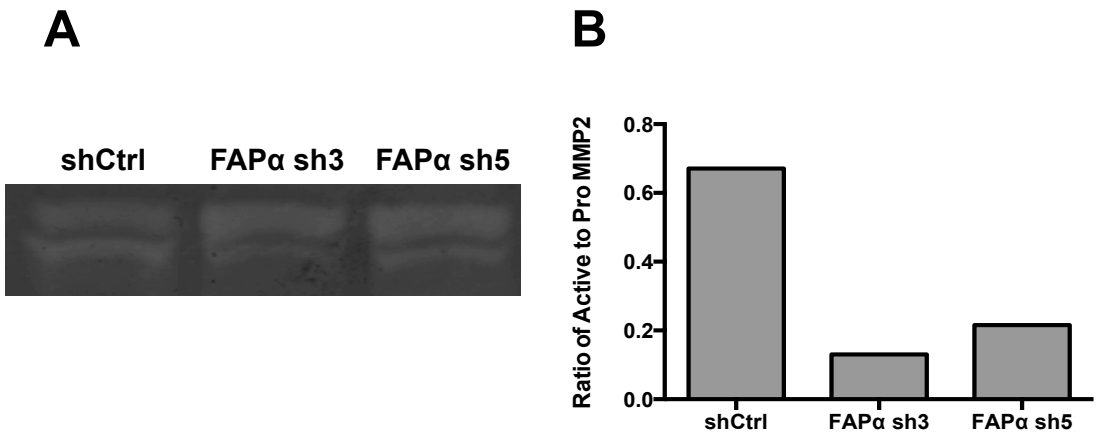

Figure 2-21: The ratio of active to pro-MMP2 is lower in FAPα knockdown cells. A: Gelatin zymography showing secreted pro-MMP2 (upper band), and active MMP2 (lower band). B: Ratio of active to pro-MMP2. Band intensity was quantified by densitometry.

MMP2 is activated by MT1-MMP cleavage from its pro- to active form (Deryugina and Quigley, 2006; Deryugina et al., 2001), so we next examined whether MT1-MMP was properly localizing to invadopodia in the control versus FAPα knockdown cells. Previous studies have shown that MT1-MMP co-locoalized with caveolin-1, a lipid raft marker, at the cell membrane, indicating that invadopodia are located at lipid raft sites (Yamaguchi et al., 2009). We examined MT1-MMP localization in control and FAPα knockdown SUM1315 cells by carrying out a sucrose gradient fractionation to separate the lipid raft regions from the rest of the cell membrane. Lysing cells using Triton

X-100 leaves the areas of the cell membrane that contain lipid rafts intact; these then migrate during the fractionation process according to their density. We found that MT1-MMP expression was reduced in the lipid raft fraction of FAPα knockdown cells (Figure 2-22, red boxes), whereas MT1-MMP was co-expressed along with caveolin-1 in the control cells.

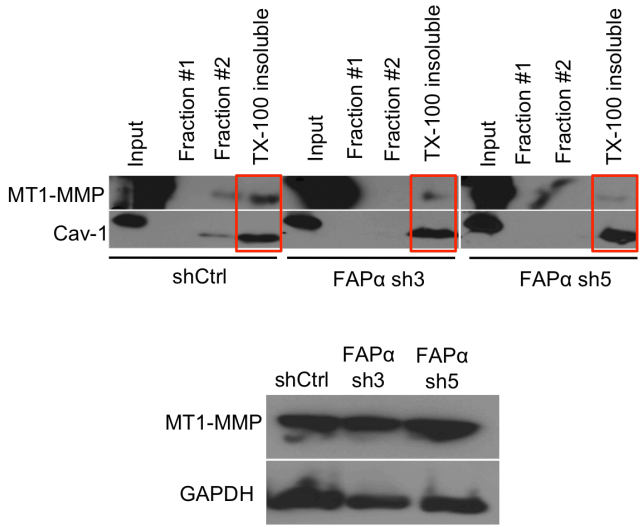

Figure 2-22: MT1-MMP is mislocalized in FAPα knockdown cells. Protein lysates from SUM1315 cells expressing the indicated shRNAs were fractionated on a sucrose gradient. The resulting fractions were then analyzed by SDS-PAGE and probed for caveolin-1 and MT1-MMP. Below, lysates from SUM1315 cells expressing the indicated shRNAs were analyzed by SDS-PAGE and probed for MT1-MMP.

The lack of MT1-MMP in the lipid raft fractions of the FAPα knockdown cells cannot be explained by a lack of overall MT1-MMP expression, nor any kind of MT1-MMP protein instability resulting from a lack of FAPα, because both control and FAPα knockdown cells express MT1-MMP (Figure 2-22). These results indicate that FAPα plays a role in MT1-MMP localization to lipid rafts, and, by extension, to invadopodia.

2.8 Discussion and Conclusions

In this study, we report a novel role for the serine protease FAPα in invadopodia-mediated ECM degradation and metastasis. We show that FAPα is induced by Twist1, which is necessary for metastasis to take place. We also show that FAPα expression is required for tumor cell metastasis, and that knockdown of FAPα significantly reduces ECM degradation. We demonstrated that abolishing the protease activity of FAPα, either through chemical inhibition or through mutation, did not affect ECM degradation. Finally, we find that FAPα is required for properly localizing MT1-MMP to invadopodia.

This study builds upon our previous work demonstrating that Twist1 is required for invadopodia formation (Eckert et al., 2011). Twist1 does not induce expression of various important invadopodia proteins, such as cortactin, Tks4/5, and MT1-MMP, which is why data showing that it does induce FAPα seemed especially worthy of additional study. FAPα and Twist1 are both upregulated in a variety of invasive cancers (data not shown), and analysis of various large human breast cancer gene expression data sets demonstrates a strong correlation between Twist1 and FAPα expression (Figure 2-23).

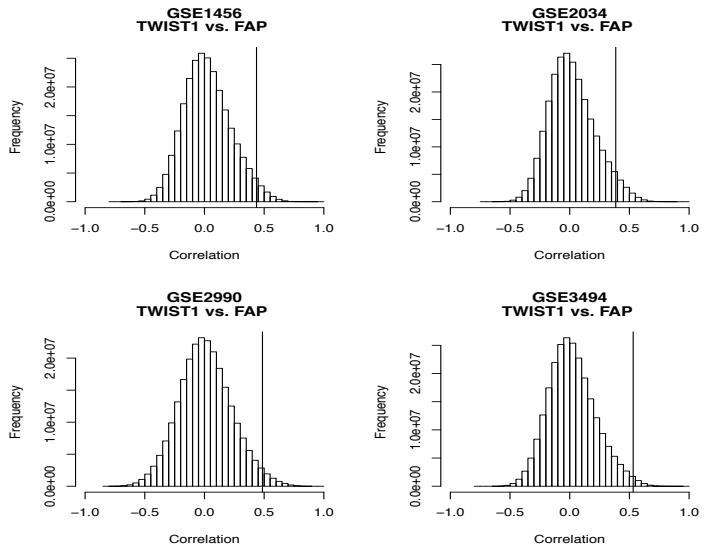

Figure 2-23: Analysis of breast cancer gene sets shows a strong correlation between Twist1 and FAPα expression. Gene sets comparing the correlation between expression of ~23,000 genes, and that of Twist1. The line in each histogram indicates where FAPa falls among the distribution of genes.

However, our results show that FAPα expression increases three days after Twist1 induction; this delayed elevation in expression strongly suggests that FAPα is not a direct transcriptional target of Twist1. Twist1 is most likely upstream of a different transcription factor responsible for regulating FAPα. A recent study indicated that FAPα is regulated through the TGFbeta pathway; FAPα mRNA levels and promoter activity increased in invasive melanoma lines upon TGFbeta treatment. TFGbeta signaling results in the activation of the Smad transcription factors, which then translocate to the nucleus and alter gene expression. Two putative Smad binding elements in the FAPα promoter were found to be required for promoter activity, indicating that TGFbeta is involved in regulating FAPα expression (Tulley and Chen, 2014).

Our data clearly show that FAPα expression is required for metastasis in vivo. Further studies need to be carried out to determine which step of the metastatic process is compromised in cells lacking FAPα. Tail-vein injection of control and FAPα knockdown cells would be ideal to determine whether it is intravasation into the circulatory system that is defective, or extravasation into the lungs. Based on our in vitro ECM degradation data, in which we demonstrate that FAPα expression is required for ECM degradation, we hypothesize that cells lacking FAPα are unable to break away from the primary tumor and breach the basement membrane.

We found that FAPα expression is required for invadopodia-mediated ECM degradation, and that lack of degradation activity can be restored by over-expression of FAPα. Interestingly, ECM degradation levels in cells overexpressing FAP do not exceed those observed in wild-type cells. This result contrasts similar experiments, in which MT1-MMP was overexpressed in cell lines that do not express it, prompting a dramatic increase in ECM degradation (Sato et al., 1994; Sounni et al., 2002). Previous studies have suggested that the gelatinase activity of FAPα may contribute to its function in invadopodia. Christiansen and colleagues showed that FAPα digests pieces of collagen I into smaller fragments following initial cleavage by MMP-1, implying that FAPα might work together with other proteases to further degrade partially cleaved ECM components, thereby “tilling the soil” in preparation for metastasis (Christiansen et al., 2007). Our data, on the other hand, clearly demonstrates that the protease activity of FAPα is not required for ECM

degradation, and that ECM degradation does not increase upon FAPα overexpression in FAPα knockdown cells, which suggests that FAPα acts in a structural rather than a proteolytic capacity in invadopodia.

We sought to determine whether the ECM degradation defect shown in the FAPα knockdown cells could be due to other gelatinases, such as secreted MMPs, being affected by FAPα suppression. We found that while MMP2 is secreted by both the control and FAPα knockdown cells in its inactive and active forms, we observed that the ratio of inactive to active MMP2 was higher in the FAPα knockdown cells compared to the control. This result indicated that a lack of active MMP2 produced by FAPα knockdown cells was most likely the reason behind the impaired ECM degradation carried out by these cells. MT1-MMP activates MMP2, and so we reasoned that MT1-MMP activity could possibly be compromised.

Previous reports have suggested that FAPα dimerizes with its homolog DPP4 (Ghersi et al., 2002; Ghersi et al., 2003), yet our data shows that DPP4 is not required for ECM degradation activity (Figure S1). We reasoned that the large extracellular domain of FAPα – including its cystein-rich domain (Chen and Kelly, 2003) – would be ideal for forming complexes with other proteins, or providing a docking site for proper protein localization, as how beta1 integrin does for FAPα (Mueller et al., 1999). Our data showing that MT1-MMP does not properly localize to lipid rafts in the absence of FAPα suggests that FAPα could be acting as a docking structure for MT1-MMP at invadopodia, thereby initiating invadopodia maturation and subsequent degradation of the ECM,

and ultimately promoting metastasis (Figure 2-24). Further studies need to be carried out to confirm whether this is indeed the case.

Metastatic disease is the most difficult aspect of breast cancer to treat, given its systemic nature and the resistance of metastatic carcinoma cells to most therapeutic agents. FAPα is an attractive therapeutic target for

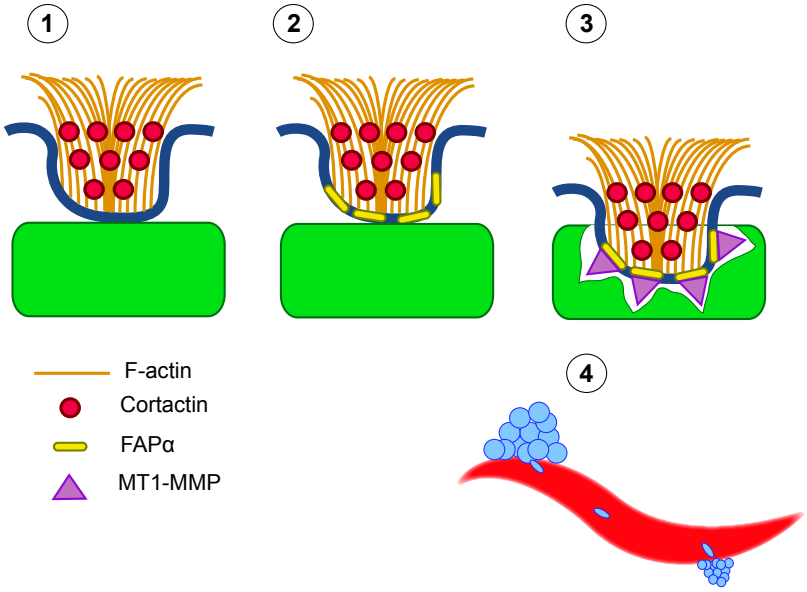

Figure 2-24: Proposed mechanism of action of FAPα at invadopodia. 1: Early invadopodia assembly involves formation of the F-actin core, and localization of cortactin. 2: FAPα localizes to the cell membrane at the tip of invadopodia. 3: FAPα provides a docking site for MT1-MMP, which, upon localization to invadopodia, allows the cell to degrade the ECM. ECM degradation is required for subsequent metastasis to take place (4).

metastasis, given that it is only expressed during wound healing and in invasive epithelial cancers (Paz et al., 2013). However, previous attempts to therapeutically target FAPα have been met with very limited success. In 2007, a small molecule inhibitor named Talabostat was developed to inhibit the protease activity of FAPα. It did not perform well in clinical trials, most likely

because of poor in vivo stability (Eager et al., 2009; Narra et al., 2007). We propose, based on our data, that Talabostat's main flaw might have been targeting the protease activity of FAPα; we have shown that inhibiting the proteolytic activity of FAPα has little effect on ECM degradation. A perhaps more effective therapeutic targeting strategy would be to target the β-propeller domain of FAPα, which could be a key domain for protein-protein interactions required for invadopodia activity. The scope of targeting FAPα extends beyond invadopodia, as FAPα also plays important roles in the tumor stroma (Kraman et al., 2010). Directing therapeutic intervention towards a single protease that affects multiple aspects of tumor progression could represent a promising approach to cancer treatment.

2.9 Materials and Methods

2.9-A Cell Lines and Cell Culture

Human mammary epithelial cells (HMLE), HMLE-TwistER, and the SUM1315 cell line were all obtained from Dr. Robert Weinberg and cultured as previously described (Yang et al., 2004). The HS578T cell line was obtained from the laboratory of Dr. Sara Courtneidge, and was cultured in DMEM with 10% FBS, and supplemented with insulin. Viral production and infection to produce stable knockdown cell lines was carried out as previously described (Eckert et al., 2011).

2.9-B Plasmids

The lentiviral shRNA constructs against Twist1 have been previously described (Yang et al., 2004). Control shRNAs in the pLKO.1 vector were obtained from the Sabatini lab (Addgene), and purchased from Sigma. ShRNAs targeting FAPα were purchased from Sigma, and have the following targeting sequences: FAPα sh3: GCATTGTCTTACGCCCTTCAA, FAPα sh5: TGATAATCTTGAGCACTATAA. ShRNAs against DPP4 were cloned into the pSP108 vector, and contain the following target sequences: DPP4 sh1: GGTCACCAGTGGGTCATAAAT, DPP4 sh3: GCTGTGAATCCAACTGTAAAG.

2.9-C Subcutaneous Tumor Implantation and Metastasis Assay

All animal care and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego. 2 million cells resuspended in 50% Matrigel were injected into the left and right flanks of adult nude mice, and allowed to grow to approximately 2 cm in diameter before mice were sacrificed. Primary tumor size was measured every 4 days. Lungs were harvested and imaged for GFP-positive tumor cells.

2.9-D Generation of FAPα Point Mutants

In order for the point mutants to be used in overexpression rescue experiments while avoiding being targeted by shRNAs, we took advantage of codon degeneracy and mutated the wild-type FAPα sequence, using site-directed mutagenesis as previously described (Heckman and Pease, 2007),

so that it would not be targeted by either shRNA. Using these mutants as a template, FAPα sh3- and FAPα sh5-resistant forms of the S624A and A657S point mutants were constructed. All primers used to construct the point mutants are listed below:

FAPα sh3-resistant WT FAPα:
Forward: GTGCATTGTCTTAAGACCAAGTAGAGTTCATAACTCT
Reverse: AGAGTTATGAACTCTACTTGGTCTTAAGACAATGCAC

FAPα sh5-resistant WT FAPα:
Forward: GGATGATAATCTTGAACATTACAAGAATTCAACTGTG
Reverse: CACAGTTGAATTCTTGTAATGTTCAAGATTATCATCC

FAPα S624A:
Forward: CATATGGGGCTGGGCCTATGGAGGATAC
Reverse: GTATCCTCCATAGGCCCAGCCCCATATG

FAPα A657S:
Forward: CCAGCTGGGAATATTACTCGTCTGTCTACACAGAG
Reverse: CTCTGTGTAGACAGACGAGTAATATTCCCAGCTGG

2.9-E Quantitative Real-Time PCR

Total RNA from cells at 80-90% confluency using the NucleoSpin RNA II kit (Macherey-Nagel), and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNAs were analyzed in triplicate using the SYBR-Green PCR Mix (Applied Biosystems). Relative mRNA levels were determined by $2^{-(Ct-Cc)}$, where Ct and Cc are the mean threshold cycle differences after normalizing to GAPDH values. Primers used for qRT-PCR are as follows:

GAPDH:
Forward: GAGAGACCCTCACTGCTG
Reverse: GATGGTACATGACAAGGTGC

FAPα:
Forward: AATGAGAGCACTCACACTGAAG
Reverse: CCGATCAGGTGATAAGCCGTAAT

DPP4:
Forward: TCCTGATGGGCAGTTTATTCTCT
Reverse: CATGTGACCCACTGTGTGTTG

Twist1:
Forward: TCCGCGTCCCACTAGCA
Reverse: AGTTATCCAGCTCCAGAGTCTCTAGAC

MT1-MMP:
Forward: GAAGCCTGGCTACAGCAATATG
Reverse: TGCAAGCCGTAAAACTTCTGC

2.9-F Western Blotting

Cells at 80-90% confluency were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 10 mM NaF, 1% Triton X-100), with 100 μM Na_3VO_4, PMSF, and the Protease Inhibitor Cocktail Set III (Calbiochem) diluted 1:200. Native proteins were run on 4-12% pre-cast gels (PAGEgel) so as not to destroy the FAPα epitope, otherwise western blots were carried out according to standard procedures. Antibodies used for western blotting include the following: FAPα (1:500, D8, Vitatex), Twist1 (1:500), MT1-MMP (1:1000, GeneTex), Caveolin-1 (1:20,000, BD Biosciences), GAPDH (1:2500, GeneTex).

2.9-G ECM Degradation Assays

ECM degradation assays were carried out as previously described (Artym et al., 2009; Eckert et al., 2011).

2.9-H Immunofluorescence (IF)

Matrix substrates were prepared using unlabled 0.2% porcine gelatin, as for the ECM degradation assays. Cells were seeded on the matrix for 3 days, and then fixed in 4% paraformaldehyde (PFA) for 15 minutes at 37°C. Cells were washed 3 times with 1X PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and then blocked with 10% goat serum in PBS for 1 hour. Samples were incubated with primary antibodies overnight at 4°C with gentle shaking, and with secondary antibodies for 2 hours at room temperature. Samples were then washed, incubated with Alexa Fluor 594-conjugated phalloidin (Invitrogen) and DAPI in PBS for 20 minutes at room temperature, then mounted with VECTASHIELD (Vector Laboratories) prior to imaging. Primary antibodies used for IF include Cortactin (1:1000, Santa Cruz Biotechnology) and FAPα (1:500, R&D Systems). Secondary antibodies used include Alexa Fluor 488 and 647 (Invitrogen).

2.9-I FAPα Enzymatic Activity Assay

Cells were plated at 90% confluency in 96-well plates, and washed 3 times with 1X PBS. The wash solution was replaced with fresh PBS, and either additional PBS or 10 μM M83 inhibitor was added. 125 μM C95 substrate was added to all wells, and then fluorescence emission was

measured at 360/460 nm excitation/emission wavelengths. The M83 inhibitor and the C95 substrate were generously provided by Dr. Patrick McKee.

2.9-J Gelatin Zymography

Conditioned media from cells plated at 80-90% confluency for 48 hours was collected and diluted 1:1 with SDS loading buffer without reducing factors or boiling, and run on a 10% gelatin zymogram gel (Invitrogen). Gels were then incubated in Zymogram Renaturing Buffer (Invitrogen) for 30 minutes at room temperature, then with Zymogram Developing Buffer (Invitrogen) at 37°C overnight. Gels were stained with hot staining solution (40% ethanol, 10% acetic acid, 0.1% Coomassie R-250) for 30 minutes at room temperature with gentle agitation, then destained with 10% ethanol and 7.5% acetic acid for 30 minutes at room temperature.

2.9-K Lipid Raft Fractionation

Cells at 80-90% confluency were washed with ice-cold 1X PBS, and collected in lysis buffer (50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA in 1% Triton X-100, with protease inhibitors). Cells were then homogenized with 10-20 strokes of a Dounce Homogenizer on ice every 10 minutes for 30 minutes. Post-nuclear supernatant was removed by centrifugation at 1000 x *g* for 10 minutes at 4°C. Equal volumes of protein lysate and 80% sucrose solution were mixed and placed in a pre-chilled 12 mL ultracentrifuge tube (Beckman), and overlaid with 6 mL 35% sucrose, and 4 mL 5% sucrose.

Samples were centrifuged for 16 hours at 36,000 rpm in an SW41 rotor at 4°C. Fractions of 1 mL each were collected from the top of the tube to the bottom, and analyzed by Western blotting.

2.9-L: Statistical Analysis

All statistical analysis was carried out using GraphPad Prism software. Unless otherwise noted, Student's T-test was carried to determine statistical significance.

2.10 Acknowledgements

Chapter 2, in full, is currently being prepared for submission for publication of the material. Pathak N., Li D.M., Jiang Y., Palmer T.D., Christiansen V.J., McKee P.A., Yang J. Fibroblast Activation Protein alpha (FAPα) regulates invadopodia activity and promotes tumor metastasis. *In preparation.* The dissertation author was the primary investigator and author of this material.

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Chapter 3

Future Perspectives and Conclusions

3.1 Regulation of FAPα expression

In the previous chapter, it was demonstrated through various methods that FAPα is a target of Twist1. Analysis of breast cancer gene sets showed a high correlation between FAPα and Twist1 expression. In addition, analysis of FAPα mRNA and protein showed a 4-fold increase 3 days after Twist1 induction, and knockdown of Twist1 results in decreased FAPα mRNA expression. Unlike other direct transcriptional targets of Twist1, such as PDGFRα (Eckert et al., 2011), there is a delay in induction of FAPα mRNA expression, indicating that FAPα is not a direct transcriptional target of Twist1. We hypothesized that Twist1 is most likely inducing transcription of a different downstream transcription factor, which then is activating expression of FAPα.

3.1A The transcription factors BACH2 and MAFB are upregulated in response to Twist1

Using a multiple sequence alignment tool from the NCBI DCODE resource (Loots and Ovcharenko, 2007), we identified two basic leucine zipper transcription factors, BACH2 and MAFB, that have two consensus binding sites on the 2.5 Kb human and mouse FAPα promoters. The Maf family of transcription factors was originally identified as an activator of erythroid

differentiation (Mignotte et al., 1989). While the Maf proteins do have a conserved basic region, they do not by themselves have any transactivation activity (Oyake et al., 1996); they therefore form heterodimers with the Bach family of proteins, and bind to the T-MARE DNA sequence motif (Oyake et al., 1996). Together, the BACH2/MAFB complex usually serves as a B cell-specific transcriptional repressor (Muto et al., 2004; Muto et al., 2002), but other transcription factors that heterodimerize with BACH2 have been shown to be transcriptional activators also (Kobayashi et al., 2000). Of particular interest to us, there are a few parallels between BACH2/MAFB and Twist1 expression. MAFB was shown to be expressed in migrating neural crest cells that eventually form the craniofacial mesoderm (McGonnell et al., 2001); a critical EMT event during embryonic development in which Twist1 also plays a role. Underscoring the importance of MAFB in craniofacial development, SNPs near the *MAFB* locus were found to be associated with cleft lip and cleft palate (Beaty et al., 2010). Mutations in the *TWIST1* gene in humans are also associated with craniofacial defects (Woods et al., 2009), indicating that Twist1 also plays a key role in craniofacial development (Yu et al., 2008). During embryogenesis, Bach2 is expressed in developing mouse limb buds, and induces expression of *fgf4*, which is a key regulator of limb bud morphogenesis (Kobayashi et al., 2000). Interestingly, Twist1 is also expressed in limb buds, and is also required for *fgf4* gene expression; Twist1$^{-/-}$ mice exhibit defects in limb bud development (O'Rourke et al., 2002). There is little evidence to suggest that BACH2 and MAFB play a role in tumor cell

metastasis, however, given that they function similarly to Twist1 during mammalian development, these two transcription factors emerged as prime candidates to activate FAPα downstream of Twist1.

To investigate the role BACH2 and MAFB play in the transcriptional regulation of FAPα, we first sought to determine whether they were transcriptional targets downstream of Twist1. Using an inducible form of Twist1, Twist-ER, in HMLE cells (Mani et al., 2008), we found that expression of both BACH2 and MAFB increases 8 hours after Twist1 induction (Figure 3-1). This result, combined with the putative BACH2/MAFB binding sites on the FAPα promoter, suggested that BACH2 and MAFB could be the transcription factors downstream of Twist1 that induce FAPα expression.

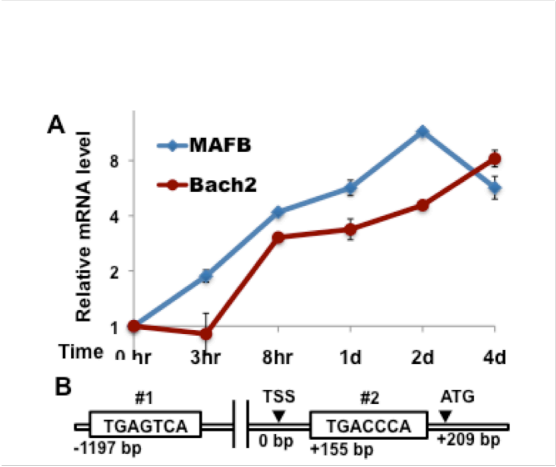

Figure 3-1: BACH2 and MAFB are induced by Twist1. A: mRNA expression of BACH2 and MAFB after Twist1 induction. B: Putative binding sites of BACH2 and MAFB on the FAPa promoter.

We next set out to determine whether knockdown of BACH2/MAFB would affect FAPα expression. We knocked down BACH2 and MAFB separately in HMLE-Twist-ER cells, induced Twist1 expression for 15 days to allow EMT to take place, and then measured FAPα mRNA levels. Unfortunately, this experiment suffered some setbacks, namely that the shRNA-mediated BACH2/MAFB knockdown was either not effective, or not stable over the course of EMT induction. We were thus unable to determine whether BACH2 and/or MAFB are the key transcription factors that regulate FAPα.

Given that FAPα is uniquely expressed in tissues that are undergoing extensive remodeling, such as during wound healing and cancer (Busek et al., 2004), establishing how FAPα is regulated would be a key step in elucidating how it functions in the context of tumor progression and metastasis. Future experiments involving BACH2/MAFB could involve creating various FAPα promoter constructs for luciferase reporter assays, to first establish whether BACH2/MAFB do indeed bind at the putative binding sites found in the FAPα promoter, and whether mutation or deletion of those binding sites would result in loss of FAPα promoter activity. Next, constructing different shRNAs targeting BACH2 and MAFB (the shRNAs used previously were purchased from Sigma) to investigate whether BACH2/MAFB suppression affects FAPα expression in the presence of Twist1 would be an ideal experiment to perform.

3.1B Other evidence of FAPα regulation

Two other published studies have explored the transcriptional regulation of FAPα. The first, by Zhang et al., initially involved cloning the mouse Fap promoter and aligning it to the human FAPα promoter sequence, which revealed several conserved regions. Further investigation of these regions using promoter fragment deletions fused to the luciferase reporter gene found that the key region of the promoter was between -245 and -119, 126 nucleotides upstream of the transcriptional start site. Additionally, the authors showed that this region contained putative EGR1 binding sites (Zhang et al., 2010), and mutation of these binding sites resulted in a decrease in FAPα promoter activity. EGR1, or Early Growth Response 1, is a zinc finger transcription factor involved in regulating a network of tumor suppressor genes (Baron et al., 2006). The authors next carried out EMSA and found an EGR1-DNA complex, indicating that EGR1 does indeed bind to the putative binding site on the FAPα promoter. Finally, the authors demonstrated that siRNA-mediated knockdown of EGR1 in human sarcoma cell lines leads to a 50% reduction in FAPα mRNA expression (Zhang et al., 2010). In this study, neither interference with EGR1 binding, nor knockdown of EGR1 resulted in complete suppression of FAPα expression, indicating that there may be other transcription factors that occupy the key promoter region that the authors identified.

The second study exploring the transcriptional regulation of FAPα involves studying the role of TGFβ -mediated signaling (Tulley and Chen, 2014). Unlike in the previous study, the authors characterized the human

FAPα promoter, and found that it was approximately 2.6 kb long, with a TATA box; the study by Zhang et al. did not identify a TATA box in the human FAPα promoter sequence because it is not conserved between the mouse and human sequences (Zhang et al., 2010). When working with more invasive melanoma cell lines, the authors found that there was a 3-fold increase in secreted TGFβ in the conditioned media as compared to less invasive melanoma cell lines or normal epidermal melanocytes. They also demonstrated that blocking TGFβ signaling, using either a chemical inhibitor targeting the TGFβ type I receptor, or by using neutralizing antibodies against TGFβ to eliminate autocrine TGFβ signaling, resulted in a decrease in FAPα mRNA expression. On the other hand, treatment with TGFβ led to an increase in FAPα mRNA expression, indicating that the TGFβ signaling pathway is possibly playing a role in regulating FAPα expression. The authors next cloned FAPα promoter fragments for use in luciferase reporter assays, and found that they were TGFβ -responsive. Furthermore, a putative Smad 3/4 binding site was found within the FAPα promoter, and ChIP assays confirmed that Smad 3/4 binding was indeed taking place in vivo in the invasive cell lines, further cementing the role of TGFβ -mediated signaling in transcriptional regulation of FAPα.

Interestingly, when the non-invasive cell lines were treated with TGFβ, no increase in FAPα mRNA was observed. However, TGFβ treatment did abolish expression of c-Ski, a known Smad transcriptional repressor that is targeted for ubiquitination and degradation in response to TGFβ (Le Scolan et

al., 2008), in all cell lines, demonstrating that TGFβ -mediated signaling is still active. These results could suggest that other additional transcription activators/repressors are required in less invasive cell lines to induce FAPα expression. Alternatively, the FAPα promoter may not be accessible in these cell lines due to epigenetic silencing. Indeed, since FAPα is expressed only under very specific circumstances, its promoter could possibly be under stable repression, similar to how epithelial genes must be silenced for a long period of time while EMT is underway (Tam and Weinberg, 2013). It may be worth comparing histone modifications in invasive and non-invasive cells: H3K9 trimethylation, for example, creates heterochromatin that is more resistant to transcriptional activation than trimethylation of H3K27 (Tam and Weinberg, 2013). The studies by Zhang et al. and Tulley and Chen show that transcriptional regulation of FAPα is complex and most likely involves redundant pathways. Our data demonstrates that Twist1 is definitely a factor in the regulation of FAPα expression, but further in-depth studies are needed to fully understand how this protease is expressed.

3.2 The role of FAPα in invadopodia dynamics

The previous chapter described experiments in which the protease activity of FAPα was abrogated, either through chemical inhibition or through mutation of the catalytic domain. Suppressing protease activity did not have an effect on ECM degradation however, suggesting that FAPα may be playing more of a structural role in invadopodia. Furthermore, MT1-MMP does not

localize to lipid raft microdomains, which are where invadopodia typically form (Albrechtsen et al., 2011; Yamaguchi et al., 2009), when FAPα is not present. Together, these results suggest that FAPα may be involved in promoting invadopodia maturation through recruitment of proteases, rather than actually contributing its protease activity towards ECM dedgradation. Along with the recruitment of proteases, invadopodia maturation also involves the active elongation of the entire structure (Schoumacher et al., 2010), and since invadopodia are dynamic and transient structures (Linder, 2007), invadopodia must be able to retract, allowing the cell to migrate through the ECM. Since the protease activity of FAPα is not required for properly functioning invadopodia, we propose that it may instead be playing a role in regulating invadopodia dynamics.

Surrounding the actin-rich core of invadopodia is a host of actin-regulatory machinery designed to regulate invadopodia dynamics. These include actin nucleators, such as the Arp2/3 complex, actin polymerization activators, such as cortactin and N-WASP, actin filament crosslinkers, and actin binders (Murphy and Courtneidge, 2011). Schoumacher and colleagues formulated a model for invadopodia elongation and maturation: first, a core consisting of dendritic and bundled actin networks is formed at the ventral surface of the cell, providing a foundation for invadopodia formation. During elongation, the actin bundles lengthen with the help of filapodial machinery. Finally, during maturation, microtubules infiltrate the structure, possibly providing tracks through which proteases can be delivered to the tip of the

elongating invadopodia (Schoumacher et al., 2010). The authors of the study that established this model also point out that MMP activity is required for invadopodia elongation; cells treated with the pan-MMP inhibitor GM6001 did not demonstrate proper lengthening. Given the fact that we observed cortactin- and F-actin-positive invadopodia in FAPα knockdown cells along with a significant reduction in ECM degradation, we reasoned that these invadopodia may be immature and have defects in their dynamic machinery.

To test whether FAPα plays a role in invadopodia dynamics, HS578T cells expressing either control or shRNAs targeting FAPα, alongside mCherry-LifeAct, a 17-amino acid that stains F-actin (Riedl et al., 2008), were seeded onto plates coated with collagen I. Confocal images were taken every 30 seconds for 30 minutes to visualize invadpodia turnover. Control cells exhibited extensive invadopodia turnover, with individual F-actin punta disappearing and then reappearing into view (HS578T shCtrl 30min Movie). FAPα knockdown cells, however, had static invadopodia that stayed present throughout the 30 minutes of image capturing (HS578T FAPa-sh3 30min Movie and HS578T FAPa-sh5 30min Movie). These data suggest that FAPα plays a role in invadopodia dynamics, most likely during the maturation and retraction phases. As noted earlier, active MMPs are required for proper elongation, with MT1-MMP located at the tip of lengthening invadopodia (Schoumacher et al., 2010). If FAPα is indeed required for proper MT1-MMP localization as discussed in the previous chapter, then perhaps the static invadopodia seen in the FAPα knockdown cells are not elongating properly. It

is important to note, however, that invadopodia are routinely visualized with more than one marker (Bowden et al., 2006), and therefore these studies need to be repeated with another fluorescently tagged invadopodia marker to ensure that the puncta observed are in fact invadopodia. We are currently working towards carrying out live cell imaging with GFP-tagged MT1-MMP.

Integrins also play an important role during invadopodia formation. As receptors that directly interact with the ECM and relay extracellular signals into the cytoplasm, they are in an ideal position to initiate invadopodia assembly. Indeed, integrins α3β1, α5β1 (Mueller et al., 1999), and αvβ3 (Deryugina et al., 2001) have been found at invadopodia in epithelial cells. Integrins α3β1 and αvβ3 serve as docking sites for FAPα and the MMP2/MT1-MMP activation complex, respectively. In addition to acting as a point of cell-matrix contact, integrins also mediate downstream signaling cascades; for example, integrin β1 has been shown to activate Arg kinase (Beaty et al., 2013), and a loss of integrin β1 expression led to reduced Src phosphorylation (Huck et al., 2010).

Given the key role that integrins play in invadopodia assembly, especially in the context of FAPα, we sought to determine whether FAPα suppression has any effect on integrin expression and distribution. HS578T cells expressing either control or FAPα-targeting shRNAs were seeded onto coverslips coated with collagen I for 3 days, after which the cells were fixed with 4% PFA and immunofluorescence (IF) was carried out for integrin β1 and two markers of invadopodia, cortactin and F-actin. The staining pattern revealed a ring of integrin β1 surrounding the invadopodia in both the control

cells and the FAPα knockdowns (Figure 3-2). Interestingly, not all invadopodia were surrounded by an integrin ring; only those that had reached a certain size. Quantification of the rings showed that FAPα knockdown cells contained a greater percentage of ringed invadopodia, compared to control cells (Figure 3-2).

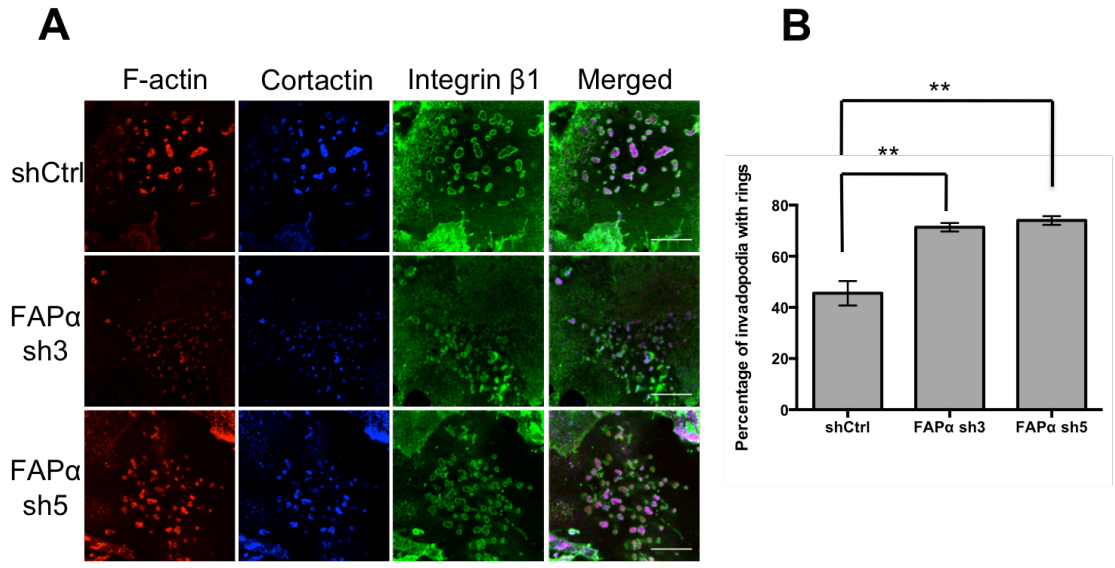

Figure 3-2: Integrin β1 rings surround invadopodia in HS578T cells. HS578T cells expressing the indicated shRNAs were stained for F-actin (red), cortactin (blue), and integrin β1 (green). Scale bar: 10 μm. FAPa knockdown cells contain a higher percentage of cells with ringed invadopodia. **p < 0.02, using a two-tailed Student's T-test.

Upon carrying out the same IF in different cell lines (HMLE-Twist, and the head and neck cancer cell line SCC61), the integrin β1 ring pattern disappeared. We instead observed that the integrin β1 looked more like dots instead of rings, and these dots were often close to, but not co-localizing with, invadopodia. We were unfortunately unable to successfully stain any of our cell lines for α-integrins, but we plan to troubleshoot this assay in the future.

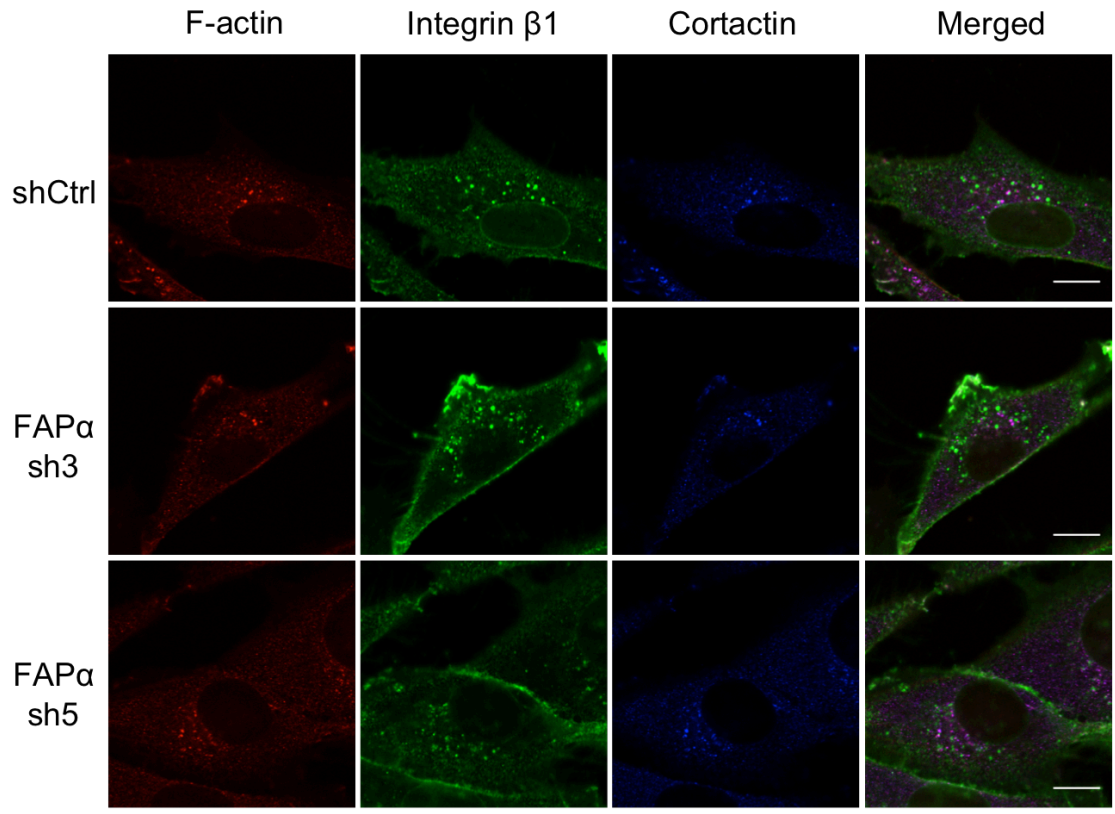

Figure 3-3: Integrin β1 forms dots in HMLE-Twist cells. HMLE-Twist cells were stained for F-actin (red), integrin β1 (green), and cortactin (blue). Integrin β1 does not form rings around invadopodia in these cells. The integrin punctae do not seem to co-localize with invadopodia. Scale bar: 10 μm.

The differences in integrin β1 staining patterns might provide clues as to invadopodia functionality. It could be that the ringed pattern provides extra adhesion to the ECM, which might prevent the invadopodia from elongating properly. This would explain why the HS578T cells – a cell line that does not degrade the ECM as effectively as other lines – exhibit this pattern. It would also explain why the invadopodia in the FAPα knockdown cells, which had a higher percentage of ringed invadopodia, are static: if the integrin-mediated adhesion is too strong around the site of invadopodia assembly, further

elongation might not be possible. Increased adhesion at the site of invadopodia formation would also most likely present a challenge for invadopodia to disengage from its sites of cell-matrix contact. The integrin dot pattern might allow for increased invadopodia turnover – and thus more ECM degradation than the integrin ring pattern.

3.3 Summary and future directions

Breast cancer metastasis, rather than the primary breast tumor, is the main cause of death in breast cancer patients. During metastasis, cells break away from the primary tumor, invade through surrounding tissues, and intravasate into the circulatory system. After using the circulatory system to travel to distant organs, they then extravasate into a new tissue environment, where they then proceed to form micrometastases that eventually develop into secondary tumors (Nguyen et al., 2009).

The basic helix-loop-helix transcription factor Twist1 is required for metastasis to take place. Twist1 enables tumor cells to undergo metastasis by inducing EMT, which results in the dissolution of cell-cell junctions and the expression of mesenchymal cell proteins, which make cells more migratory and invasive (Yang et al., 2004). As a part of the EMT process, Twist1 increases the invasive capabilities of tumor cells by inducing formation of invadopodia, actin-rich subcellular structures that serve to localize proteases to cell-matrix points of contact (Eckert et al., 2011). Formation of invadopodia,

along with Twist1 expression, is required in order for tumor cells to undergo metastasis.

Twist1 is involved in inducing expression of the type II serine protease FAPα, which is expressed exclusively during wound healing and in tissues undergoing extensive remodeling (O'Brien and O'Connor, 2008). FAPα localizes to invadopodia, and suppression of FAPα expression dramatically reduces the number of cells that disseminate from the primary tumor to the lungs in a mouse model of metastasis. FAPα knockdown cells are unable to effectively degrade the ECM, but cells treated with an FAPα inhibitor or expressing a catalytically inactive form of FAPα are still able to carry out ECM degradation, indicating that FAPα may be playing a structural role in invadopodia. Cells that do not express FAPα also fail to properly localize MT1-MMP to lipid raft sites, where invadopodia are known to form (Yamaguchi et al., 2009), suggesting that FAPα might be required for protease recruitment in invadopodia. Ongoing studies of FAPα include elucidating the mechanistic details of its transcriptional regulation, since FAPα does not appear to be a direct transcriptional target of Twist1. We are also exploring FAPα's potential role in invadopodia dynamics.

There are still several questions that remain regarding FAPα and its role in invadopodia assembly and function. FAPα is localized to invadopodia, but how and when during invadopodia formation it arrives at the actual structure is still very much unknown. The trafficking of proteases is a key step in invadopodia maturation. It is known that MT1-MMP is delivered to

invadopodia through multiple potential routes, such as through late endosomes via N-WASP (Steffen et al., 2008; Yu et al., 2012), or via clatherin-mediated endocytosis (Murphy and Courtneidge, 2011). FAPα trafficking to invadopodia needs to occur earlier than that of MT1-MMP, if it is indeed functioning as a docking site for proteases, and thus different mechanisms of delivery may be involved. While it has been established that proteases localize to invadopodia late in invadopodia assembly (Artym et al., 2006; Murphy and Courtneidge, 2011), the timing of FAPα trafficking to invadopodia remains to be confirmed. Since it is most likely playing a structural role in invadopodia formation, FAPα probably localizes to invadopodia early during assembly.

While a previous study claimed that FAPα heterodimerizes with DPP4 at invadopodia (Ghersi et al., 2006), data from the previous chapter demonstrates that DPP4 expression is not required for proper invadopodia function. The question of whether FAPα interacts with any other proteins during invadopodia assembly thus remains to be answered. The extracellular domain of FAPα is very large, and consists of several cysteine residues that could potentially form disulfide bridges that would serve to connect FAPα to other proteins. Establishing which proteins interact with FAPα would also provide a greater understanding of FAPα's role in invadopodia assembly. We are currently pursuing this avenue of inquiry through immunoprecipitation and mass spectrometry experiments.

Along a similar vein, the specific domain of FAPα responsible for allowing ECM degradation to take place is still not known. To answer this

question, we attempted to construct deletion mutants of FAPα, along with chimeric proteins that had various domains of FAPα replaced with the DPP4 equivalent, but were unable to express these mutant proteins in cells. Nevertheless, we will continue to investigate this avenue of research, because elucidating the domain of FAPα necessary for its function in invadopodia has important therapeutic applications. Prior therapies targeting FAPα have solely focused on inhibiting its protease activity (Eager et al., 2009; Narra et al., 2007), which we have shown to be ineffective at abrogating ECM degradation. A better tactic, perhaps, would be to use monoclonal antibodies to target the domain of FAPα responsible for protein-protein interactions, or the region required for its potential function as a docking site for MT1-MMP. Even though the development of anti-metastasis therapeutics is still in its infancy, targeting markers of invadopodia, which could prevent the formation of deadly secondary tumors, is a promising place to start.

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HS578T FAPa-sh3 30min Movie

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