UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Fibroblast Activation Protein in Invadopodia and Tumor Metastasis

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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The Thesis of Dong Mei Li is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2014

DEDICATION

This thesis is dedicated to my family, whose unwavering love, support, and encouragement allow me to achieve what I have accomplished thus far.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BM: Basement Membrane

CTC: Circulating Tumor Cell

DMEM: Dulbecco's Modified Eagle Medium

ECM: Extracellular Matrix

EMT: Epithelial-Mesenchymal Transition

FAP: Fibroblast Activation Protein

GFP: Green Fluorescent Protein

HMLE: Human Mammary Epithelial cells immortalized with Large T antigen

IF: Immunofluorescence

MET: Mesenchymal-Epithelial Transition

MMP: Matrix Metalloprotease

MT1-MMP: Membrane-Tethered Matrix Metalloprotease

SCC: Squamous carcinoma cell

shRNA: small hairpin RNA

LIST OF FIGURES

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I would like to thank Dr. Jing Yang whose support and guidance have made this entire effort possible. I have learned an immense amount about scientific research, and Dr. Yang's encouragement and mentorship have been invaluable throughout my learning process.

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ABSTRACT OF THE THESIS

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Tumor metastasis, the leading cause of death among cancer patients, is a complex multi-step process during which tumor cells disseminate from

their primary site to distant organs. The transcription factor Twist1 is overexpressed in many aggressive human cancers and is a potent inducer of tumor metastasis. Mechanistically, Twist1 activates a developmental program known as the Epithelial-Mesenchymal Transition (EMT), during which carcinoma cells lose cell-cell junctions and become more migratory and invasive. Twist1 can also induce the formation of invadopodia, actin-rich membrane protrusions that recruit various proteases to degrade the extracellular matrix (ECM). Various studies show that invadopodia are crucial for tumor invasion during metastasis. We have identified the membrane-associated serine protease Fibroblast Activation Protein (FAP) to be a critical gene induced upon Twist1 activation to promote invadopodia function. However, the precise role of FAP at invadopodia and its function in tumor metastasis is not well understood.

This study presents data demonstrating that FAP is essential for invadopodia-mediated ECM degradation and breast tumor metastasis in vivo. Mechanistically, I found that FAP is required for the proper localization of matrix metalloproteinase MT1-MMP to invadopodia in order to generate mature and functional invadopodia for EMT degradation. Together, these results indicate that FAP is a critical regulator of invadopodia function and tumor metastasis. Given the unique expression of FAP in human cancers, FAP could be a promising target for anti-metastasis therapeutics.

Introduction

Breast cancer

Cancer, a disease characterized by the uncontrolled growth of abnormal cells, is the second most common cause of death in the United States and is a disease that anyone could be susceptible to. In 2014, approximately 1.6 million cases will be diagnosed and over half a million people are expected to die of cancer in the US (American Cancer Society, 2014). Although the relative survival rate for all cancers diagnosed has increased over the years due to earlier diagnoses and improved treatments, there is still much unknown in cancer biology.

One particularly prevalent type of cancer that affects individuals worldwide is breast cancer, as one in eight women will develop breast cancer during their life times. About 232,670 new cases of invasive breast cancer are expected to be diagnosed among women and 2,360 new cases are expected in men during 2014 (American Cancer Society, 2014). In these individuals, approximately 90% of breast cancer related deaths would be due to the metastatic dissemination of the disease (Cummings et al., 2014). While early detection, surgical intervention, and adjuvant treatment can effectively treat localized primary tumors, secondary tumors from metastasis are often resistant to treatment and causes lethality. Breast cancer in particular metastasizes most often to the lung, bone, brain, and liver (Weigelt et al., 2005).

Metastasis

Metastasis is a highly complex multistep process during which tumor cells disseminate from the primary tumor to establish secondary tumors at a distal site. In order to metastasize, a tumor cell must accomplish each step in the metastatic cascade (Figure 1-1): lose cell-to-cell adhesions to gain the ability to move and invade into surrounding tissues, intravasate and travel through the circulatory or lymphatic system to a distal organ, extravasate out of the circulatory or lymphatic system into the organ tissue, and establish secondary tumors in the new organ (Nguyen et al., 2009).

During the development of organ systems, epithelial layers are formed by cells connected laterally by epithelial junctions, allowing for the assembly of cell-cell adhesions within the sheets of epithelial cells. These sheets of epithelial cells establish an apical-basal polarity, where epithelial cells can only move laterally along the basement membrane (BM), a specialized extracellular matrix (ECM) that protects and organizes the polarized epithelial tissues as well as acts as a barrier against cell invasion. Tumor cells gain motility and invasiveness due to the modifications to its shape and attachment to other cells in order to breach the BM, along with the aid of matrix metalloproteinases (MMPs).

Once the BM has been breached, the tumor cells can intravasate into the circulatory or lymphatic system, allowing them to spread widely around the body. These circulating tumor cells then face multiple obstacles that drastically

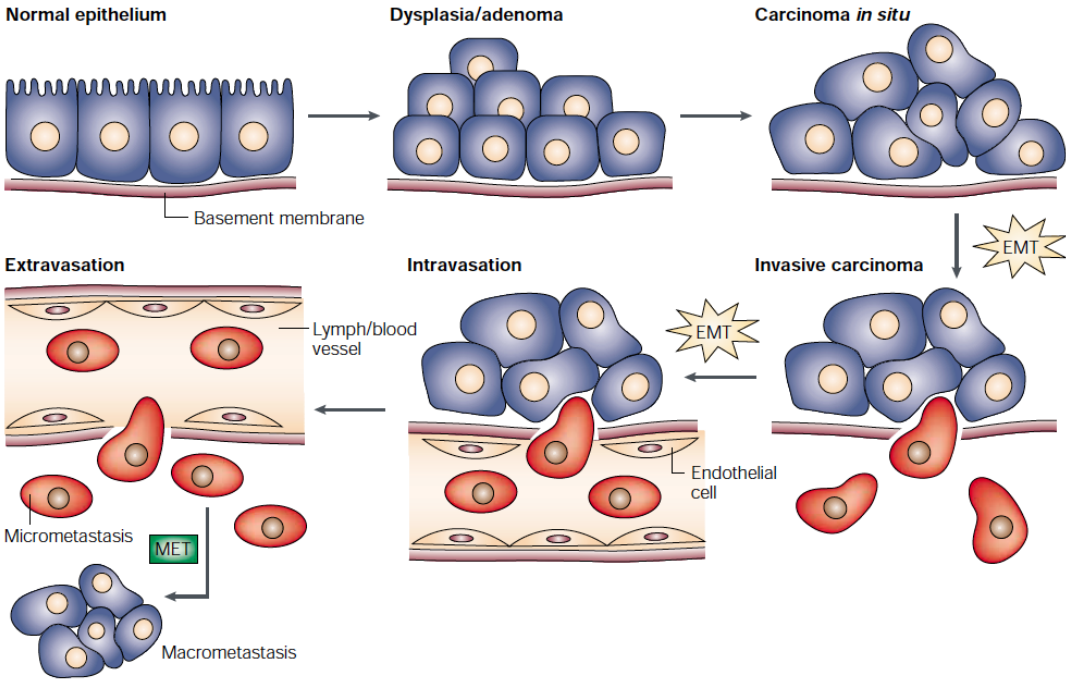

Figure 1-1: Multi-step progression of tumor cell metastasis.
Adapted from Thiery, J.P. 2002. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2:442-454.

reduce the number of tumor cells that are capable of establishing secondary tumors in distal organs. First, the tumor cells must undergo an intravasation process in order to enter the circulatory or lymphatic system for systemic dissemination. While a developmental program known as Epithelial-Mesenchymal Transition (EMT) is thought to help modulate the migratory and invasive properties of tumor cells, the precise mechanism of how tumor cells are capable of intravasation is still vague. Next, the circulating tumor cells must possess the proper capabilities to survive in the vasculature by avoiding anoikis, a form of programmed cell death induced when anchorage-dependent cells are detached from the ECM. Recent studies have shown that the circulating tumor cells rely on maintaining their mesenchymal state by associating with platelet cells to increase TGF-β production (Labelle et al., 2011). Then, the circulating tumor cells must extravasate to the organ of interest. Extravasation has been shown as a relatively rapid process where EMT has also been implicated in the promotion of extravasation and the initial establishment of the tumor cells in distal organs (Stoletov et al., 2010).

Once the circulating tumor cells have extravasated to a distal site, the resulting metastases show an epithelial character, suggesting a reversal of EMT known as Mesenchymal-Epithelial Transition (MET). A previous study conducted by Tsai et al has shown that the loss of an EMT-inducing signal at a distal site prohibited cell proliferation and metastases formation (Tsai et al., 2012). This suggests that only epithelial tumor cells could establish and generate metastases but the cooperation between both epithelial and

mesenchymal tumor cells is necessary for the formation of metastatic tumors (Tsai and Yang, 2013). The journey within the blood/lymphatic vessels, the hostile new tissue the tumor cells must extravasate into, and the ability to colonize and proliferate to establish secondary tumors are all factors that contribute to the selective process of metastasis and thus metastasis latency – the time between organ infiltration and colonization (Nguyen et al., 2009).

Epithelial-Mesenchymal Transition (EMT)

Epithelial-Mesenchymal Transition (EMT) is a crucial developmental regulatory program that has been designated as the first step of metastasis (Yang et al., 2004). While epithelial cells cannot cross into the ECM because of its anchorage to the BM and apical-basal polarity, elongated migratory mesenchymal cells from the epithelium can invade into the ECM as individual cells due to their lack of polarity (Yang and Weinberg, 2008). This transition of epithelial to mesenchymal cells allows for the phenotypic and molecular changes necessary for metastasis like breaking down cell-cell adhesion, becoming more motile, and invading through the ECM (Thiery and Sleeman, 2006).

EMT was first discovered during embryonic development where the formation of mesenchymal cells was more appropriate for the migration and establishment in areas for organ formation (Thiery, 2002). Later, EMT was observed in other normal developmental processes like gastrulation, neural crest development, and wound healing. In gastrulation, the primitive epithelial

cells move to the midline of the embryo to form the primitive streak before undergoing EMT to detach from the epithelial layer and form the mesoderm and endoderm of the embryo (Nakaya and Sheng, 2008). For neural crest development, the epithelial cells in the midline of the neural tube undergo EMT and become migratory neural crest cells that can differentiate to form different tissue subtypes (Acloque et al., 2009). Besides these developmental processes, EMT has also been observed in wound healing as cells moved into the wounded site to fill in the gap in the epithelium (Weinberg, 2007).

There are strong resemblances between the metastatic cascade and the normal developmental processes, suggesting that metastatic tumor cells are able to re-activate EMT to change their own morphology, motility, and ability to invade and disseminate throughout the body. For example, carcinomas progress through the metastatic cascade as the cancerous epithelial cells develop changes capable of invasion after undergoing EMT. Certain molecular markers categorize these changes due to EMT like the loss of E-cadherin, a crucial cell-cell adhesion molecule, and gain of N-cadherin, a cadherin that promotes cell motility and invasiveness (Cavallaro, 2004; Hanahan and Weinberg, 2011).

Invadopodia

Several crucial steps of metastasis following EMT, like invasion, intravasation, and extravasation, are thought to involve ECM degradation and remodeling. Recently, specialized subcellular proteolytic structures known as

invadopodia have been shown to be critical for ECM degradation (Linder, 2007). The structure of invadopodia consists of an actin-rich core, surrounded by important protein components, such as cytoskeletal modulators, adhesion proteins, scaffolding proteins, and signaling molecules (Murphy and Courtneidge, 2011). As invadopodia are actin-rich membrane protrusions located on the basal side of transformed cells, invadopodia recruit various matrix proteases to degrade the ECM, thereby allowing the epithelial-turned-mesenchymal tumor cells to invade through the BM and break away from the primary tumor (Eckert et al., 2011). While other actin-based protrusions like lamellipodia and filopodia are present in normal cells, invadopodia are distinctively present in invasive cancer cells (Linder, 2007).

Using intravital imaging techniques on metastatic tumor xenograft in mice, a number of studies have revealed invadopodia formation during both the intravasation and extravasation steps of tumor metastasis (Eckert et al., 2011). In addition, suppressing invadopodia function by inhibiting essential invadopodia components like cortactin, Tks5, or MT1-MMP, prevents breast cancer metastasis in vivo (Paz et al., 2014). Altogether, the data suggest that invadopodia-mediated ECM degradation is essential for tumor invasion and metastasis.

Given that the central function of invadopodia is to recruit proteases for matrix remodeling, invadopodia contain many proteases like matrix metalloproteinases (MMPs), the ADAM (A Disintegrin And Metalloproteinase) family, and membrane-bound serine proteases (Murphy and Courtneidge,

2011). MMPs are extracellular proteases that can degrade and remodel the BM, allowing tumor cells to invade into the surrounding tissues around the primary tumor (Kessenbrock et al., 2010). One such protease shown to be crucial in degrading the ECM for invasion is MMP14, also known as MT1-MMP, and its downstream targets, MMP2 and MMP9. MT1-MMP cleaves collagen fibers, which is the most abundant protein in the ECM as well as an important structural component in blood vessels, to allow tumor cells to form a track for cellular migration and invasion (Tam et al., 2002).

These proteases have been implicated in cancer progression and metastasis, implying that MMPs inhibition could suppress ECM degradation and tumor metastasis. However, numerous metalloproteinase inhibitors have failed in clinical trials and some MMPs have been shown to have anti-tumorigenic effects (Freije et al., 2003; Overall and Kleifeld, 2006). While the approach of broadly inhibiting MMPs might not be a feasible approach to prevent tumor metastasis, inhibiting the molecular mechanism regulating invadopodia assembly might be an alternative to effectively inhibit matrix degradation and tumor invasion.

The role of Twist1 in EMT, Invasion, and Metastasis

The tumor invasion-metastasis cascade contains multiple steps that tumor cells must encounter and survive in order for successful metastasis. These steps generally begin with the induction of EMT and then invasion, followed by metastasis. While EMT can be triggered by environmental

stimulus like hypoxia, inflammation, oncogenic stress, and metabolic stress, the changes that occur during EMT are controlled by transcriptional modifications of multiple genes involved in cell-cell adhesion, cell migration, and invasion (Figure 1-2). Several transcription factors have been identified as EMT inducing, meaning that the ectopic expression of the gene was sufficient to induce EMT in epithelial cells.

One such transcription factor that has been extensively studied for its role in EMT is the Twist1 transcription factor. Twist1, a basic helix-loop-helix transcription factor, was first identified as a critical gene for proper gastrulation and giving rise to mesoderm formation in Drosophila (Leptin, 1991). During mammalian development, Twist1 is involved in neural tube closure and myogenic differentiation (Chen and Behringer, 1995). It has been demonstrated that the induction of Twist1 is required for EMT to take place, and is thus necessary for tumor cell metastasis (Yang et al., 2004). This finding was supported by a number of studies that correlated Twist1 expression with invasive cancers: Twist1 is expressed in breast cancer (Vesuna et al., 2006), hepatocellular carcinoma (Lee et al., 2006), head and neck cancer (Yuen et al., 2007), cervical, ovarian, bladder, and gastric cancers (Kajiyama et al., 2006; Shibata et al., 2008; Vecchi et al., 2007; Zhang et al., 2007). In many of these cancers, the high levels of Twist1 correlated with increased rate of metastasis, poor overall prognosis, and poor progression-free-survival.

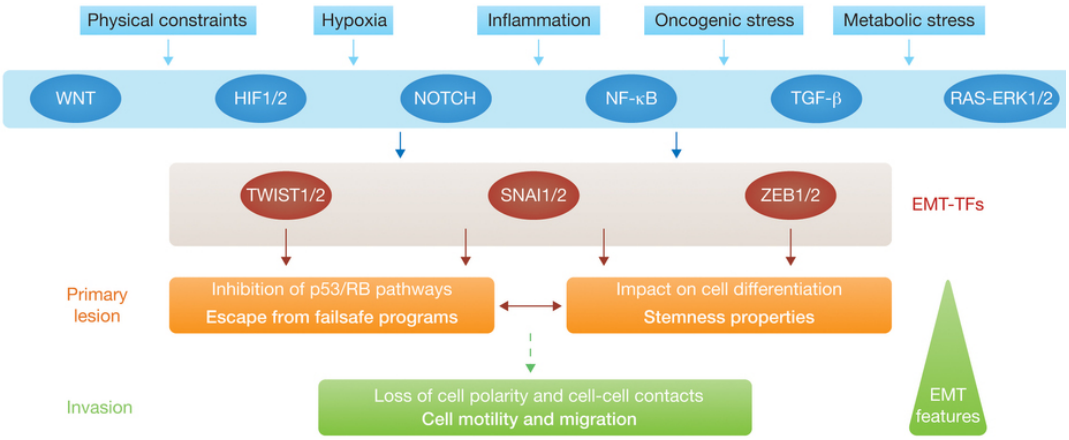

Figure 1-2: Schematic overview of transcription factors involved in EMT.
Adapted from Puisieux, A., Thomas, B., and Caramel, J. 2014. Oncogenic roles of EMT-inducing transcription factors. *Nature Cell Biology*. 16: 488-494.

In addition, a study accomplished by Eckert et al demonstrated that Twist1 is a key mediator in the formation of invadopodia through the induction of growth factor PDGFRα and that both PDGFRα expression and invadopodia assembly were required for carcinoma cell metastasis to occur (Eckert et al., 2011). This suggests that the regulation of invadopodia-mediated ECM degradation is pivotal for invasion metastasis.

Fibroblast Activation Protein (FAP)

Previous data from our lab have found that Twist1 induces the expression of Fibroblast Activation Protein (FAP) and that FAP accumulates at invadopodia. In addition, FAP is exclusively expressed in wound healing and invasive epithelial cancers (O'Brien and O'Connor, 2008). Given that studies have already established Twist1 as a necessary component for the induction of EMT, invadopodia formation, and consequent tumor metastasis, FAP and its unique expression could have significant implications in the tumor invasion-metastasis cascade, specifically in invadopodia and consequently the regulation of metastasis.

FAP was first identified in 1986 in cultured fibroblasts by using monoclonal antibody F19, and subsequently named as F19 Cell Surface Antigen (O'Brien and O'Connor, 2008). FAP, a 170-kDa transmembrane glycoprotein, consists of a small cytoplasmic domain, a glycosylated β-propeller domain rich in cysteine residues, a single-pass transmembrane domain, and the catalytic domain (Chen and Kelly, 2003). FAP has both

exopeptidase and endopeptidase activity, and has been shown as localized at invadopodia (Ghersi et al., 2006). Although the role of FAP at invadopodia is unclear, some studies insinuate that FAP's gelatinase activity may contribute to invadopodia's degradation activity. For example, it has been shown that after MT1-MMP cleaves collagen I, FAP digests collagen I into smaller fragments, suggesting that FAP cooperates with other proteases to cleave ECM components (Christiansen et al., 2007).

FAP is a promising target for inhibiting tumor metastasis because FAP expression is not only restricted to tissues undergoing wound healing and invasive epithelial cancers but also has been identified through immunohistochemistry of tumor cells and stromal fibroblasts of various aggressive cancer types including breast, gastric, colonic, and cervical cancers (O'Brien and O'Connor, 2008). Furthermore, FAP is expressed in the stroma of epithelial malignancies where increased expression of FAP is associated with more aggressive tumor growth (Henry et al., 2007). FAP and its presence at invadopodia as well as its correlations with aggressive cancers warrant further detailed study in the invasion-metastasis cascade as it has the potential of leading to an effective method to block tumor invasion and metastasis.

While Twist1 activates EMT, cells undergo morphological changes and become motile in order to become invasive. Then, Twist1 is also necessary for invadopodia formation, in order for invadopodia-mediated ECM degradation to occur and allow the newly epithelial-turned-mesenchymal cells to invade the

vasculature and establish the subsequent metastases at distal sites. Since FAP is a protease induced by Twist1 and found at invadopodia, FAP is a promising target to investigate whether and how the role of FAP in different cell types contributes to tumor development and whether the role of FAP at invadopodia is necessary for the tumor-invasion-metastasis cascade.

In this study, we present data on how FAP is necessary for the tumor invasion-metastasis cascade as the loss of FAP impaired invadopodia-mediated ECM degradation, leading to a reduction of metastases in a mouse breast tumor model. Our data suggest that FAP acts as a stabilizing protein at invadopodia in order to aid in ECM degradation and remodeling.

CHAPTER 1

Fibroblast Activation Protein (FAP)

Promotes Tumor Cell Metastasis

FAP expression is required for Twist1-mediated tumor metastasis

Previous studies have shown that Twist1 is required for EMT and metastasis as well as invadopodia assembly (Eckert et al., 2011; Yang et al., 2004). Thus, it was imperative to examine genes downstream of Twist1 and their possible involvement with invadopodia. Through a DNA microarray analysis with an inducible Twist1 (Twist-ER) construct (Mani et al., 2008), it was found that the mRNA and protein expression of Fibroblast Activation Protein (FAP) increased dramatically post Twist1 induction (Figure 2-1A, 2-1B) (Pathak, 2014).

Given that Twist1 is required for tumor cells to metastasize from the mammary gland to the lungs and that Twist1 induces FAP expression, we sought to determine whether FAP plays a role in metastasis in vivo. We first looked into an exogenous system where Twist1 was overexpressed in order to ensure that the expression of FAP would be induced. HMLER-Twist cells expressing either a control shRNA (short hairpin RNA), or one of two independent shRNAs targeting FAP were injected subcutaneously into nude mice (Figure 2-2A, 2-2B). These HMLER-Twist cells were labeled with GFP and transformed with oncogenic Ras to induce the growth of tumors upon injection into mice.

While FAP suppression did not significantly affect the primary tumor growth rate or tumor size (Figure 2-2C, 2-2D), FAP knockdown severely affected the ability of tumor cells to metastasize to the lungs. Compared to the HMLER-Twist cells expressing the control non-target shRNA, the FAP

knockdown cells failed to metastasize to the lungs by an average of 400-fold, even though the primary tumors had similar growth rates and weights (Figure 2-2E, 2-2F). These data establish that the induction of FAP expression is crucial for the ability of tumor cells to metastasize to the lungs.

FAP expression is required for spontaneous tumor metastasis

Next, we wanted to see if these results could be replicated in an endogenous model, where FAP is endogenously expressed instead of purposely induced like the exogenous model. The 4T1 mouse breast tumor cell line was employed because of its close similarity to human breast cancer as well as its ability to spontaneously produce highly metastatic tumors that can metastasize to both the lungs and liver (Aslakson and Miller, 1992).

We first performed a preliminary trial with 15 mice to assess whether FAP is really a necessary component for metastasis and if the 4T1 cell line was an appropriate system to utilize. 4T1 mouse cells expressing either a control shRNA, or one of two independent shRNAs targeting FAP were injected into the mammary fat pads of BALB/C mice (Figure 2-3A). Tumors quickly grew and the mice were sacrificed within a month post injection due to the highly metastatic characteristic of the 4T1 cell line.

As the 4T1 cells are known to spontaneously metastasize to the lungs, the formation of visible nodules on the lungs was determined to be the metastatic spread of 4T1 cells. The tumors across the control shRNA and independent shRNAs targeting FAP groups were around 2 grams in weight,

but the amount of lung metastases in each group differed drastically (Figure 2-3B). The FAP knockdown cells definitively had less metastasis to the lungs when compared to the cells expressing the control shRNA. The preliminary trial held promising results that suggested FAP knockdowns seemed to suppress lung metastases when compared to the control shRNA (Figure 2-3C, 2-3D). In addition, the 4T1 cell line was an ideal system to utilize, as it closely mimicked human breast cancer that would spontaneously give rise to visible white nodules as metastases in lungs.

Thus, we repeated the in vivo experiment with the 4T1 cell line and increased the number of mice from 15 to 30. In addition, I generated new 4T1 cells to express a different control shRNA as well as two independent shRNAs targeting FAP (Figure 2-4A). These new 4T1 cells expressing the control shRNA or shRNAs targeting FAP and the 4T1 parental cells were injected into the mammary fat pads of BALB/C mice.

Although the relative mRNA expressions of FAP in the FAP knockdown cells were about 40% and 50% suppressed compared to the cell expressing the control shRNA, respectively, the phenotype of metastasis to the lungs was apparent. Again, the FAP suppression did not significantly affect primary tumor weight, an average of 1.5 grams (Figure 2-4B), but greatly affected the tumor cells' ability to metastasize to the lungs. While the 4T1 cells expressing a control shRNA generated multiple small nodules throughout the lungs, both the FAP knockdown cells failed to metastasize to the lungs, with the cells expressing 50% FAP expression to have much less metastasis (Figure 2-4C,

2-4D). This data, in combination with the results from the prior in vivo experiments, indicate that FAP expression is indeed crucial for metastasis in vivo as the FAP knockdown cells had a decreased ability of metastasizing to the lungs for both the exogenous and endogenous models.

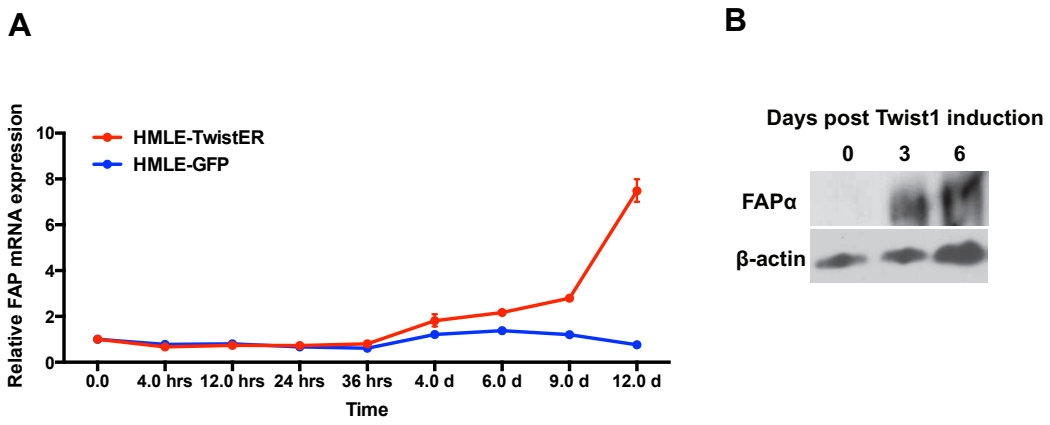

Figure 2-1: FAP is induced by Twist1.
A: The mRNA levels of FAP were measured in HMLE-TwistER cells undergoing Twist1 induction, compared to control cells not induced by Twist1.
B: Cell lysates from induced HMLE-TwistER cells were analyzed and probed for FAP and β-actin.

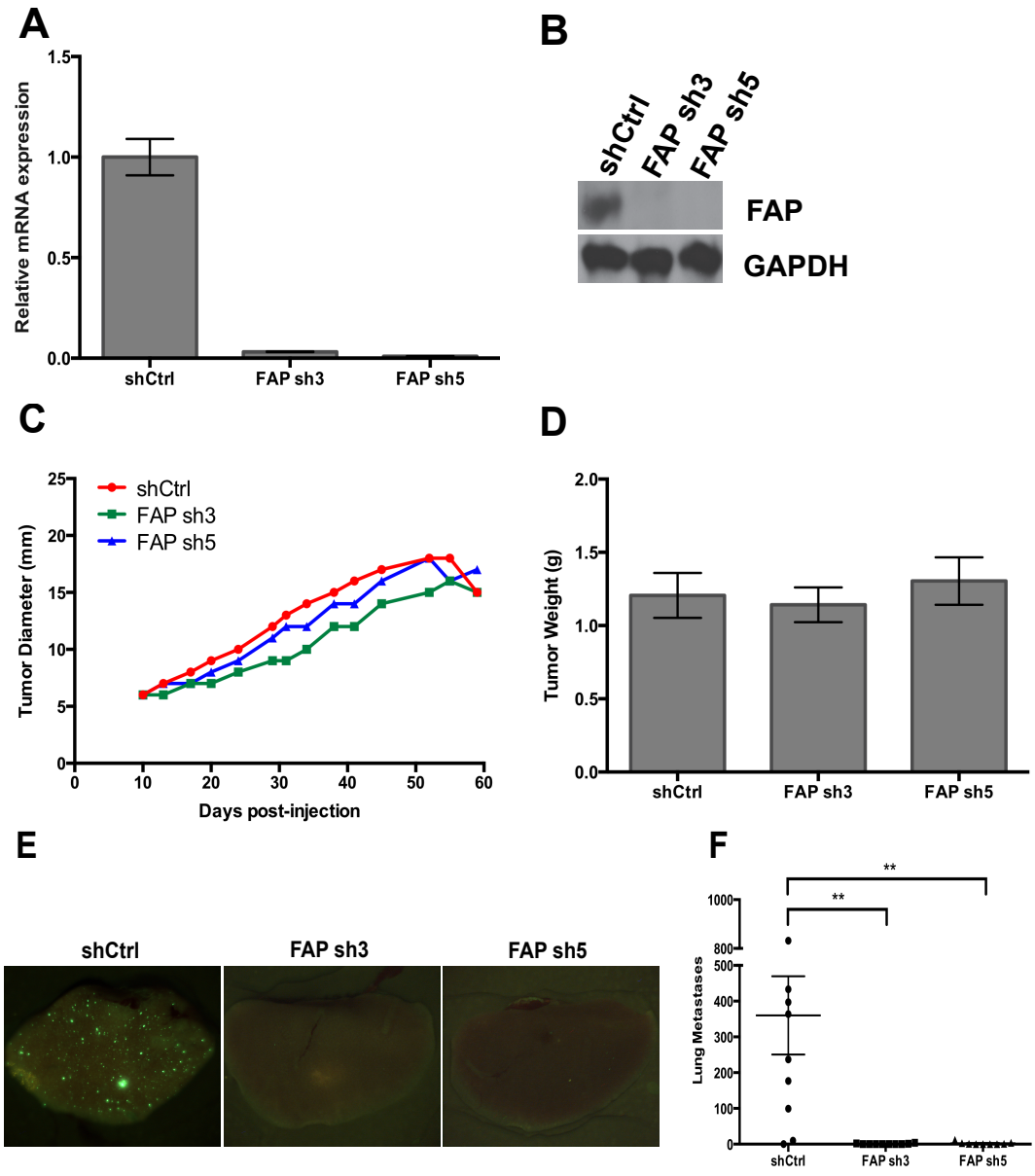

Figure 2-2: FAP expression is required for Twist1-mediated metastasis with HMLER-Twist cells.
A: The mRNA expression of FAP in HMLER-Twist cells expressing two independent shRNAs targeting FAP.
B: FAP protein expression in HMLER-Twist cells.
C: Tumor growth rate is not affected by FAP knockdown. (not significant)
D: Tumor weight is not affected by FAP knockdown. (not significant)
E: Representative images of lungs of nude mice injected with HMLER-Twist cells expressing indicated shRNAs.
F: Quantification of the GFP-positive cells in each individual lung. N=10 mice per group. *p < 0.01.

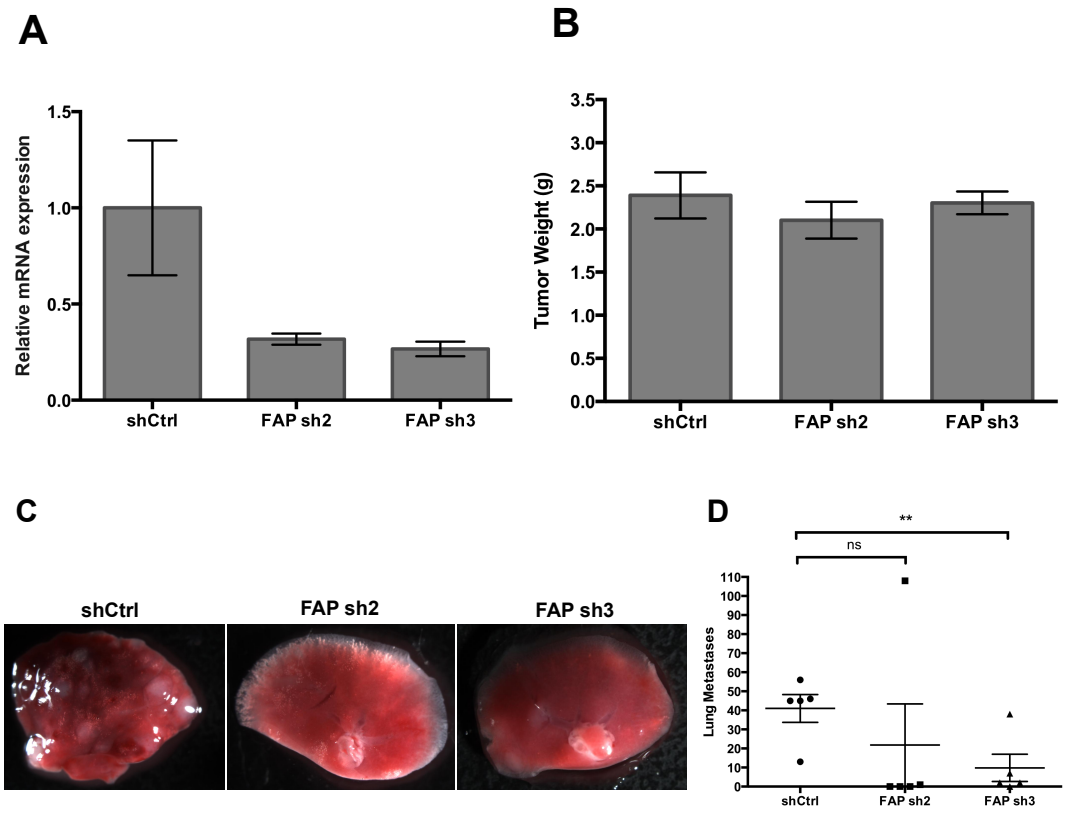

Figure 2-3: FAP expression is required for spontaneous metastasis with 4T1 cells (preliminary trial).
A: The mRNA expression of FAP in 4T1cells expressing two independent shRNAs targeting FAP.
B: Tumor weight is not affected by FAP knockdown. (not significant)
C: Representative images of lungs of BALB/C mice injected with 4T1 cells expressing indicated shRNAs.
D: Quantification of the lung metastases (visible nodules) in each individual lung. N=5 mice per group. *p < 0.01.

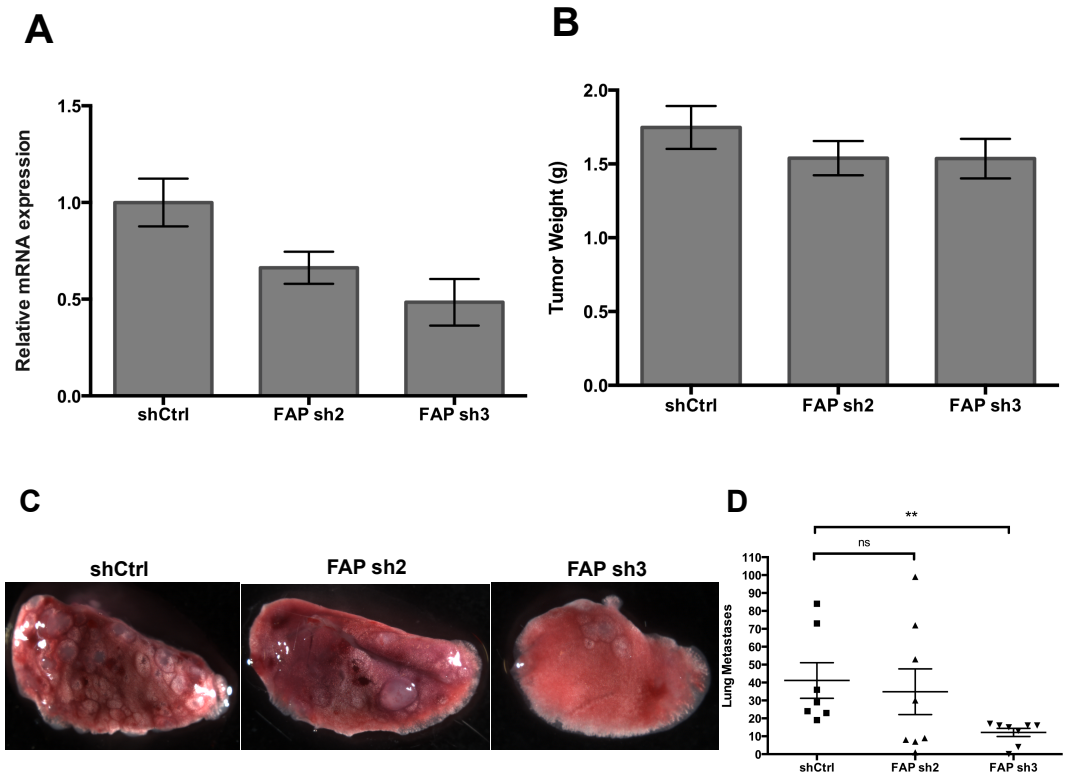

Figure 2-4: FAP expression is required for spontaneous metastasis with 4T1 cells.
A: The mRNA expression of FAP in 4T1cells expressing two independent shRNAs targeting FAP.
B: Tumor weight is not affected by FAP knockdown. (not significant)
C: Representative images of lungs of BALB/C mice injected with 4T1 cells expressing indicated shRNAs.
D: Quantification of the lung metastases (visible nodules) in each individual lung. N=8 mice per group. *p < 0.01.

CHAPTER 2

FAP Regulates Invadopodia Activity

FAP expression is required for invadopodia-mediated ECM degradation

Besides Twist1 expression, previous studies have shown that proper invadopodia formation is also required for tumor metastasis (Eckert et al., 2011). Other studies have also shown that FAP is located at invadopodia of endothelial cells, seen by co-localization with F-actin and actin-binding protein cortactin, markers used to properly identify invadopodia (Bowden et al., 1999; Ghersi et al., 2006). Furthermore, FAP is present at invadopodia that are actively degrading the ECM (Pathak, 2014). In conjunction with that Twist1 is known to induce invadopodia, FAP is a Twist1-responsive gene, and that FAP is localized at active invadopodia, we sought to determine how FAP is involved at invadopodia.

Invadopodia-mediated ECM degradation requires both the proper formation of invadopodia as well as the ability of the invadopodia to degrade the ECM (Murphy and Courtneidge, 2011). Structural components like actin and cortactin must converge for invadopodia formation, and after the subcellular protrusions have formed, proteases must localize to the maturing invadopodia in order to degrade the ECM. To investigate whether the suppression of FAP would affect the formation of invadopodia formation or the process of invadopodia-mediated ECM degradation, we utilized the HMLER-Twist cells that exogenously express Twist1 to ensure that FAP expression is present and can be suppressed with shRNA.

Since FAP has been shown to be at invadopodia that are actively degrading the ECM, we first examined if FAP suppression would affect

invadopodia-mediated ECM degradation. HMLER-Twist cells expressing a control shRNA or one of two independent shRNAs targeting FAP were seeded onto an Oregon Green-conjugated gelatin matrix, where areas of localized invadopodia-mediated ECM degradation would show up as dark patches. The co-localization of F-actin and FAP that overlap with dark areas of degradation demonstrated the need of FAP for invadopodia-mediated ECM degradation (Figure 2-5A). FAP knockdown cells had a decrease of 80-90% invadopodia-mediated ECM degradation when compared to the HMLER-Twist cells expressing a control shRNA (Figure 2-5B). These data indicate that the suppression of FAP severely decreases the ability of invadopodia to degrade the ECM.

FAP expression is required for stabilization of invadopodia

To further investigate the effects of FAP on invadopodia, we employed live-cell imaging to visualize the activity of invadopodia in SCC61 cells. The SCC61 cell line, derived from human squamous cell carcinoma, was chosen because of its capability of forming invadopodia (Murphy and Courtneidge, 2011). I first generated SCC61 cells that expressed a control shRNA and an independent shRNA targeting FAP (Figure 2-6B).

As the distinct invadopodia are marked by the co-localization of F-actin and cortactin, we assessed the effects of the SCC61s expressing control shRNA or FAP knockdown on invadopodia formation. While the absence of FAP did not affect the formation of invadopodia, as invadopodia was present

in both the SCC61 cells expressing a control shRNA or an shRNA targeting FAP, the live-cell imaging of these cells demonstrated two distinct phenotypes. Invadopodia in the FAP knockdown cells were revealed to have a 2-fold increase in the distance traveled in the cells when compared to the SCC61 cells expressing a control shRNA (Figure 2-6A, 2-6D). This suggests that during the invadopodia lifetime (Figure 2-6C), invadopodia are unstable in the absence of FAP. In conjunction with previous data showing that the suppression of FAP decreased invadopodia-mediated ECM degradation and tumor metastasis, the live-cell imaging results suggests that FAP could play a role in stabilizing the invadopodia.

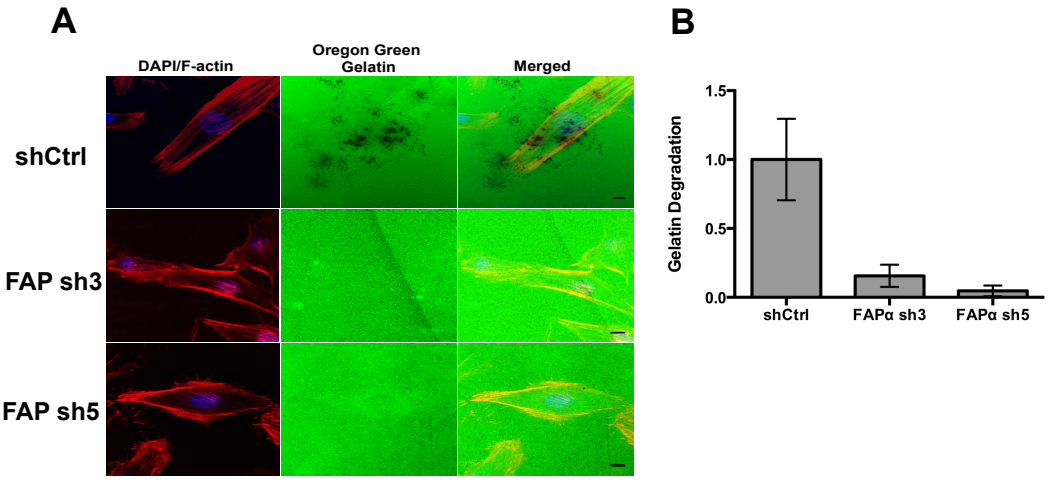

Figure 2-5: Suppression of FAP impairs invadopodia-mediated ECM degradation

A: HMLER-Twist cells were seeded on Oregon Green labeled gelatin for 16 hours. Nuclei were stained with DAPI (blue) and F-actin with phalloidin (red). Scale bar: 10 um.

B: Quantification of Oregon Green gelatin degradation. *$p < 0.02$.

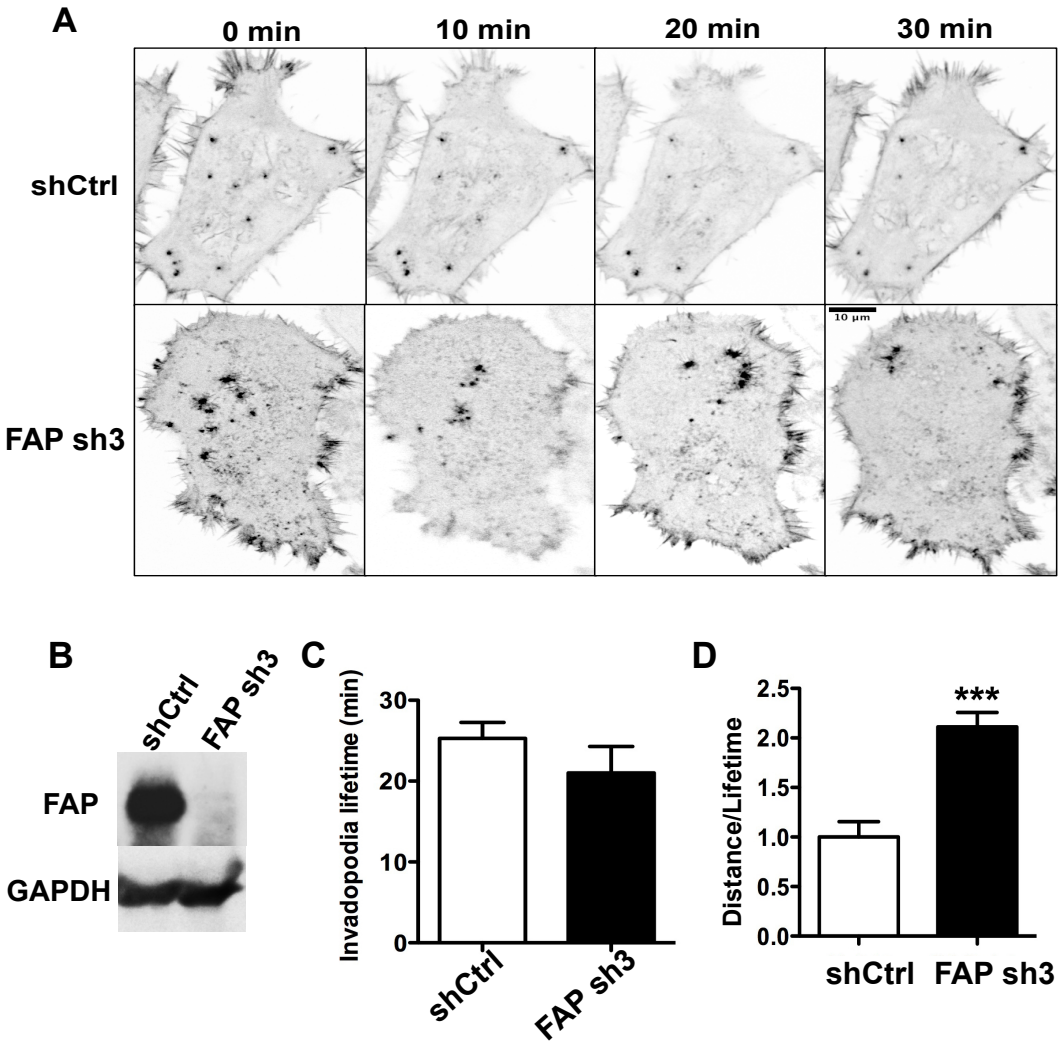

Figure 2-6: FAP expression is required for stabilization of invadopodia.

A: Representative live-imaging images revealed the activity of invadopodia. Images of cells were captured every 2 minutes. Images were inverted in contrast to highlight the movement of the invadopodia, as indicated by the dark puncta.

B: FAP protein expression in SCC61 cells.

C: Quantification of invadopodia lifetime.

D: Quantification of distance traveled by invadopodia over the lifetime of the invadopodia.

CHAPTER 3

FAP Recruits MT1-MMP to Invadopodia

FAP expression is required for recruitment of MT1-MMP

While the suppression of FAP did not affect initial invadopodia assembly, the phenotype of knocking down FAP on invadopodia stability and function is very similar to the phenotype caused by the suppression of the matrix metalloproteinase MT1-MMP. MT1-MMP knockdown only moderately affected the initial invadopodia assembly but greatly suppressed invadopodia-mediated ECM degradation (Artym et al., 2006). Therefore, we tested whether the suppression of FAP affects MT1-MMP function at invadopodia.

MT1-MMP, also known as MMP14, plays a huge role in ECM remodeling by degrading several ECM components like collagen I to enhance the invasiveness of tumor cells (Christiansen et al., 2007; Sounni et al., 2002). The co-localization of F-actin and cortactin has been found to initiate the accumulation of MT1-MMP at invadopodia (Artym et al., 2006). Immunofluorescence showed that the co-localization of F-actin and cortactin was intact for both the SCC61 cells expressing a control shRNA and FAP knockdown, as there were visible subcellular protrusions (Figure 2-7A). However, there was a definitive difference in the accumulation of MT1-MMP to invadopodia as the FAP knockdown cells had a remarkable decrease in the localization of MT1-MMP to invadopodia when compared to the SCC61 cells expressing a control shRNA (Figure 2-7B). The fluorescence intensity between the SCC61 cells expressing a control shRNA and FAP knockdown had a 2-fold decrease, and the immunofluorescence data was further supported by the decreased amount of MT1-MMP at invadopodia at FAP knockdown cells when

compared to the control cells versus the amount of MT1-MMP at the periphery of the cells (Figure 2-7C, 2-7D). These results suggest that while FAP does not affect the formation of invadopodia, FAP plays a role in the recruitment of MT1-MMP to invadopodia for the sake of invadopodia-mediated ECM degradation.

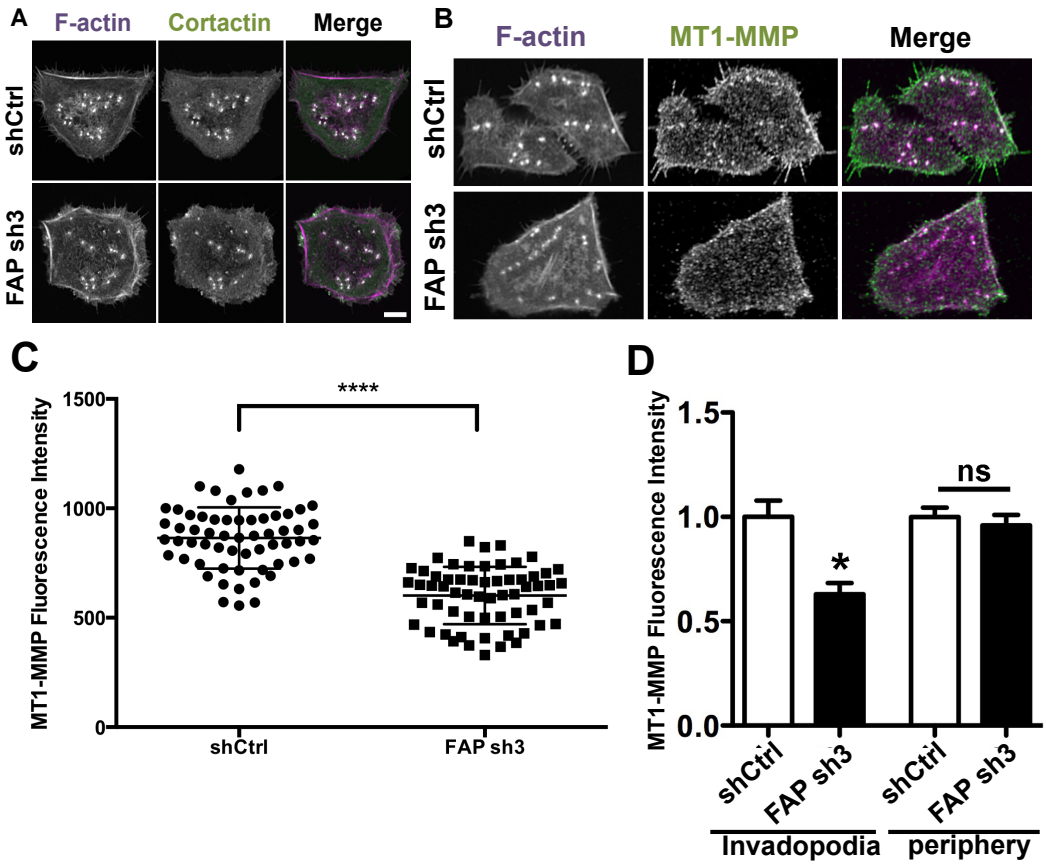

Figure 2-7: FAP is required for MT1-MMP localization to invadopodia.
A: SCC61 cells were stained for F-actin (purple) and cortactin (green).
B: SCC61 cells were stained for F-actin (purple) and MT1-MMP (green).
C: Quantification of co-localization of MT1-MMP and cortactin. *p < 0.01.
D: Quantification of MT1-MMP at the invadopodia and periphery of the cells to show the decreased localization of MT1-MMP at invadopodia. *p < 0.01.

Conclusions and Future Perspectives

Tumor metastasis is a highly complex and dynamic process that is a cause of 90% of cancer-related deaths (Chaffer and Weinberg, 2011). During metastasis, a cancer cell from the primary tumor is capable of invading into the vasculature, survive, and translocate to a distal site and establish secondary tumors. In breast cancer, the metastatic dissemination is usually the cause of death because of heterogeneity of the metastatic cells and the subsequent inability to target the metastatic cells (Cummings et al., 2014). While previous reports suggested the inhibition of specific proteases involved with the tumor invasion-metastasis cascade, it is important to distinguish and elucidate the role of a single protease that is associated with the tumor invasion-metastasis cascade so that therapeutic treatments can specifically target that one protease.

In this study, we present a novel role for the serine protease FAP in invadopodia-mediated ECM degradation and tumor metastasis. Based upon previous studies that show Twist1 is necessary for metastasis and FAP expression is induced by Twist1 (Yang et al., 2004)(Pathak 2014), we show that the expression of FAP is not only required for tumor cell metastasis in vivo, but that the suppression of FAP significantly reduces invadopodia-mediated ECM degradation. In addition, we demonstrated that while FAP suppression does not affect the formation of invadopodia, FAP expression is required for the stabilization of invadopodia and the proper localization of MT1-MMP to invadopodia. Conclusively, we propose that FAP plays a stabilization role in

invadopodia in order to allow FAP to recruit MT1-MMP to invadopodia for invadopodia-mediated ECM degradation, and subsequent metastasis.

The tumor invasion-metastasis cascade not only requires the tumor cells to be capable of becoming migratory and invasive through EMT, but also that invadopodia-mediated ECM degradation is successful so that those tumor cells can intravasate into the vasculature. Eckert et al has shown that Twist1 is required for the formation of invadopodia, and since Twist1 induces FAP, we investigated what role FAP held at invadopodia (Eckert et al., 2011)(Pathak, 2014).

Our in vivo experiments have demonstrated the necessity of FAP expression for tumor metastasis to the lungs in both an exogenous and endogenous model, indicating that FAP is a crucial protease involved with the tumor invasion-metastasis cascade. Previous studies have indicated that metastasis is associated with invadopodia-mediated ECM degradation. Thus, we sought to elucidate the role of FAP at invadopodia.

Invadopodia-mediated ECM degradation required both the proper invadopodia assembly as well as the invadopodia's ability to degrade. Eckert et al has shown that Twist1 is required for the formation of invadopodia, and since Twist1 induces FAP, we investigated whether FAP was necessary for invadopodia formation (Eckert et al., 2011)(Pathak, 2014). Interestingly, while our results found that the FAP did not affect the initial steps of invadopodia formation, invadopodia's stability in the cells is compromised in the absence of FAP, as invadopodia moved at a much faster rate and longer path when

compared to a control. This suggests that FAP expression is needed for stabilizing the structure of invadopodia.

We also determined that FAP expression was required for invadopodia-mediated ECM degradation. As invadopodia-mediated ECM degradation is vital to the success of metastasis and FAP expression is required for both, we sought to determine the mechanism of FAP at invadopodia.

For successful invadopodia-mediated ECM degradation, MT1-MMP has been identified by previous studies that it accumulates at invadopodia and is a vital metalloproteinase in ECM degradation (Artym et al., 2006). Our data shows that MT1-MMP is not properly localized to invadopodia in the absence of FAP, providing a clue to why FAP expression is needed for invadopodia-mediated ECM degradation. Previous data from our lab has demonstrated that although FAP is a protease, its proteolytic activity was not required for invadopodia-mediated ECM degradation, suggesting that FAP acts more in a structural capacity than proteolytic at invadopodia (Pathak, 2014). Looking at the structure of FAP and its extremely large extracellular domain, we speculate that FAP would be ideal in providing a docking site for proper protein/protease localization at invadopodia in order to initiate the maturation of invadopodia and subsequent degradation of the ECM. This could possibly explain why in the absence of FAP, MT1-MMP localization to invadopodia is compromised because MT1-MMP cannot dock properly to FAP at invadopodia and thus, the invadopodia-mediated ECM degradation is dramatically decreased.

Collectively, our data suggests that the role of FAP is to act as a stabilizing protease of invadopodia in order to recruit MT1-MMP for invadopodia-mediated ECM degradation, thus allowing for the metastatic dissemination of tumor cells to the lungs as seen in the exogenous and endogenous mouse breast tumor models (Figure 3-1). Further studies will need to be conducted to fully explain the mechanism of how FAP stabilizes invadopodia.

The identification of FAP's role in tumor metastasis has the potential of leading to an effective method to block tumor invasion and metastasis. Many of the inhibitors for matrix metalloproteinases performed well in vitro but failed in clinical trials due to the broad spectrum of inhibition, leading to other side effects that could be detrimental for survival (Overall and Kleifeld, 2006). However, because FAP is specifically expressed and accumulated at the surface of metastatic tumor cells due to its accumulation in invadopodia and our in vivo data has explicitly demonstrated that the suppression of FAP was sufficient to prevent metastasis, FAP is definitely a promising target for anti-metastasis treatments.

For example, the development and usage of FAP blocking antibodies could be a feasible approach. As FAP is a membrane-associated protease, and that previous data has demonstrated that the proteolytic activity of FAP is unnecessary for invadopodia-mediated ECM degradation, using antibodies to block FAP from possibly becoming a docking site for other proteases like MT1-MMP have the potential to inhibit the amount of invadopodia-mediated ECM

degradation and consequently the amount of metastasis. In addition, FAP is only expressed during wound healing and in invasive epithelial cancers (O'Brien and O'Connor, 2008), further enhancing its attractiveness as a therapeutic target with fewer side effects.

Focusing therapeutic intervention towards a single protease that has been shown to affect multiple facets of the tumor invasion-metastasis cascade could represent a promising approach to anti-metastasis treatment.

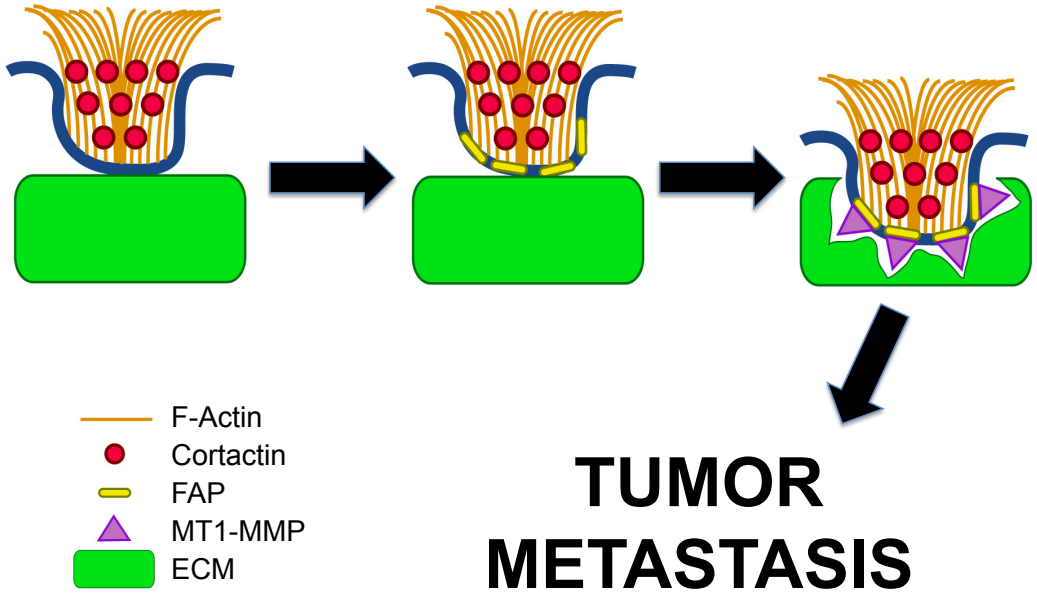

Figure 3-1: Proposed mechanism of action of FAP at invadopodia and metastasis.
The accumulation of actin and cortactin will recruit FAP to invadopodia. FAP will then recruit MT1-MMP to invadopodia for invadopodia-mediated ECM degradation, leading to subsequent tumor metastasis.

Materials and Methods

Cell Lines and Cell Culture

Cell lines used: HEK293T, 4T1, SCC61, HMLER

All cell lines were incubated at 37°C with 5% CO_2.

The HEK 293T cell line is a human embryonic kidney cell line utilized for transfection of plasmids in order to extend the temporal expression of the gene. The HEK 293T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

The 4T1 cell line is a mouse breast tumor cell line isolated from a BALB/C mouse that closely mimics human breast cancer. The 4T1 cells spontaneously metastasize to the lungs, liver, lymph nodes, and brain, forming visible nodules in those organs. The 4T1 cells were cultured in DMEM supplemented with 5% fetal bovine serum, 5% calf serum, non-essential amino acids, penicillin, and streptomycin.

The SCC61 cell line is a squamous cell carcinoma cell line isolated from a patient with head and neck cancer. The SCC61 cells were cultured in DMEM supplemented with 10% fetal bovine serum, hydrocortisone, penicillin, and streptomycin.

The human mammary epithelial cell line with Ras (HMLER) is an immortalized cell line that was transformed into carcinoma cells by the introduction of SV40 large-T antigen, telomerase catalytic subunit, and H-Ras oncoprotein. These cells were cultured in Mammary Epithelial Growth Medium mixed 1:1 in DMEM/F12 supplemented with epidermal growth factor (Sigma), hydrocortisone, insulin, L-glutamine, penicillin, and streptomycin.

To passage cells, plates with cell monolayers were rinsed with DPBS and incubated with 0.5-2 mL of 0.15% Trypsin in EDTA at 37°C for 5-10 minutes. The trypsin was inactivated by adding the serum media at a 1:5 ratio. Cells were spun down at 1500 rpm for 3 minutes and then resuspended to necessary concentrations.

Plasmids

The control shRNAs in the pLKO.1 vector were obtained from the Sabatini lab (Addgene), and purchased from Sigma. The small hairpin RNAs (shRNA) targeting FAP were purchased from Sigma, and have the following targeting sequences:

Human FAP sh3: GCA TTG TCT TAC GCC CTT CAA
Human FAP sh5: TGA TAA TCT TGA GCA CTA TAA

Mouse FAP sh2: CCG GCC CTT TGC TAA TTC AAG TGT ACT CGA GTA CAC TTG AAT TAG CAA AGG GTT TTT G
Mouse FAP sh3: CCG GCC ATA TAT ATA TTC CGC GTA ACT CGA GTT ACG CGG AAT ATA TAT ATG GTT TTT G

Lentiviral Infection

10^6 HEK 293T cells were first plated onto 6 cm dishes in serum media. After 24 hours, the HEK 293T cells were transfected with the following plasmids to make a lentivirus: 0.9 µg of the pCMVΔ8.2R gag/pol expression vector, 0.1 µg VSV-G expression vector, and 1 µg of the transfer vector. 2 µg total of DNA were transfected using the TransIT-LT1 Transfection Reagent (Mirus) and incubated at 37°C overnight. Media was changed the day after transfection while target cells were seeded at about 20% confluency onto 6 cm dishes.

Viral supernatants were harvested at 48 and 72 hours post transfection and filtered through a 0.45 µm filter. Target cells were then infected with the filtered viral supernatant and 6 µg/mL protamine sulfate and incubated for six hours. Cells were selected with appropriate drugs as followed:

4T1: Puromycin at 12 µg/mL for selection and 6 µg/mL for maintenance.

SCC61: Puromycin at 5 µg/mL for selection and 2 µg/mL for maintenance.

Total RNA Isolation

RNA isolation was performed using the NucleoSpin RNA II kit (Macherey-Nagel). Cell monolayers were cultured for 2 days until 80-90% confluency. 350 µL of Buffer RA1 and 3.5 µL of β-mercaptoethanol were added to each plate. The cells were then scraped off with a spatula into an eppendorf tube and vortexed vigorously before filtering through a Nucleospin® Filter at 11,000 x g

for 1 minute. 350 µL 70% ethanol was added to the homogenized lysate and mixed by pipetting up and down at least 10 times.

Each sample was loaded onto a NucleoSpin® RNA column and centrifuged at 11,000 x g for 30 seconds. 350 µL Membrane Desalting Buffer was added to the samples next and centrifuged at 11,000 x g for 1 minute.

95 µL of DNase reaction mixture (consists of 10 µL reconstituted rDNase and 90 µL Reaction Buffer for Dnase) was added directly to the center of the silica membrane of the column and incubated at room temperature for 15 minutes. To inactivate the rDNase, 200 µL of Buffer RAW2 was added and centrifuged at 11,000 xg for 30 seconds. The column was then washed with 600 µL Buffer RA3 and centrifuged at 11,000 x g for 30 seconds, followed by 250 µL Buffer RA3 and centrifuged at 11,000 x g for 2 minutes.

The RNA was eluted in 60 uL RNase-free water and centrifuged at 11,000 x g for 1 minute. The RNA concentration was measured using a nano-photometer (Implen) and stored at -80°C until use.

Reverse Transcription

The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 2µg total of RNA was added to the reaction mix, which consisted of RT buffer, dNTP, Random Primers, and

Reverse Transcriptase from the kit. The reaction was ran in a Biorad Thermal Cycler at the follow cycle twice: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using SYBR®-Green PCR Master Mix (Applied Biosystems). The qRT-PCR mix includes SYBR Green 1 Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. 6 µL of the SYBR®-Green mix with desired primers were mixed with 4 µL of cDNA input.

cDNA samples were analyzed in triplicates and relative mRNA levels were determined by $2^{-(Ct-Cc)}$, where Ct and Cc values are the mean threshold cycle differences after normalizing to HPRT (housekeeping gene) values. Primers used for qRT-PCR are as follows:

Human HPRT:
Forward: GCTATAAATTCTTTGCTGACCTGCTG
Reverse: AATTACTTTTATGTCCCCTGTTGACTGG

Mouse HPRT:
Forward: GTTAAGCAGTACAGCCCCAAA
Reverse: AGGGCATATCCAACAACAAACTT

Human FAP:
Forward: AATGAGAGCACTCACACTGAAG
Reverse: CCGATCAGGTGATAAGCCGTAAT

Mouse FAP:
Forward: GTCACCTGATCGGCAATTTGT
Reverse: CCCCATTCTGAAGGTCGTAGAT

Total Protein Extraction

Cells were harvested at 70-80% confluency after being in culture for 2 days. The cells were first washed with ice-cold PBS and then lysed in lysis buffer (50mM Tris-HCl at pH 7.5, 150 mM NaCl, 10mM NaF, 1% Triton X-100), with 100μM Na_3VO_4, 100μM PMSF, and the Protease Inhibitor Cocktail Set III (Calbiochem) diluted 1:200. Whole lysates were scraped down with a spatula and collected. Samples were pulse sonicated at 3 seconds intervals, with 3 seconds break between each sonication. This was repeated 3 times for each sample. Samples were then spun down at 14,000 x g for 15 minutes. Samples were snap frozen in liquid nitrogen and stored at -80°C until use.

Determining Protein Concentration

Protein concentrations were determined using the Lowry assay (Bio-Rad). 1 mL of reagent A was mixed with 20 μL of reagent S to create a working reagent. 100 μL of the working reagent was mixed with 2 μL of the protein sample. 800 μL of reagent B was added to the sample and incubated at room temperature for 15 minutes.

Protein concentrations were measured by using a nanospectrometer (Implen). 900 µL of the samples were aliquoted into the cuvette. Bovine Serum Albumin of concentrations 0, 5, 10, and 20 mg/mL was used to create a standard curve.

Western Blotting

Proteins were denatured at 70°C for 10 minutes with 50mM DTT and 5X Loading Sample Buffer before running on 4-12% pre-cast gels (PAGEgel) to ensure the denaturing of FAP dimer. The SDS PAGEgel was ran at 70V for 30 minutes and then at 120V until loading dye reaches the bottom. Protein was then transferred onto a nitrocellulose membrane at 200V for 120 minutes.

The membrane is then blocked with 5% milk for an hour. Primary antibodies were diluted in 2% BSA in PBS with 0.1% Tween20 (PBST) incubated with the membranes for overnight at 4°C with agitation. Post primary antibody incubation, the membrane was washed 3 times for 10 minutes each with PBST. After the washes, the membrane was incubated with the appropriate secondary HRP conjugated antibody in 5% milk for an hour at room temperature. The membrane was then washed 3 times for 10 minutes each with PBST and 3 times for 10 minutes each with 1X PBS.

The membrane was visualized with Amersham ECL Select^{TM} Western Blotting Detection Kit (GE) and exposed onto Kodak Biomax Film.

Antibodies utilized for the western blotting include FAP (1:500, D8, Vitatex) and GAPDH (1:2500, GeneTex).

Tumor Implantation and Metastasis Assays

The Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego, approved all animal care and experiments.

Subcutaneous

2 million cells of each condition were resuspended in 50% Matrigel (Becton Dickson) to 15 µL aliquots and injected into the left and right flanks of adult nude mice. The mice were monitored for 5 days post injection and weighed biweekly. Tumors were measured every 4 days and allowed to grow to approximately 2 cm before mice were sacrificed. Lungs were harvested and imaged for GFP-positive tumor cells. Tumors were harvested and fixed in 4% paraformaldehyde for 24 hours before soaking in 70% ethanol.

Mammary Fat Pad

100,000 cells of each condition were resuspended in 50% Matrigel (Becton Dickson) into 15 µL aliquots and injected into the left and right mammary fat pads of BALB/C mice. The mice were monitored for 5 days post injection and weighed biweekly. Tumors were monitored and allowed to grow for a month before mice were sacrificed. Lungs were harvested and imaged for nodules.

Tumors were harvested and fixed in 4% paraformaldehyde for 24 hours before soaking in 70% ethanol.

ECM Degradation Assays

Preparation of glass coverslips

Round 12mm diameter coverslips were etched with 20% Nitric Acid for 30 minutes and washed thoroughly with deionized water for 2 hours to remove all remaining nitric acid. The coverslips were then rinsed 3 times with 70% ethanol, 1 time with 100% ethanol, and then stored in 100% ethanol at room temperature until use.

Preparation of the Oregon-Green gelatin coated coverslips

All steps listed below must be carried out in a sterile environment. Acid etched coverslips were first dried and placed into wells of a 24-well tissue culture plate before incubation with 50 µg/mL poly-L Lysine (Sigma-Aldrich) for 20 minutes. The coverslips were then washed 3 times with PBS before incubation with 0.5% gluteraldehyde for 15 minutes. The coverslips were washed 3 times with PBS again after gulteraldehyde incubation.

A 1:5 dilution of Oregon-Green gelatin (Life Technologies) and 0.2% porcine gelatin was made and warmed in a 37°C water bath before use. 15 µL droplets of the diluted Oregon-Green gelatin mixture were aliquoted onto a sheet of parafilm. Using a needle and forceps, the coverslips were gently

picked up and inverted onto the drops of gelatin for 90 seconds. After, the coverslips were moved back into the wells and gently washed 3 times with PBS.

5 mg/mL of sodium borohydride was then added to each well to quench the residual reactive groups and incubated for 15 minutes with agitation to prevent coverslips from floating to the top. The coverslips were then gently washed 5 times with PBS or until no more bubbles were seen. 500 µL of serum media was added to each well. The plate is then stored at 37°C with 5% CO_2 until use.

Seeding Cells

Cells were trypsinized and suspended as mentioned above. The number of cells was counted using a hemocytometer and then aliquoted to the appropriate number of cells onto each Oregon-Green gelatin coated coverslip in the 24 well tissue culture plate and incubated at 37°C with 5% CO_2 for 6 hours.

Fixing and Staining

Coverslips were then fixed with warm 4% paraformaldehyde at 37°C with 5% CO_2 for 20 minutes. Fixed cells were gently washed 3 times with PBS. After that, cells were permeabilzied with 0.1% TritonX in PBS for 15 minutes at room temperature. Permealized cells were washed 3 times with PBS and then

incubated with Alexa Fluor594-conjugated phalloidin (Invitrogen) overnight at 4°C on a shaker. The next day, the coverslips were washed 3 times with PBS 10 minutes each and then mounted with Mounting Medium for Fluorescence with DAPI (Vector).

Imaging

Cells were imaged by the Nikon upright fluorescent microscope and acquired by Metamorph 7.6 imaging software (Molecular Devices). Images were then processed by ImageJ analysis software.

Immunofluorescence

Cells were cultured on glass coverslips for 2 days, and then fixed with 4% paraformaldehyde (PFA) at 37°C for 20 minutes, washed with PBS 3 times before permeabilizing with 0.25% Triton X-100 in PBS for 10 minutes. The cells were blocked with 1% BSA in PBST for 1 hour, and labeled with primary antibodies overnight with gentle shaking at 4°C. Then the cells were labeled with secondary fluoro-chrome-conjugated antibodies for 1 hour. The samples were incubated with Alexa Fluor 594-conjugated phallodin (Invitrogen) and DAPI in PBS for 20 minutes at room temperature before imaging. Confocal images were acquired using an Olympus FV1000 with 488- and 568-laser lines. Images were analyzed using ImageJ analysis software.

Primary antibodies used for immunofluorescence include Cortactin (1:500, clone 4F11, Millipore) and MMP14 (1:500, Genetex). Secondary antibodies used include Alexa Fluor 488 and 647 (Invitrogen).

Live-imaging

The SCC61 cells used for live-imaging were stably expressing Lifeact-mCherry. Cells were cultured on 35mm Mateck glass bottom dishes (BD). 7 cells per condition were imaged for 30 minutes at 2 minute intervals in a climate controlled environment at 37°C and 5% CO_2. Confocal images were acquired using Olympus FV1000 with the 568-laser line and analyzed using ImageJ analysis software.

Statistical Analysis

All statistical analysis was carried out using the Prism software.

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