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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of Proteases in Twist Induced Tumor Invasion

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

in

Biology

by

Thinzar Helmi Min Lwin

Committee in Charge:

Professor Yang Xu, Chair
Professor Jing Yang, Co-Chair
Professor Colin Jamora

2009

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This Thesis of Thinzar Helmi Min Lwin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2009

Dedication

This thesis is dedicated to my parents (Dr Min Lwin and Dr Tin Tin Htwe) and Paul Mitsuhashi whose love, guidance, unwavering support and generosity have allowed me to achieve what I have accomplished thus far. This thesis is also dedicated to the friends whose perspectives and encouragement have kept me motivated throughout difficult challenges.

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List of Abbreviations

ADAM12: A disintegrin and metalloprotease 12

DPPIV: Di-peptidyl peptidase 4

ECM: Extracellular matrix

EMT: Epithelial to mesenchymal transition

FAPa: Fibroblast activation protein alpha (also known as seprase)

FITC: Fluorescein isothiocyanate

HMEC: Human mammary epithelial cell

IF: Immuno Fluorescence

RNAi: RNA interference

shRNA: small hairpin RNA

siRNA: small interfering RNA

Tks5: Tyrosine kinase substrate 5

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Acknowledgements

I would like to thank Dr Yang whose guidance has made this entire effort possible. Three years ago I had never held a pipette and today I am about to finish up a master's thesis. I have learned an immense amount about scientific research and Dr. Yang's keen mentorship has been invaluable throughout the entire learning process.

I would also like to thank Mark Eckert for all his help in conducting the complicated assays necessary for the visualization of matrix degradation and invadopodial structures. I am grateful for his valuable suggestions and assistance in trouble shooting difficult assays. The FITC gelatin degradation will define both our scientific careers in this lab.

I would like to thank all the members of the Yang lab whose conversations, insights and friendships have made my experience in basic science research an unforgettable one. Thank you for patiently answering my many questions and explaining concepts in cancer biology.

ABSTRACT OF THE THESIS

The role of Proteases in Twist induced Tumor Invasion

by

Thinzar Helmi Min Lwin

Master of Science in Biology

University of California, San Diego, 2009

Professor Yang Xu, Chair

Professor Jing Yang, Co-Chair

Metastasis is a complex multi-step process by which cancer cells disseminate from a primary site to distant organs. The transcription factor Twist has been shown to play a key role in metastasis in a mouse mammary tumor metastasis model. It has also been implicated in invasive human cancers and correlated with increased incidence of metastasis and poor disease progression in vivo. One cellular function of Twist that contributes to metastasis is to promote

Epithelial-Mesenchymal-Transition, allowing cancer cells to lose cell to cell adhesion and gain motility. However, the role of Twist in other steps of the metastatic cascade has not yet been examined. My project focuses on the role of Twist in promoting tumor invasion and the formation of invasive cellular structures called invadopodia. Fluorescent gelatin degradation assays as well as immunofluorescence assays demonstrate that Twist is necessary and sufficient for local matrix degradation and invadopodia formation. It was unclear the mechanism by which Twist induced invadopodia formation. I hypothesize that Twist may regulate proteases that are associated with invadopodia, playing a key role in invadopodia formation and matrix degradation. My study showed that Twist expression specifically upregulated the expression of three proteases associated with invadopodia: ADAM12, FAPa, and DPPIV. I further investigated the role of ADAM12 studying matrix degradation and invadopodia formation. We found that expression of ADAM12 is necessary for proper matrix degradation and functional invadopodia formation. Together, these results indicate that induction of ADAM12 plays a critical role in Twist-induced matrix degradation and local invasion.

Introduction

A metastasis is a cancerous lesion that arises in areas of the body distant from the primary tumor site. The process by which this secondary tumor arises is a complicated series of events during which cancer cells disseminate from the primary mass to colonize secondary sites. The formation of metastases is the leading cause of death in patients with solid tumors, while primary tumors are responsible for only about 10% of all deaths from cancer (Sporn, 1996). With advancements in surgical technology, most primary tumors can often be removed without major complications. The remaining 90% of deaths from cancer arise from metastasis.

Metastasis is the final step in tumor progression in which a localized tumor cell transforms into an invasive cell. This process involves a wide range of genetic and phenotypic changes. Although a leading cause of death from cancer, this metastatic signaling cascade is one that is still not fully understood. What is known about this pathway is that a series of events are activated such that polarized epithelial cells are transformed into motile cells with the ability to invade through the basement membrane and stroma to migrate to distant locations (Weinberg, 2007).

Multi-Step Metastasis Model

The specific details of the metastatic cascade are unique to each cell type, but most cancer cells follow similar paths when they become invasive and metastatic. A multi-step model of metastasis describes each stage cancer cells

undergo in order to metastasize to secondary sites (Figure I1). They must first lose cell to cell adhesions in order to gain motility, detach from the primary tumor and invade into the surrounding tissue. Cancer cells then intravasate through the endothelium of blood or lymphatic vessels and enter the circulating blood stream or lymphatic system. Some of these cells survive the journey, and even smaller subsets are able to extravasate out of the vessel to arrive at the new site. A few are able to succeed in proliferating and grow into secondary tumors in the new environment (Thiery, 2002) (Fidler, 2003).

Epithelial-to-Mesenchymal Transition (EMT)

Epithelial cells typically are organized as sheets, single or stratified, of cuboidal or columnar cells. The cells that make up epithelial tissue have apical and basolateral polarities and are connected to each other by cell to cell junctions. Mesenchymal cells on the other hand do not have a well defined morphology. They are often spindle shaped and exhibit an invasive-migratory phenotype. The changes that result in a metastasis require the cell to undergo phenotypic and molecular remodeling. Epithelial cells from a carcinoma lose their cell to cell junctions and become motile mesenchymal-like cells. Epithelial characteristics such as adherent junctions and cytokeratin intermediate filaments are lose in exchange for a mesenchymal profile that features acquisition of N-Cadherin expression, Vimentin intermediate filaments and Fibronectin secretion (Weinberg, 2007).

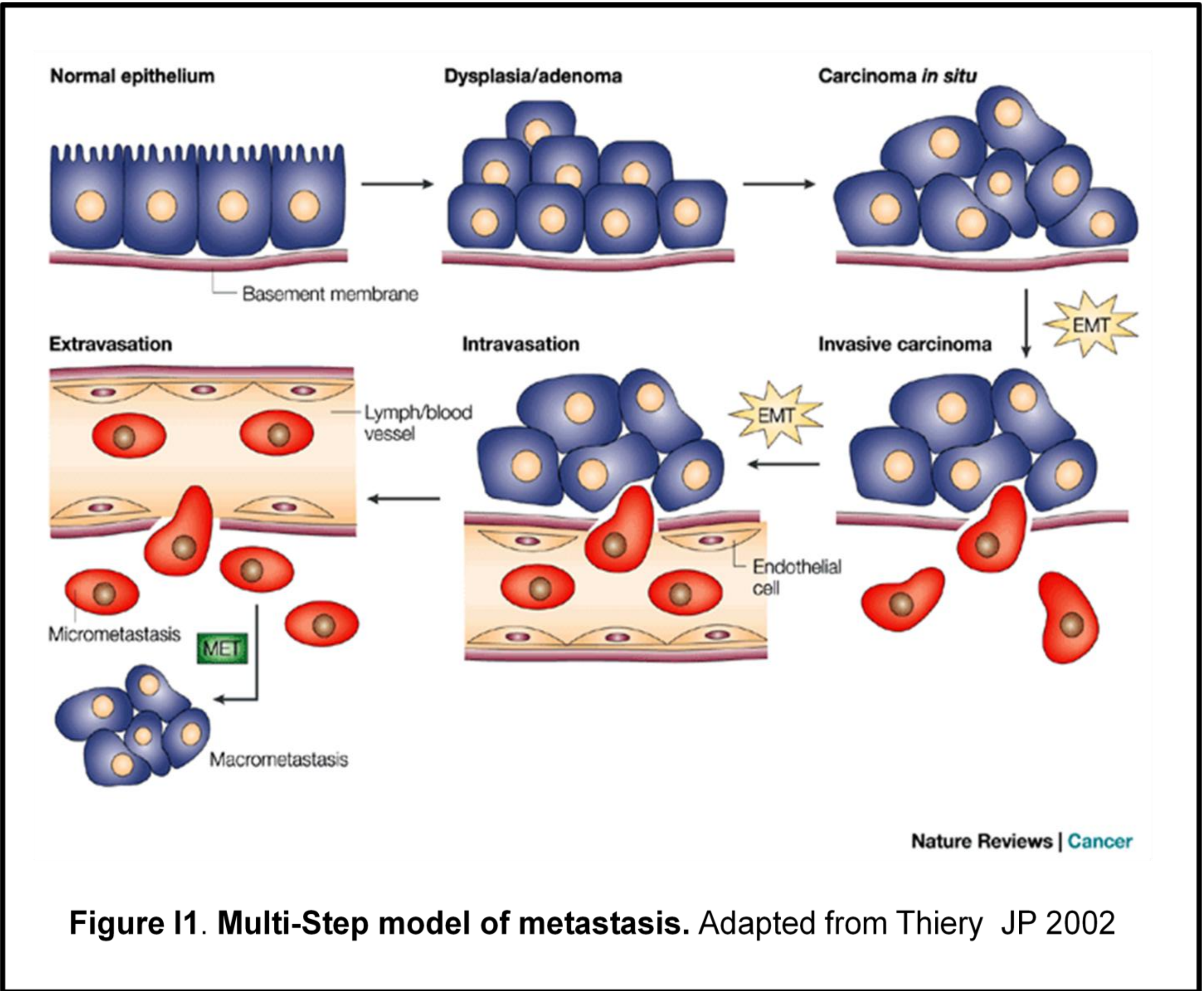


Figure I1. **Multi-Step model of metastasis.** Adapted from Thiery JP 2002

The switch from an epithelial to mesenchymal phenotype in cancer cells is one that is not yet completely understood, but some clues can be drawn from studies of conserved developmental pathways (Baum, Settleman, and Quinlan, 2008).

The transformation from epithelial to mesenchymal phenotype in cancer cells very closely resembles the changes that developing cells undergo during stages of chordate embryogenesis. During embryogenesis, sheets of epithelial cells lose their epitheloid morphology and gaining mesenchymal features in an evolutionarily conserved process called the epithelial-to-mesenchymal transition (EMT).

EMT is observed in several normal developmental processes including gastrulation, neural crest development, and wound healing(Thiery, 2002; Thiery, and Sleeman, 2006). During gastrulation, cells undergo EMT and detach themselves from the ectoderm, invade through the basement membrane, and migrate inward, to form the mesoderm. Cells within the dorsal neural epithelium also undergo EMT to give rise to a highly migratory population of cells called the neural crest. EMT is also observed in wound healing to allow cells to move into the wounded site to fill in the gap in the epithelium (Weinberg, 2007).

Strong resemblances between the tumor invasive processes and normal developmental processes highly suggest a common mechanistic model. It is believed that metastatic cancer cells are able to re-activate the latent EMT program and use it to change their own morphology, motility, and ability to invade and disseminate.

Role of Twist and other Transcription Factors in EMT

The changes that occur during EMT are governed by modifications in the expression of many genes. Several transcription factors have been identified as being EMT inducing- that is, ectopic expression of the gene is sufficient to induce EMT in epithelial cells.

Some transcription factors that have been shown to promote EMT are Snail, Slug, SIP1, and Twist. These transcription factors are highly active during key steps of embryogenesis and have been associated with invasive carcinomas. The intricate interplay of these signals has not yet been completely elucidated (Figure I2).

Snail, Slug, and SIP1, all of which are zinc-finger containing transcriptional repressors, directly bind to the E-boxes near the transcriptional stat site of the E-cadherin gene. So the major role of these three transcription factors is thought to suppress E-Cadherin tnrascription, thus allowing the dissociation of cell adhesion during EMT. Several studies have also showed that expression of such transcription factors occurs in human breast tumors. Unlike the Snail.Zlug Zn-finger transcription factors, Twist does not bind directly to the E-caherin promoter to suppress its transcription. Currently the signaling pathways Twist regulates to promote EMT and tumor metastasis are under extensive investigation. Recent studies have reported upregulation of Twist in numerous human cancers.

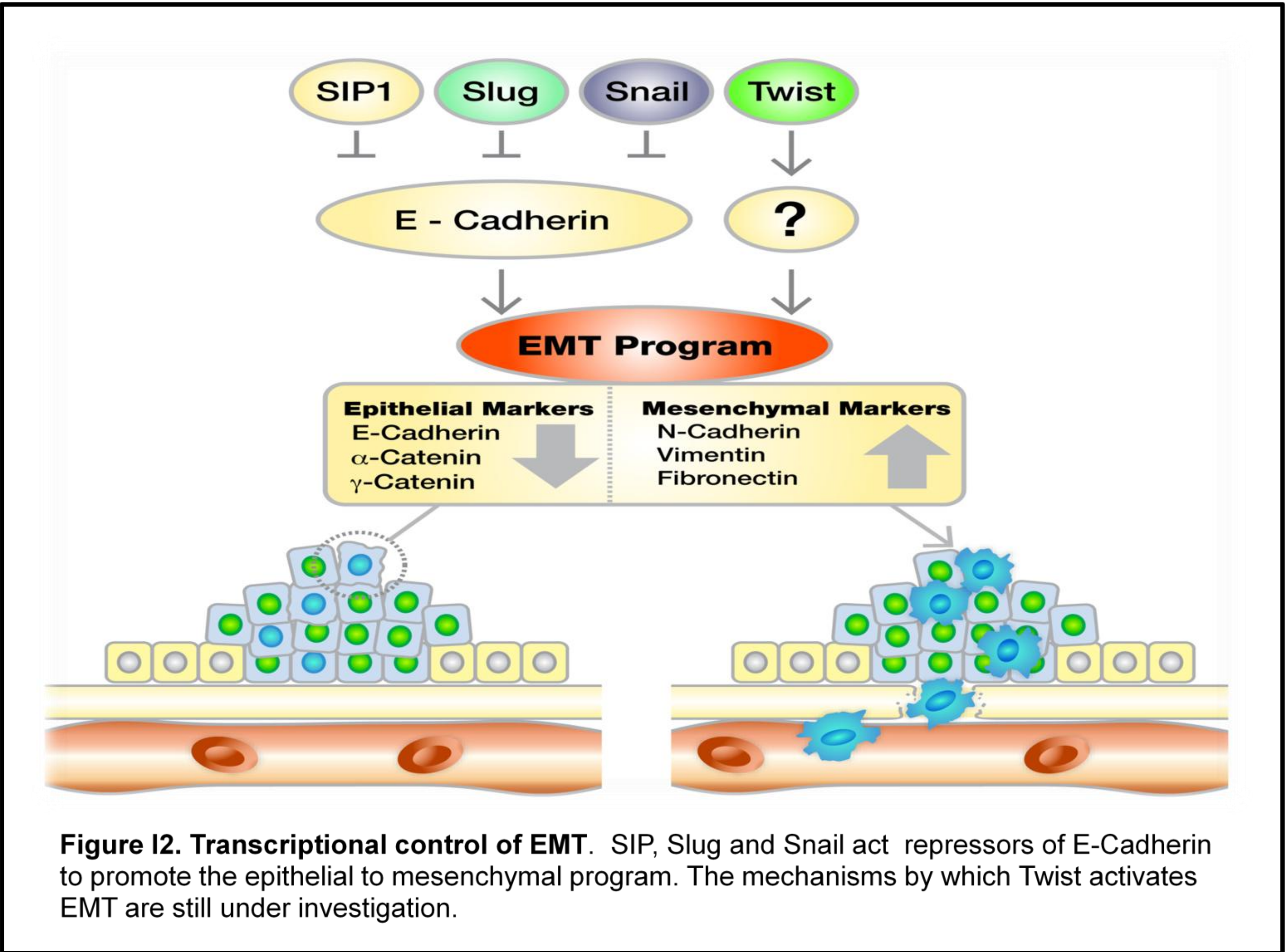


Figure I2. Transcriptional control of EMT. SIP, Slug and Snail act repressors of E-Cadherin to promote the epithelial to mesenchymal program. The mechanisms by which Twist activates EMT are still under investigation.

Role of Twist in Invasion and Metastasis

Twist, a basic helix loop helix transcription factor, is an important regulator of EMT. It was originally identified as a critical gene for proper gastrulation and determining mesoderm formation in *Drosophila* (Castanon, and Baylies, 2002). In vertebrates, Twist is expressed in neural crest cells and is necessary for cranial neural tube closure and myogenic differentiation.

Twist was implicated in cancer metastasis and EMT as being a highly upregulated gene in tumors that are able to metastasize, but not in tumors that are unable to metastasize. Its expression was not detected in normal epithelial cells. Suppression of Twist expression in highly metastatic mouse breast tumor cells did not affect formation of primary mammary tumors, but were prevented them from forming large macroscopic metastases in the lung (Yang, Mani, et al, 2004). Twist overexpression in epithelial cell lines was able to promote EMT phenotypes including loss of cell to cell adhesion, gain of spindle shaped fibroblastic morphology, and scattering of cells. In these cells with Twist overexpression, epithelial adherens junction proteins such as E-Cadherin and α, β, and γ-catenins were no longer detectable while mesenchymal markers such as fibronectin, vimentin, and N-Cadherin were upregulated. Ectopic expression of E-Cadherin was unable to revert the cells to an epithelial phenotype, indicating the function of Twist as a potent transcriptional regulator, modulating other targets in the EMT process.

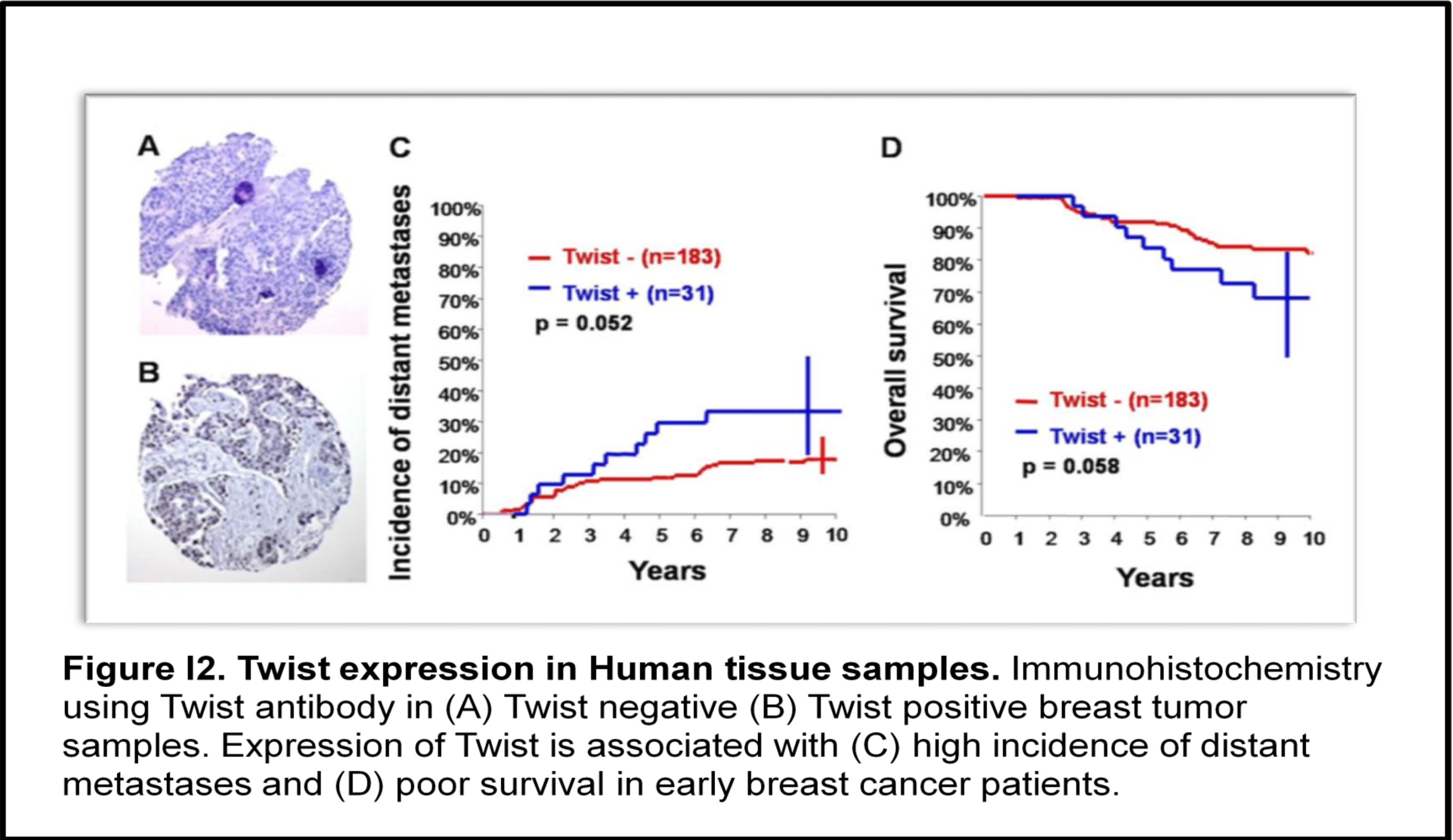


Figure I2. Twist expression in Human tissue samples. Immunohistochemistry using Twist antibody in (A) Twist negative (B) Twist positive breast tumor samples. Expression of Twist is associated with (C) high incidence of distant metastases and (D) poor survival in early breast cancer patients.

Preliminary data from our lab of early stage breast cancer patients also support the relationship between high levels of Twist expression and high incidence of distant metastases as well as decreased survival outcome (Figure I3). This preliminary data is supported by a number of studies that correlate the expression of Twist with invasive cancers. High levels of Twist have been shown to be present in breast cancer samples by qRT-PCR and immunohistochemistry (Vesuna, van Diest, et al, 2008). It is upregulated in malignant malignant prostate cancers (90%), but seen in only a small fraction of non-malignant tissue (6.7%) (Valsesia-Wittmann, Magdeleine, et al, 2004). Twist overexpression is associated other types of invasive cancers such as hepatocellular carcinoma (Lee, Poon, et al, 2006), seen in head and neck (Yuen, Chan, et al, 2007), cervical (Shibata, Kajiyama, et al, 2008), ovarian (Kajiyama, Hosono, et al, 2006), bladder (Zhang, Xie, et al, 2007) and gastric cancers (Vecchi, Nuciforo, et al, 2007). In many of these cases, high levels of Twist correlate with increased incidence in metastasis, poor overall prognosis and poor progression free survival.

Invadopodia: A Specialized Subcellular Structure for Matrix Degradation

Twist has been shown to be able to promote the EMT change in cell morphology from epithelial to mesenchymal. It has also been shown to be necessary for metastasis of cancer cells in a mouse mammary tumor metastasis model. The question of whether or not Twist is necessary for other stages of the multi-step metastasis model have not yet been answered. My project specifically

deals with the role of Twist in invasion.

To invade through basement membrane and extracellular matrix, cells need to gain the ability to remodel the ECM which is achieved through the upregulation of various proteases. These proteases are concentrated at actin rich membrane protrusions on the basal side of an invading cell and have been named invadopodia. These subcellular protrusions extend into the ECM, forming complex invaginations. Invadopodial structures have been associated with active sites of degradation by correlative light microscopy (Baldassarre, Pompeo, et al, 2003). Invadopodia are found on selective cells at the invading front of a tumor and their initiation, assembly and cycling is a very active field of study.

Invadopodial structure consists of an actin filament rich core containing the actin assembly machinery with signaling and functional proteins concentrated at the membrane. Molecular characterization of invadopodia has uncovered different components of the structure, organized by function (Artym, Zhang, et al, 2006):

1) The cytoskeletal modulators: Actin and regulators of its branching and polymerization.

2) The adhesion proteins: Integrins that mediate invadopodia interactions with the ECM.

3) The signaling proteins: Kinases and GTP-ases that control cross talk with other pathways to create, maintain, and cycle the structure.

4) The membrane associated proteases: Mediating ECM degradation at points of protrusion.

5) Scaffolding proteins: Mediating interactions between other proteins.

Twist and its reported role in EMT and metastasis of human epithelial cells as well as its correlations with metastatic cancers warrant further detailed study in the metastatic cascade. While it remains to be further examined whether Twist plays a role in other steps of the cascade beyond the EMT and metastasis, it is know that Twist can play a role in inducing the EMT change. Invasion is a key step for the ability of tumor cells to migrate in vivo and invadopodia are dynamic structures that are specialized for matrix degradation. Preliminary studies have also established an important connection between invadopodia formation by breast cancer cells and the rate of metastasis in nude mouse models (Weaver, 2008). Therefore my project is aimed at exploring the link between Twist, invadopodia, and extracellular matrix degradation.

Chapter 1

Is Twist necessary for local invasion?

Twist has been shown to be essential for mouse mammary carcinoma cells to metastasize from the mammary gland to the lung. One cellular function of Twist is to contribute to metastasis is to activate the EMT process, thus promoting epithelial tumor cells to lose their epithelial phenotype and gain mesenchymal characteristics. Is Twist also necessary for other steps of metastasis?

To form distant metastases, cancer cells must first free themselves from the primary tumor and invade through the encapsulating basement membrane. The invasion and migration of cancer cells by controlled degradation of extracellular matrix is a hallmark of metastatic cancer cells. Twist is necessary for cells to gain the mesenchymal features needed for the cells to extract themselves and gain motility. It is not yet known whether Twist expression can regulate the ability of cancer cells to invade through the basement membrane.

This chapter will address if Twist expression is necessary for local matrix degradation and invadopodia formation. The tumor microenvironment is supported by the extracellular matrix (ECM). A cancer cell must activate a series of proteolytic cascades to traverse the basement membrane and degrade ECM to invade. Is Twist expression necessary to degrade this matrix? If so, will exogenous Twist expression induce a non invasive cell to degrade a matrix? Invadopodia are sub-cellular structures that have been shown to mediate matrix

degradation by concentrating protease activity at the cell-matrix contact. Are these structures present in Twist expressing cells? Is Twist necessary for the formation of these structures?

Results

Twist is necessary and sufficient for matrix degradation

To assess the role of Twist in invasion and invadopodia, different types of cells with high and low expressions of Twist are examined in the FITC gelatin degradation assay. The FITC gelatin degradation assay is a sensitive system for directly measuring matrix degradation. Cells are seeded on glass coverslips coated with a thin FITC-labeled gelatin matrix. Areas of degradation appear as dark areas of degraded fluorescent matrix underneath the cells. Staining with Phalloidin for F-actin and DAPI for nuclei is used.

The cell lines used are the 4T1 and the HMEC cell lines. The 4T1 cell line is a mouse mammary tumor cell line that has high endogenous Twist expression. It is highly metastatic and will form visible lung and liver metastases when implanted in the mammary fat pad. To this highly invasive cell line, shRNA constructs of Twist or the control shRNA vector were introduced to observe whether its invasive characteristics were affected when expression of Twist were reduced. The knock down of Twist levels by the shRNA constructs have been verified in previously published data (Figure 1.1) (Yang, Mani, et al, 2004). The HMEC cell line is a primary human epithelial cell line that has been immortalized with large T Antigen and telomerase. HMEC cells have no endogenous Twist expression. To this non-invasive cell line, a Twist overexpressing construct was introduced to test whether heightened levels of Twist will induce invasive characteristics.

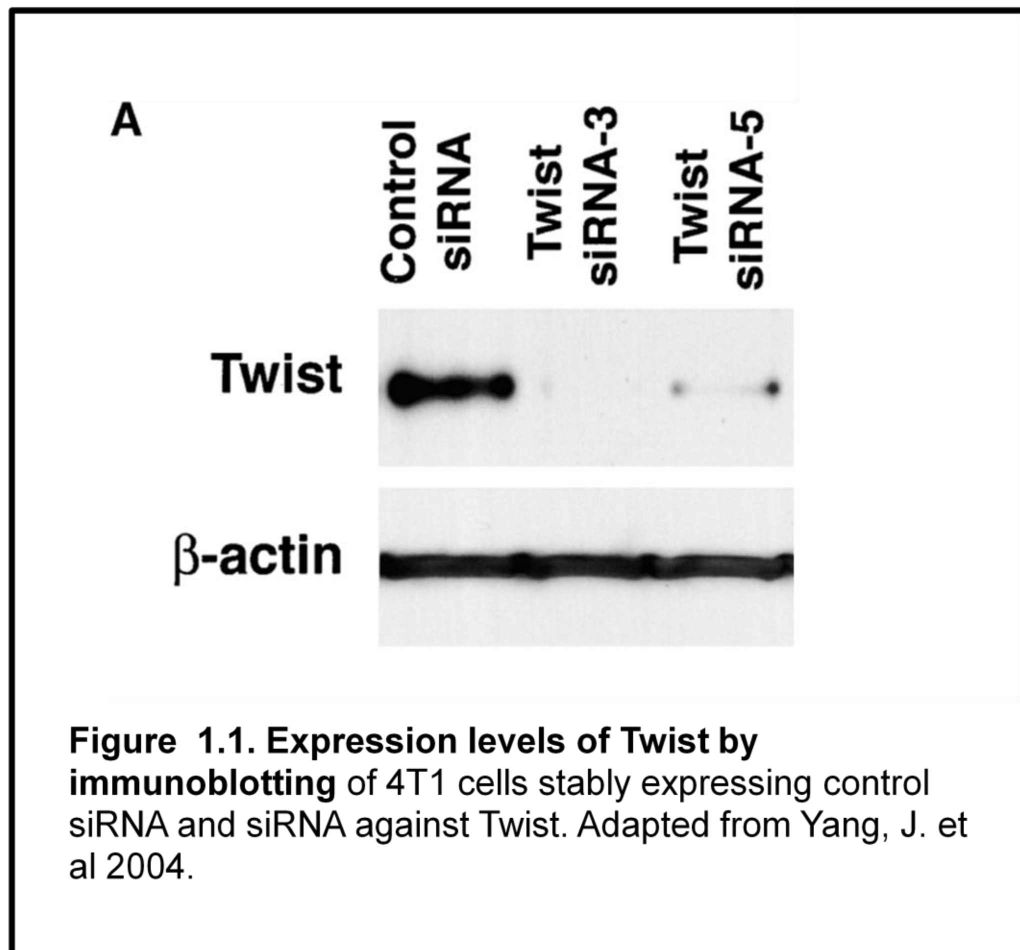


Figure 1.1. Expression levels of Twist by immunoblotting of 4T1 cells stably expressing control siRNA and siRNA against Twist. Adapted from Yang, J. et al 2004.

After incubating the 4T1 control cells and 4T1 siTwist cells on FITC gelatin coated coverslips for 8 hours, the 4T1 control cells showed extensive areas of degradation underneath the cells whereas the 4T1 siTwist cells showed decreased amounts of gelatin degradation (Figure1.2a and Figure 1.2b). Quantification of the areas of degradation show that the average area of degradation by 4T1 control cells is 10 times greater than the average area of degradation by 4T1 siTwist cells(Figure 1.2c). The quantification of the areas of degradation was performed with ImageJ. At least 10 fields were taken for each sample. The area of degradation per field was normalized by the number of cells in each field. The relative area of degradation for 4T1 control cells was set as 1 and the values for the 4T1 siTwist cells were compared against it. This experiment indicate that Twist is required for the ability of 4T1 cells to degrade ECM.

After incubating the HMEC cells and HMEC Twist expressing cells on the FITC gelatin coated coverslips for 8 hours, the HMEC Twist cells showed increased amounts of gelatin degradation (Figure 1.3a and Figure 1.3b). Quantification of the areas of degradation showed that the average area of degradation by HMEC Twist cells was 10 times greater than the average area of degradation by HMEC only cells. (Figure 1.3c). The relative area of degradation

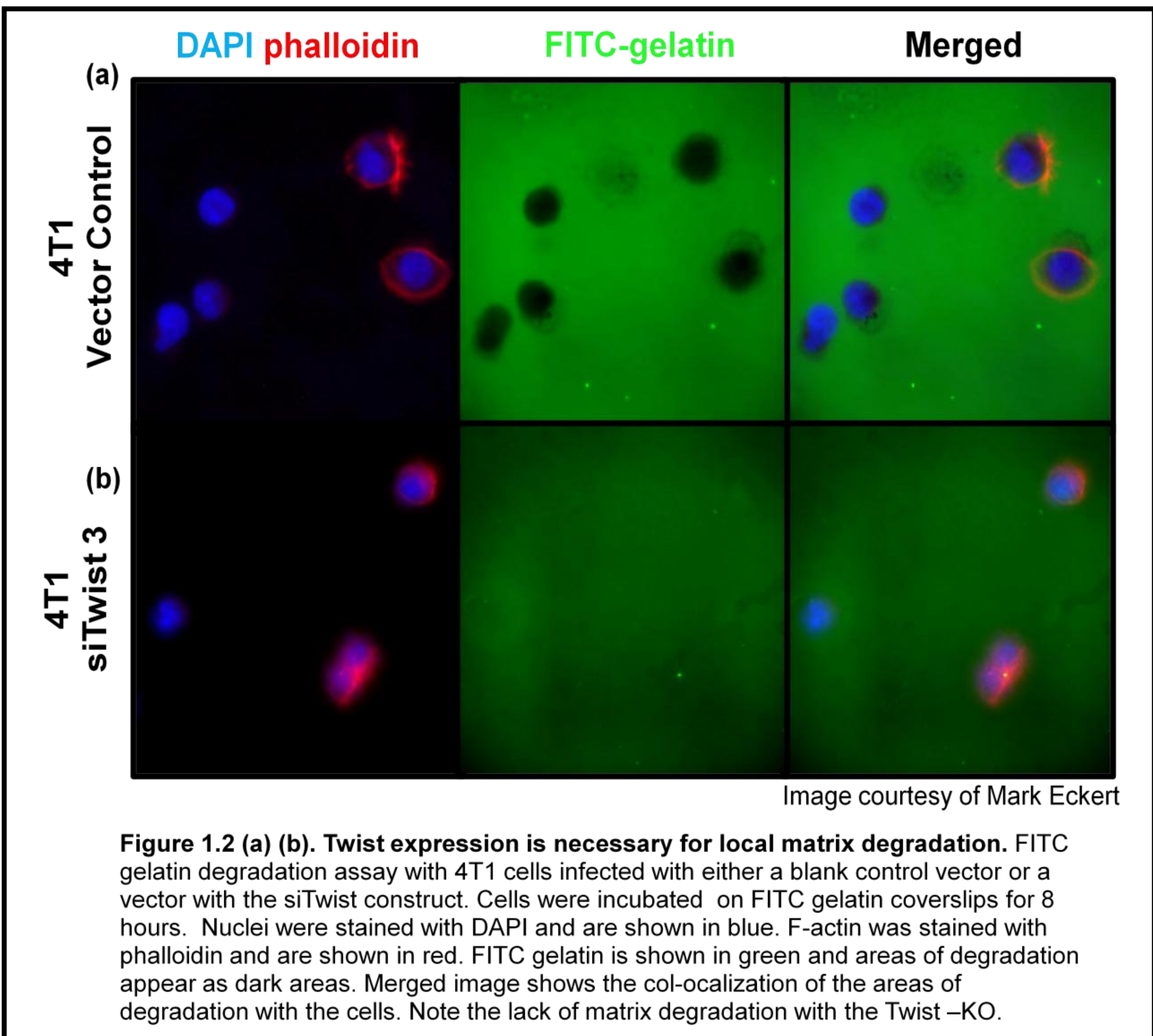


Figure 1.2 (a) (b). Twist expression is necessary for local matrix degradation. FITC gelatin degradation assay with 4T1 cells infected with either a blank control vector or a vector with the siTwist construct. Cells were incubated on FITC gelatin coverslips for 8 hours. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. FITC gelatin is shown in green and areas of degradation appear as dark areas. Merged image shows the col-ocalization of the areas of degradation with the cells. Note the lack of matrix degradation with the Twist –KO.

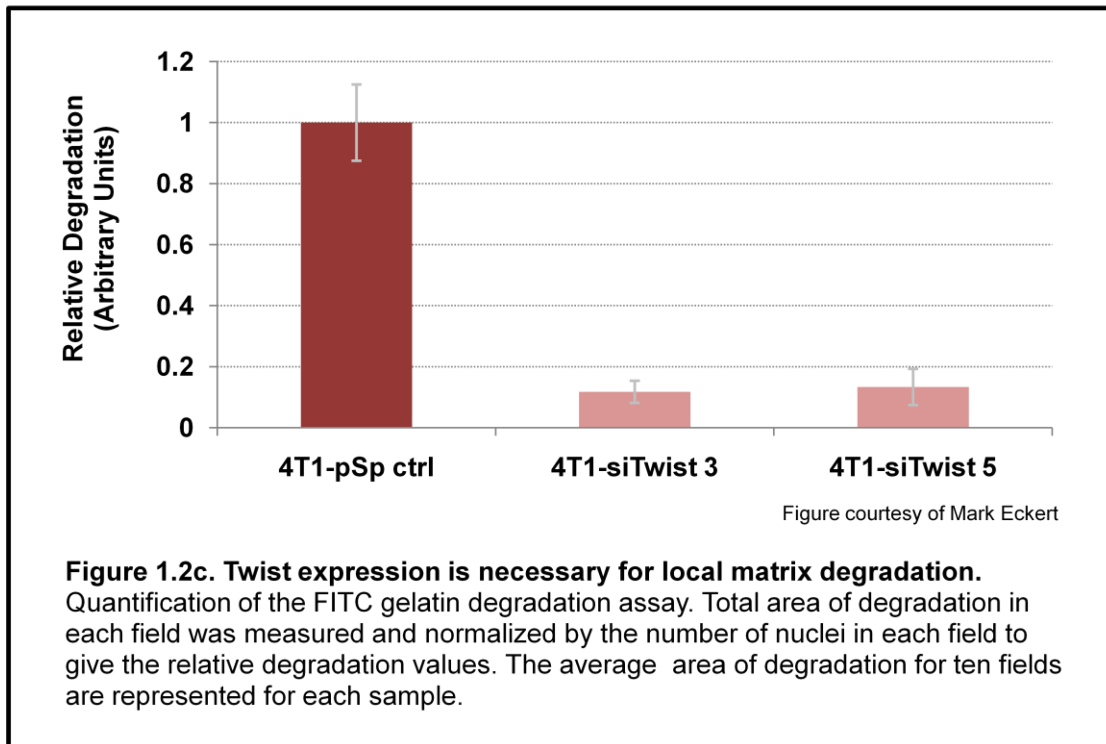


Figure 1.2c. Twist expression is necessary for local matrix degradation. Quantification of the FITC gelatin degradation assay. Total area of degradation in each field was measured and normalized by the number of nuclei in each field to give the relative degradation values. The average area of degradation for ten fields are represented for each sample.

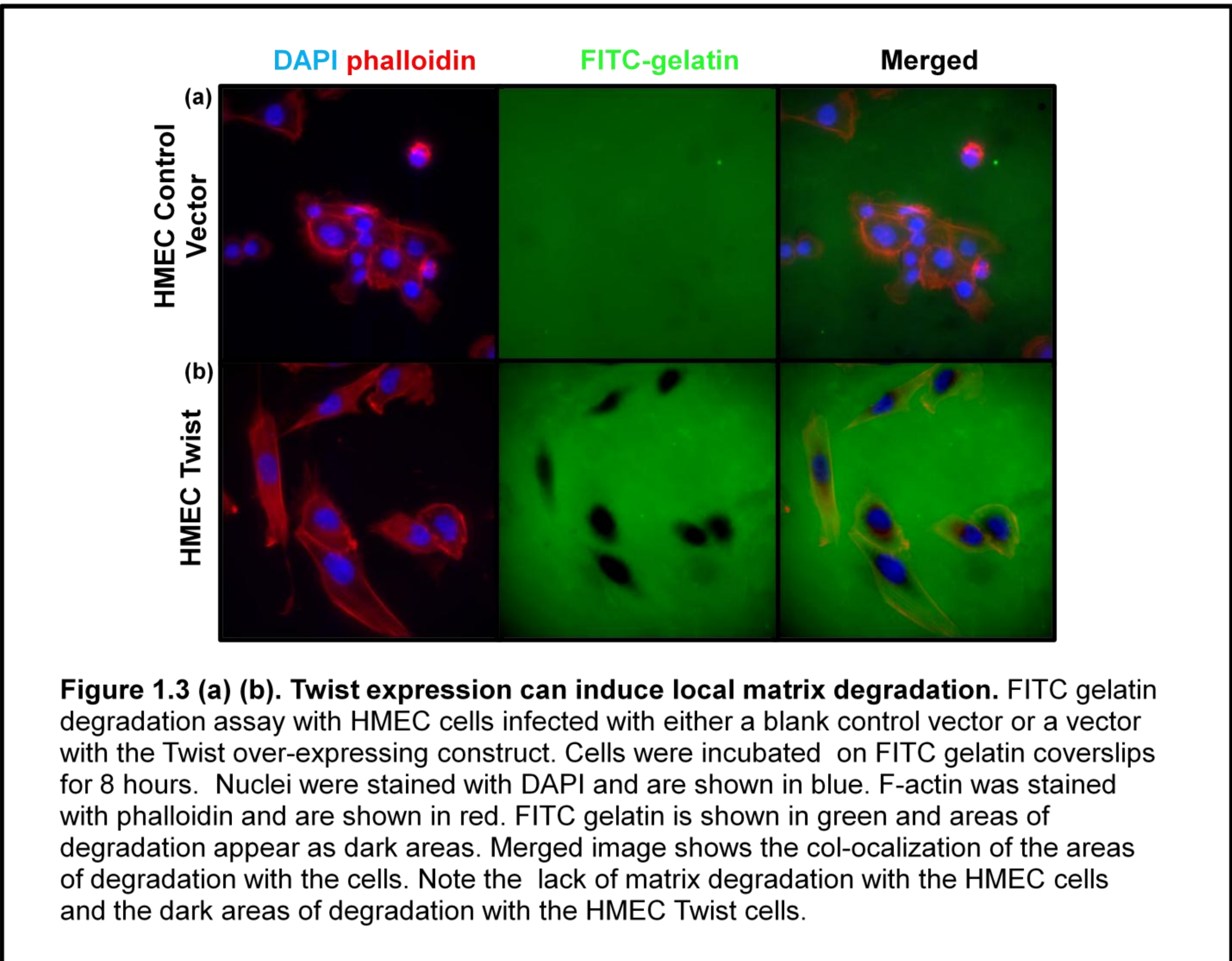


Figure 1.3 (a) (b). Twist expression can induce local matrix degradation. FITC gelatin degradation assay with HMEC cells infected with either a blank control vector or a vector with the Twist over-expressing construct. Cells were incubated on FITC gelatin coverslips for 8 hours. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. FITC gelatin is shown in green and areas of degradation appear as dark areas. Merged image shows the col-ocalization of the areas of degradation with the cells. Note the lack of matrix degradation with the HMEC cells and the dark areas of degradation with the HMEC Twist cells.

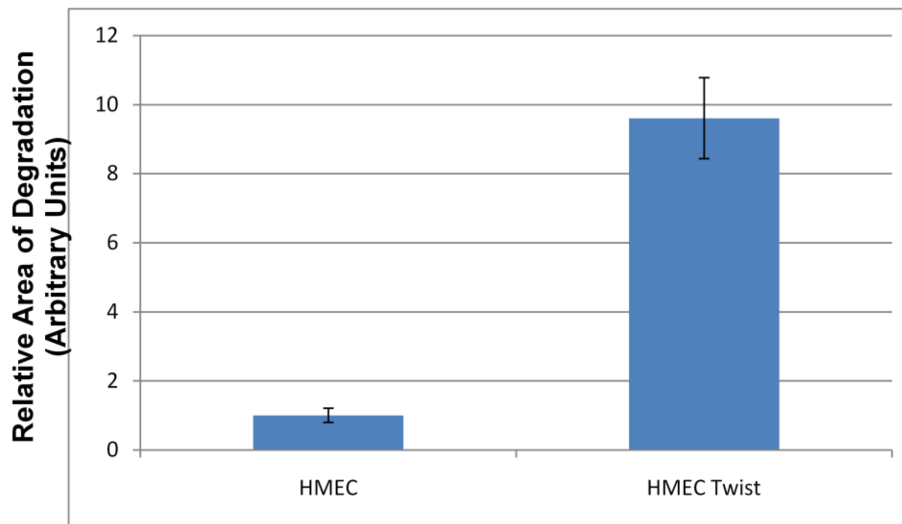


Figure 1.3c. Twist expression can induce local matrix degradation. Quantification of the FITC gelatin degradation assay. Total area of degradation in each field was measured and normalized by the number of nuclei in each field to give the relative degradation values. The average area of degradation for ten fields are represented for each sample.

for HMEC Twist cells was set as 1 and the values for the HMEC cells were compared against it (this sentence should be in figure legend). This data further demonstrate that expression of Twist is sufficient to promote ECM degradation.

Twist is necessary and sufficient for the formation of invadopodia

Invadopodia are actin-based membrane protrusions that are specialized at mediating matrix degradation. Since Twist is necessary and sufficient for matrix degradation, we then asked whether Twist is also necessary for the formation of invadopodia and whether exogenous Twist expression could induce the formation of invadopodia.

An immunofluorescence analysis was performed to examine the formation of these invadopodial structures. Phalloidin staining for F-actin shows punctuate aggregates which usually co-stain with a number of other markers that have been shown to be upregulated in invadopodia.(no paragaph break)Cortactin, phosphor-tyrosine, Arp2/3, β-actin, Tks5, and some proteases are commonly used markers for invadopodia. Here, Cortactin staining along with phalloidin staining was used in the following immunofluorescence assay. Cortactin is an actin assembly protein that has been shown to be enriched in invadopodia. It is required for stabilization of branched actin and is important for the formation of invadopodia (Clark, Whigham, et al, 2007). Loss of cortactin decreases the number of actin/cortactin rich invadopodia observed and decreases the amount of matrix degradation. Cortactin is a good marker for invadopodia since it has been shown to be present early on during the formation of invadopodia (Artym,

Zhang, et al, 2006).

After being cultured for 3 days on gelatin-coated coverslips, 4T1 cells showed punctuate cortactin staining which co-localizes with punctuate F-actin staining when merged (Figure 1.4a). The 4T1 siTwist cells on the other hand showed diffuse cortactin and F-actin staining (Figure 1.4b). A fraction of the 4T1 siTwist cells showed punctuate cortactin and F-actin staining, but the cortactin puncta failed co-localize with F-actin, indicating that loss of Twist prevents the formation of invadopodia.

After being cultured for 3 days on gelatin coated coverslips, HMEC cells did not show punctuate cortactin staining co-localizing with punctuate F-actin staining when merged (Figure 1.5a). The HMEC Twist cells on the other hand showed strong punctate cortactin and F-actin staining that co-localized when merged (Figure 1.5b). (no paragraph break).

To further confirm the idea that exogenous Twist expression is able to induce expression of invadopodia in HMEC cells, a Z-stack was taken of the HMEC Twist cells. Invadopodia are usually seen at the basal side of the invading cell. This Z-stack showed that the cortactin and F-actin puncta that co-localized were indeed localized at the basal surface of the cells.

Together, these results demonstrate that Twist is both necessary and sufficient for invadopodia formation, which mediates the degradation of extracellular matrix.

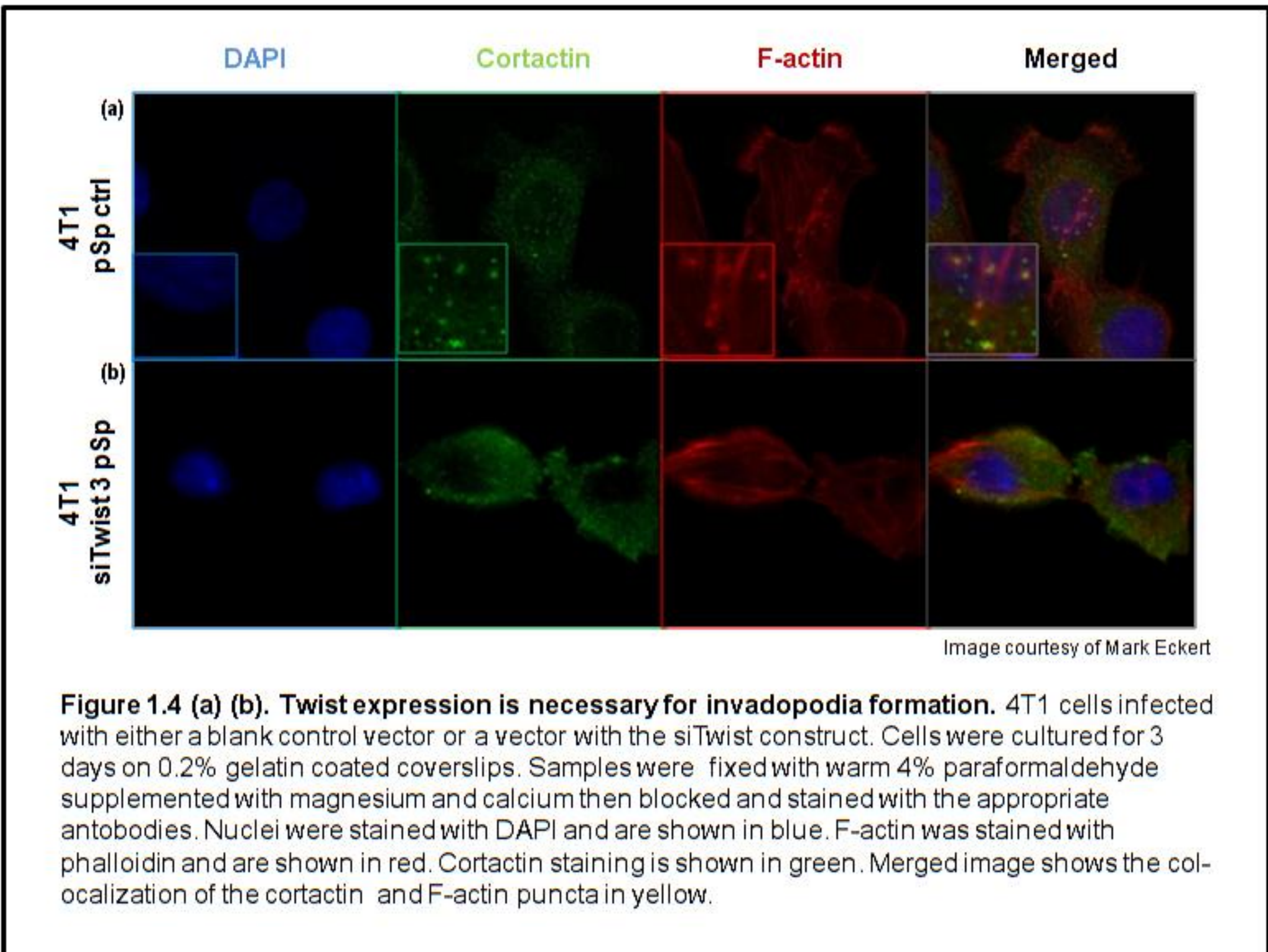


Figure 1.4 (a) (b). Twist expression is necessary for invadopodia formation. 4T1 cells infected with either a blank control vector or a vector with the siTwist construct. Cells were cultured for 3 days on 0.2% gelatin coated coverslips. Samples were fixed with warm 4% paraformaldehyde supplemented with magnesium and calcium then blocked and stained with the appropriate antobodies. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. Cortactin staining is shown in green. Merged image shows the col-ocalization of the cortactin and F-actin puncta in yellow.

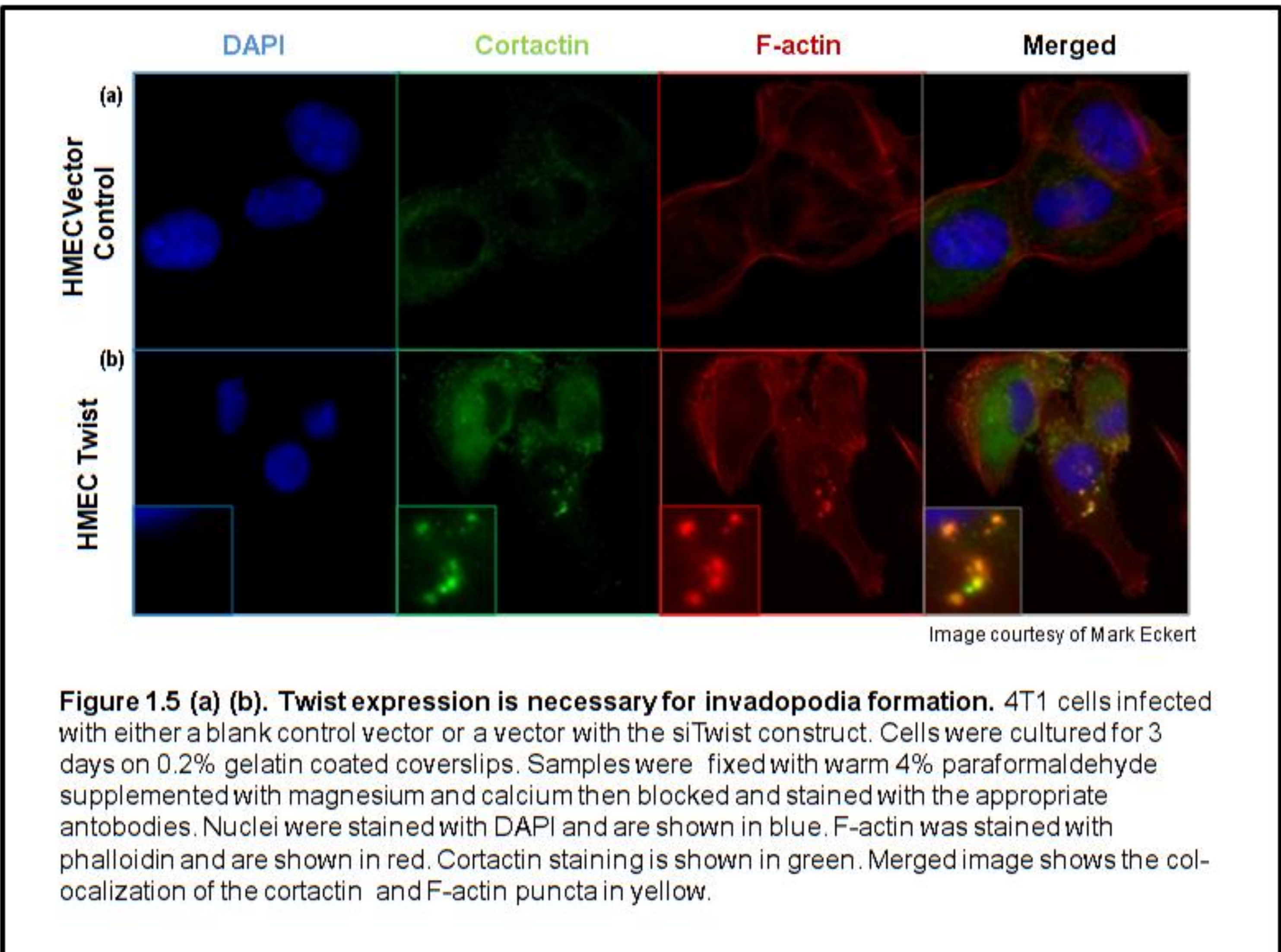


Figure 1.5 (a) (b). Twist expression is necessary for invadopodia formation. 4T1 cells infected with either a blank control vector or a vector with the siTwist construct. Cells were cultured for 3 days on 0.2% gelatin coated coverslips. Samples were fixed with warm 4% paraformaldehyde supplemented with magnesium and calcium then blocked and stained with the appropriate antobodies. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. Cortactin staining is shown in green. Merged image shows the col-ocalization of the cortactin and F-actin puncta in yellow.

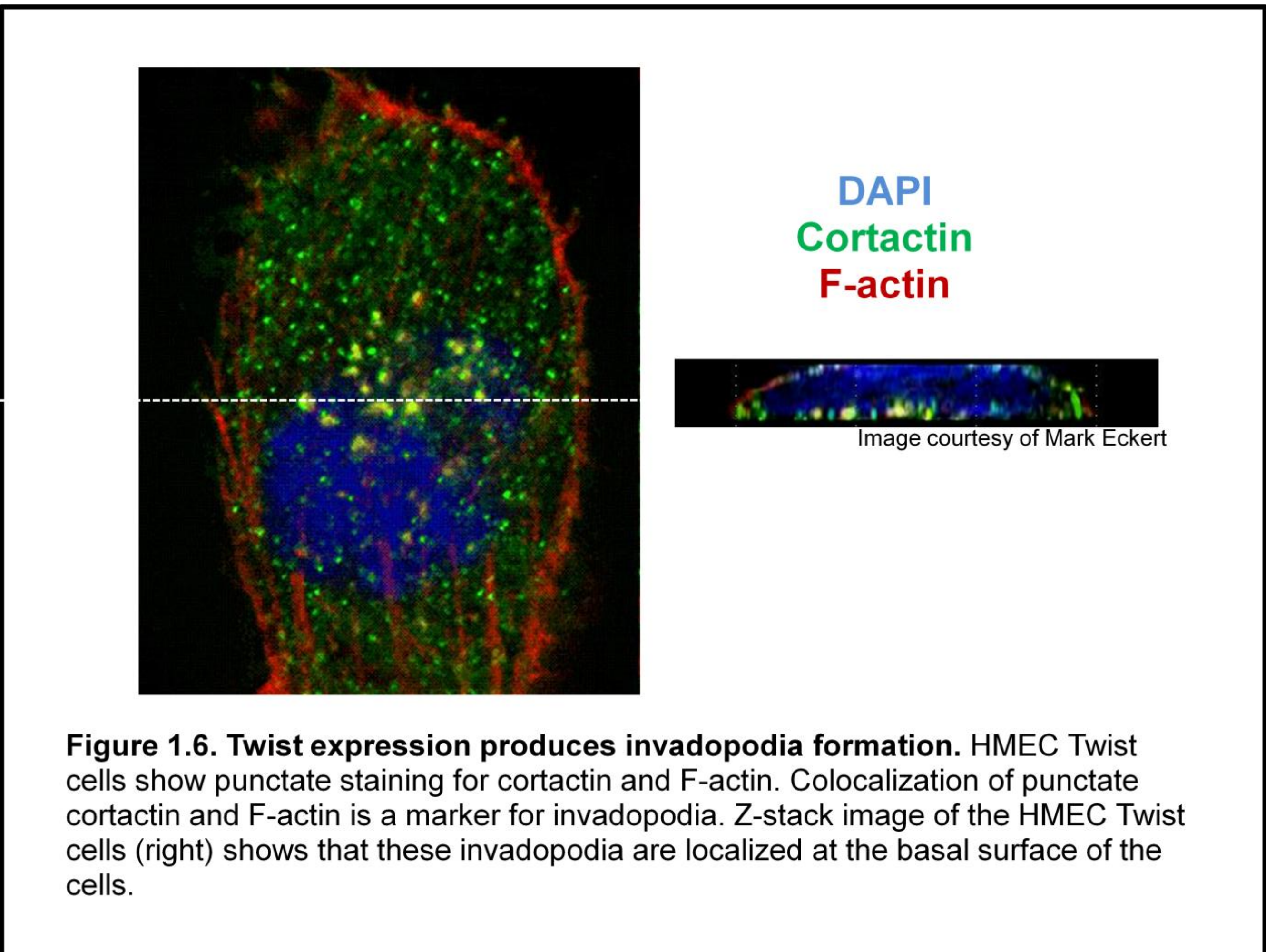


Figure 1.6. Twist expression produces invadopodia formation. HMEC Twist cells show punctate staining for cortactin and F-actin. Colocalization of punctate cortactin and F-actin is a marker for invadopodia. Z-stack image of the HMEC Twist cells (right) shows that these invadopodia are localized at the basal surface of the cells.

Discussion

Twist is capable of inducing EMT, which contributes its ability to promoter metastasis. However, its role in other steps of the metastatic cascade has not yet been elucidated. This study showed that Twist is necessary for matrix degradation and invadopodia formation in both murine and human mammary cell lines. Ablation of Twist expression reduced the average area of degradation in highly invasive and metastatic 4T1 cell lines tenfold. Introducing expression of Twist in HMEC cells increased the average area of degradation tenfold. This supports the idea that Twist also plays a key role the invasion step of the metastatic cascade.

To date, most studies on the inducing signals of invadopodia focused on Src tyrosine kinase and growth factor signaling. Indeed, no transcription regulation of invadopodia has been identified. Our identification of Twist as the first transcription factor in regulating invadopodia formation further highlights the critical role of Twist in invasion and metastasis.

Twist is responsible for matrix degradation via invadopodia formation, but it is unclear how Twist can induce invadopodia formation. While there are many studies reported on the assembly machinery of invadopodia and the recruitment of proteases, there is little known about the signals that initiate the formation of the structure. The importance of proteases in physiological ECM remodeling and tumor cell invasion has been recognized and an idea that has been proposed is that proteases may play a role in the initiation of invadopodia formation.

Small amounts of proteolysis by membrane bound proteases release peptides generated from matrix degradation. These peptides may in turn act as signaling molecules. Experimental evidence for this is supported by fluorescent microscopy detecting lysosomes at active sites of degradation. In this study, lysosomes were localized at invadopodial protrusions on fibroblasts cultured on biotinylated collagen (Lee, Overall, et al, 2006). The degraded matrix may be taken up and processed into signaling peptides in these lysosomes. Certain membrane bound proteases can also cleave growth factors and cytokine precursors into active forms, creating further signaling molecules for invadopodial initiation and assembly (Huovila, Turner, et al, 2005).

Twist expression can mediate invadopodia formation and local matrix degradation. The mechanisms as to how Twist regulates components of invadopodia to initiate the formation of the structure are still unclear. Proteases seem to be an appealing target to control initiation of invadopodia formation. The question of whether Twist can upregulate expression of proteases that have been associated with invadopodia is one that is worth examining further.

Chapter 2

How does Twist promote local invasion?

In Chapter 1, we show that expression of Twist is both necessary and sufficient for cells to migrate and invade through basement membrane and degrade gelatin matrix in vitro. Twist achieves this be inducing the formation of invadopodia. This chapter focuses on understanding how Twist controls the formation of invadopodia and ECM degradation.

Our lab has created a system in which Twist expression is fused with a modified hormone-binding domain of the estrogen receptor (Twist-ER). In this system, Twist can be activated by the addition of 4-Hydroxy Tamoxifen. The Twist-ER construct was expressed in HMEC cells. Within 10 days of induction with 4-OHT, the cells underwent EMT, characterized by changes in morphology, cell scattering, loss of epithelial markers, and gain of mesenchymal markers. This phenotype is identical to the EMT phenotype in HMEC cells after wild-type Twist expression (Figure 2.1).

A gene expression profiling analysis of the time course of Twist activation in HMEC Twist ER cells was performed. The microarray analysis showed that Twist activation induces up-regulation of several membrane-associated proteases. The membrane associated proteases that were highly upregulated and selected for further study were A Disintegrin and Metalloprotease(ADAM) family member ADAM12, serine proteases Fibroblast Activation Protein alpha (also known as Seprase), and Di-Peptidyl Peptidase 4 (also known as Cluster of

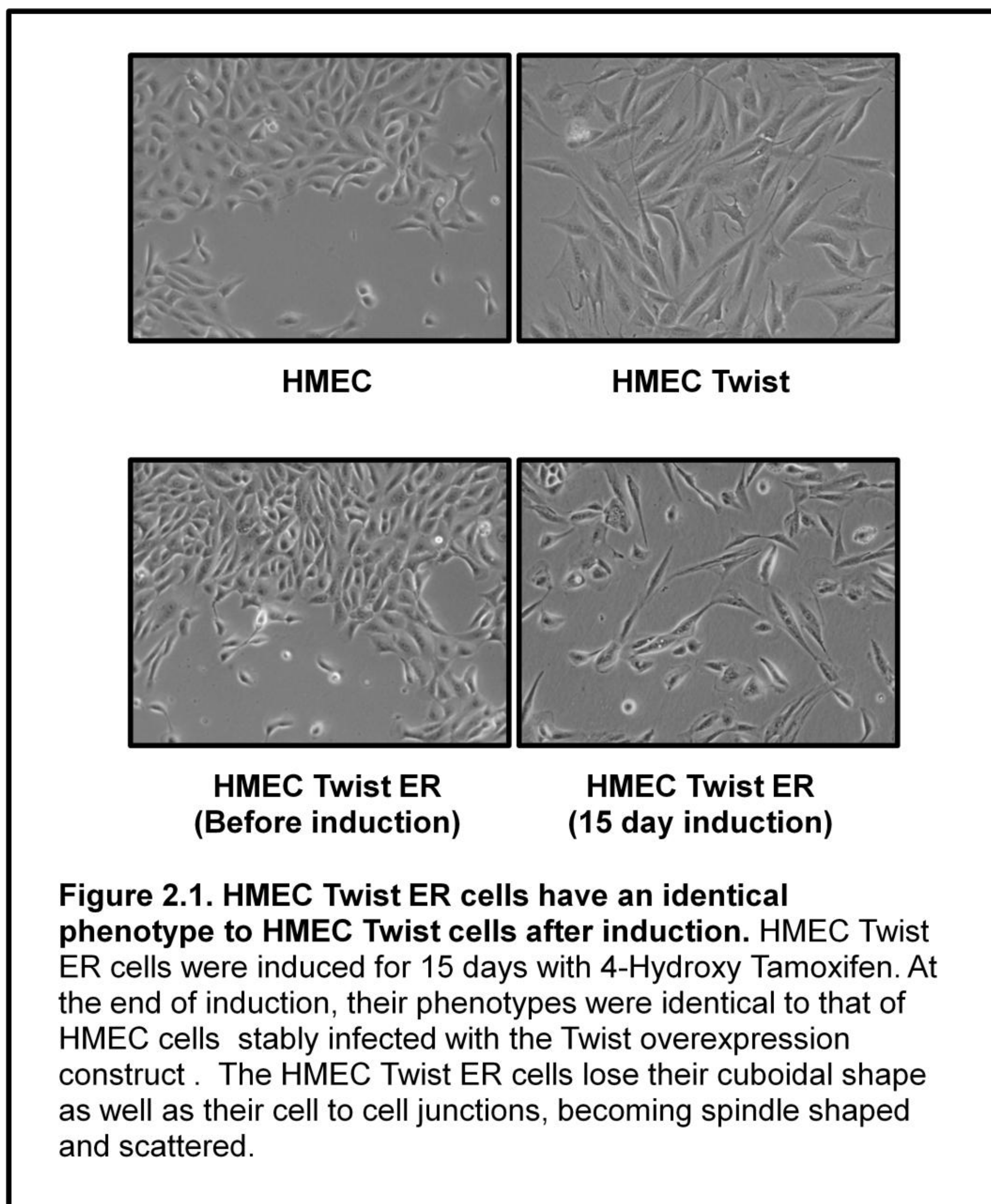


Figure 2.1. HMEC Twist ER cells have an identical phenotype to HMEC Twist cells after induction. HMEC Twist ER cells were induced for 15 days with 4-Hydroxy Tamoxifen. At the end of induction, their phenotypes were identical to that of HMEC cells stably infected with the Twist overexpression construct . The HMEC Twist ER cells lose their cuboidal shape as well as their cell to cell junctions, becoming spindle shaped and scattered.

Differentiation 26). All three proteases were also implicated in proteolytic activity

at invadopodia. Levels of ADAM12 were increased ~3.3 fold, levels of were FAPa increased ~6 fold and levels of DPPIV were increased ~53 fold in the microarray analysis. In contrast, Levels of most secreted MMP proteases were not affected by Twist activation (Data not shown).

ADAM12 is a member of the ADAMs family of metalloproteases that have been implicated in growth factor shedding and cell migration as well as a number of other processes (Seals, and Courtneidge, 2003). ADAM12 has been shown to be associated with Tks5/FISH, a scaffolding protein that is localized at invadopodia (Abram, Seals, et al, 2003a). Its overexpression is implicated in a number of cancers and cancer cell lines (Wu, Croucher, and McKie, 1997).

FAPa/Seprase is a member of the serine integral membrane peptidase family. It cleaves proline residues and gelatin in ECM components. FAPa is detected in fetal mesenchymal tissues and during wound healing (O'Brien, and O'Connor, 2008). No expression of FAPa is detected in normal tissue, but its expression is detected in a number of invasive carcinomas. FAPa can complex with a number of integrins and other proteases at the invadopodia of malignant cells (Mueller, Ghersi, et al, 1999). The gelatinase activity of FAPa has been linked to malignant tumors (Chen, Kennedy, et al, 2006).

DPPIV is also a member of the serine integral membrane peptidase family with prolyl peptidase activity. Its expression is implicated in a variety of functions which include regulation of inflammatory and immunological responses as well as signal transduction and apoptosis (Kikkawa, Kajiyama, et al, 2005). It was originally identified as a T cell differentiation antigen and its functional role in T

cells has been studied extensively. DPPIV and FAPa have been shown to form a complex localized at the invadopodia of fibroblasts (Chen, and Kelly, 2003). This complex plays a role in the migration and invasion of endothelial cells on a collagen matrix (Ghersi, Zhao, et al, 2006).

These membrane-associated proteases are critical in mediating ECM degradation and are highly enriched at invadopodia. They target ECM macromolecules such as collagen, fibronectin, and laminins for digestion (Chen, 1996). They can function in signaling by cleaving pro-enzymes into their active isoforms. Their matrix degradation product can also function as signaling peptides. They may also play a critical role in mediating the formation of invadopodia.

Our microarray data showed that the membrane associated proteases ADAM12, FAPα/Seprase, and DPPIV/CD26 were upregulated upon Twist activation. The goal of this chapter is to evaluate the microarray data via quantitative Real-Time PCR and also to test whether the upregulation of proteases is a Twist specific effect.

To examine whether the upregulation of protease expression is directly controlled by Twist, HMEC cells were stably infected with different constructs. The constructs that were introduced were the following: Twist expressing, Snail expressing, Snail expressing + siTwist, control GFP expressing + siTwist. Levels of Twist were examined in these samples to ensure proper expression or ablation (Figure 2.2a). We ask whether the expressions of the proteases are under the

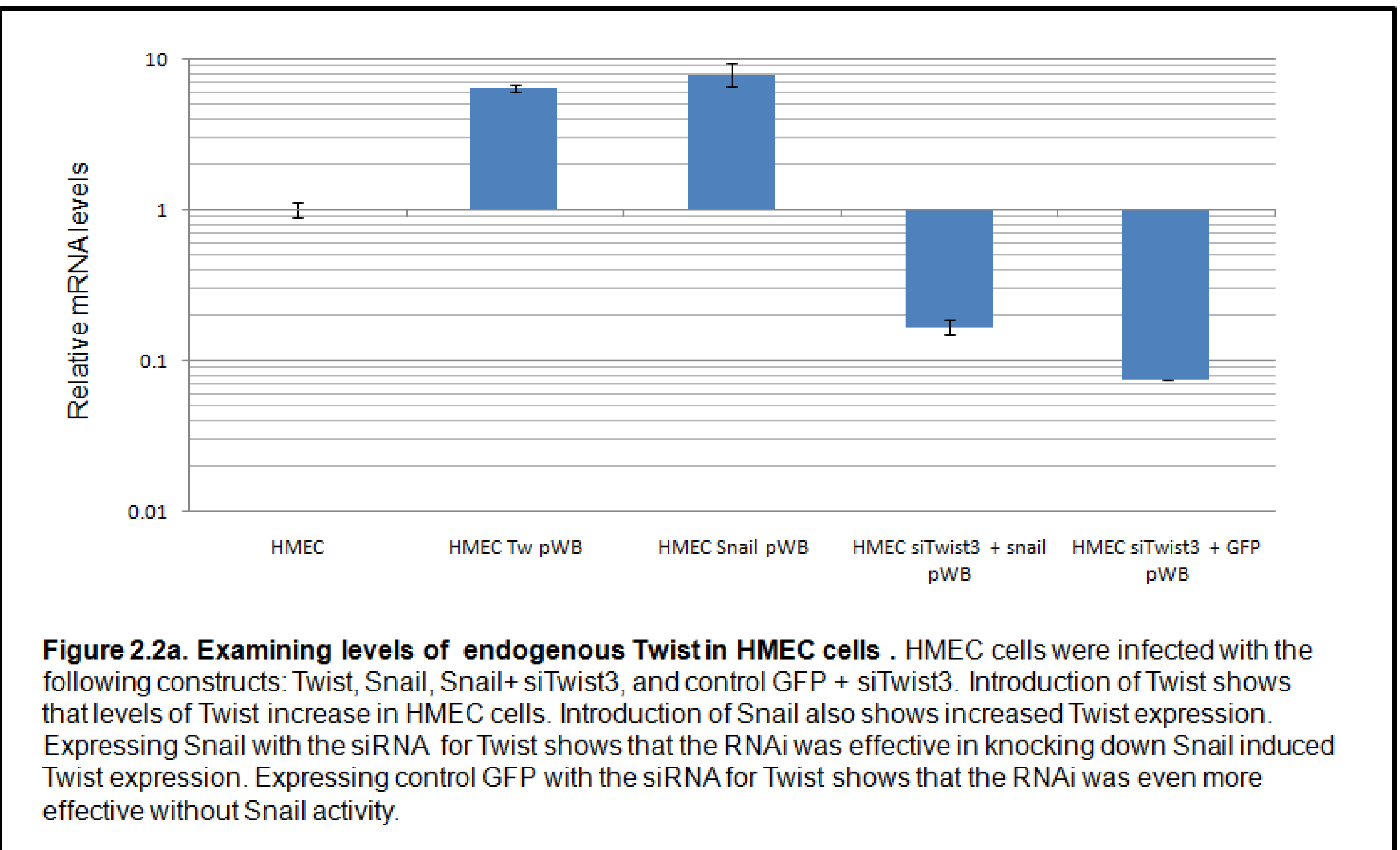


Figure 2.2a. Examining levels of endogenous Twist in HMEC cells . HMEC cells were infected with the following constructs: Twist, Snail, Snail+ siTwist3, and control GFP + siTwist3. Introduction of Twist shows that levels of Twist increase in HMEC cells. Introduction of Snail also shows increased Twist expression. Expressing Snail with the siRNA for Twist shows that the RNAi was effective in knocking down Snail induced Twist expression. Expressing control GFP with the siRNA for Twist shows that the RNAi was even more effective without Snail activity.

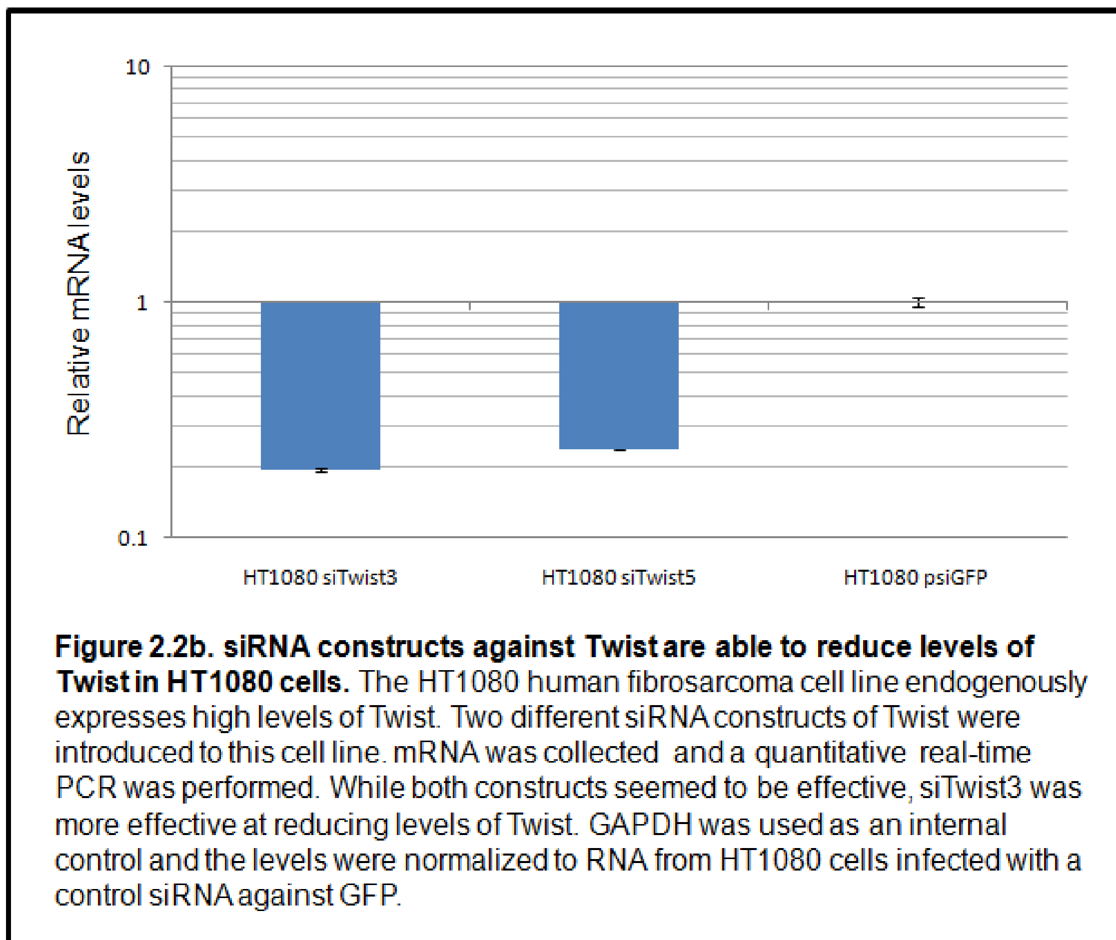


Figure 2.2b. siRNA constructs against Twist are able to reduce levels of Twist in HT1080 cells. The HT1080 human fibrosarcoma cell line endogenously expresses high levels of Twist. Two different siRNA constructs of Twist were introduced to this cell line. mRNA was collected and a quantitative real-time PCR was performed. While both constructs seemed to be effective, siTwist3 was more effective at reducing levels of Twist. GAPDH was used as an internal control and the levels were normalized to RNA from HT1080 cells infected with a control siRNA against GFP.

specific control Twist or whether any transcription factor that regulates EMT can also induce high levels of protease expression.

Snail is an important transcription factor regulating the EMT process. By directly binding to E-boxes near the start of the E-cadherin gene, it can downregulate E-cadherin transcription and induce EMT. Studies from our lab shows that the transcription factor Snail can upregulate expression of Twist. Figure 2.2a confirms that expression level of Twist was increased in HMEC cells overexpressing. If Twist expression is responsible for expression of the proteases, then Snail expression can also induce expression of the proteases to a certain extent. If the expression of these proteases are Twist specific, then shRNA against Twist in Snail expressing cells should reduce the expression of these proteases. If the expression of these proteases are not Twist specific and can be induced by any inducer of EMT, then shRNA of Twist in Snail expressing cells should not have an effect on the expression of these proteases.

To further demonstrate that the upregulation of these three proteases is dependent on Twist activity, HT1080 cells were stably infected with the siRNA construct against Twist. Knock down of Twist levels was confirmed via quantitative Real-time PCR (Figure 2.2b). The HT1080 cell line is a highly invasive human fibrosarcoma cell line that endogenously expresses high levels of Twist and can form invadopodia potently. The levels of proteases will be examined when levels of Twist are ablated in these HT1080 cells. This will clarify whether expression of proteases is a Twist dependent activity in a highly invasive human tumor cell line.

Results:

Twist activation induces upregulation of three proteases

To confirm the microarray data, a quantative Real-Time PCR was performed to measure the mRNA expression levels of these three proteases. RNA samples were collected at the indicated time points from Twist ER cells treated for 15 days with 4-Hydroxy Tamoxifen. Samples were run in triplicates and the exprssion levels were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The changes in levels of proteases throughout the time examined were compared to the starting levels before induction.

Twist activation with 4-OHT induced an increase in expression of ADAM12 starting after 1 day of treatment (Figure 2.3). A steady increase in ADAM12 levels started after 1 day and continued until the end point. The control cells showed no such increase in levels of ADAM12 expression. The levels of ADAM12 increased 100 fold after 15 days of Twist activation

Twist activation with 4-OHT induced an increase in expression of FAPa in both Twist ER cells (Figure 2.4). Twist ER cells showed increases in FAPa levels that were detectable right after 3 hours (or 0.13 days) of treatment. The steady increase in FAPa levels continued until 15 days. The control cells showed no increase in FAPa levels after 4 days of 4-OHT treatment. The FAPa levels in these control cells never reaches the level of induction reached by Twist while FAPa levels increased more than 10 fold by 15 days in the Twist ER cells.

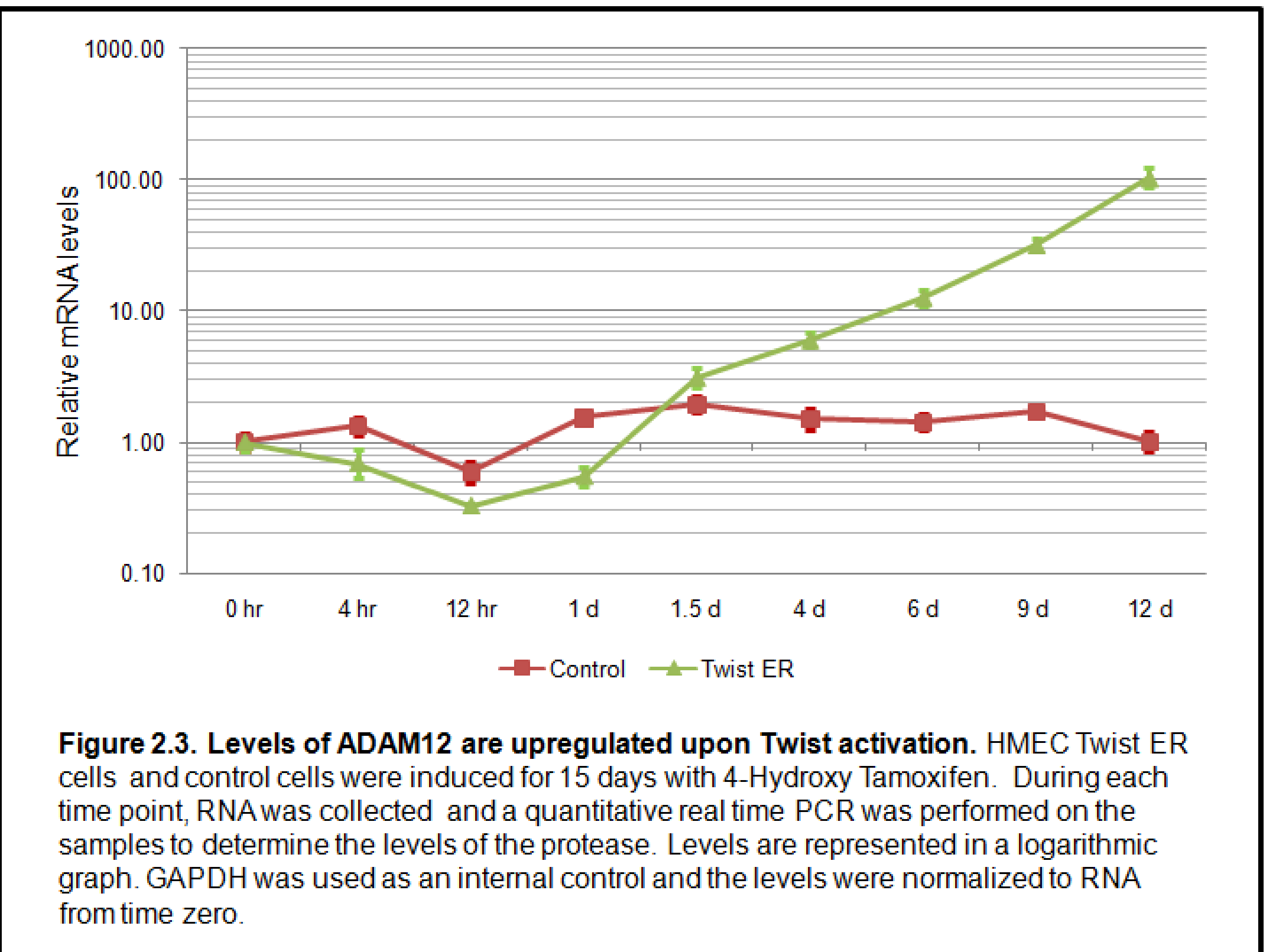


Figure 2.3. Levels of ADAM12 are upregulated upon Twist activation. HMEC Twist ER cells and control cells were induced for 15 days with 4-Hydroxy Tamoxifen. During each time point, RNA was collected and a quantitative real time PCR was performed on the samples to determine the levels of the protease. Levels are represented in a logarithmic graph. GAPDH was used as an internal control and the levels were normalized to RNA from time zero.

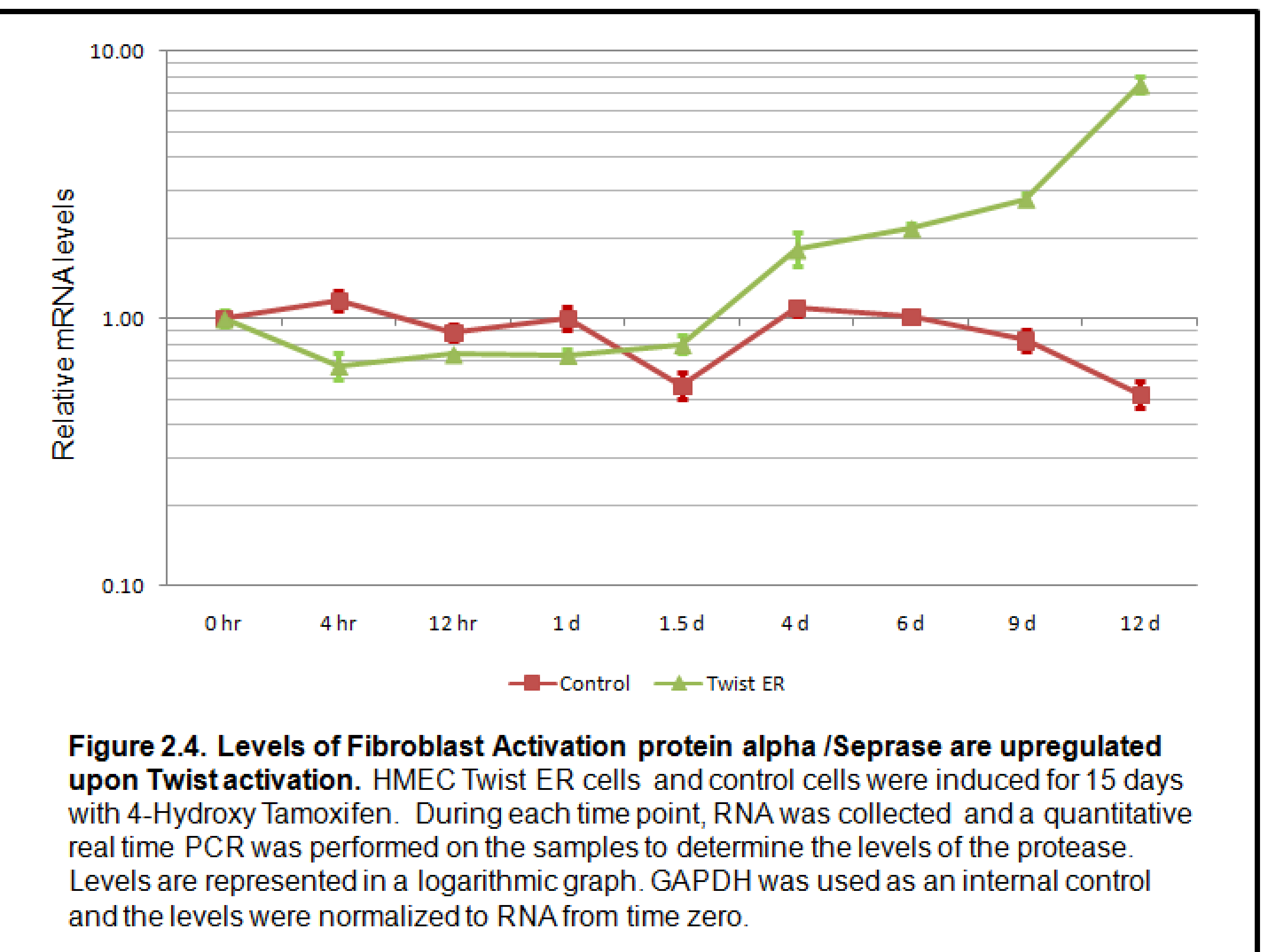


Figure 2.4. Levels of Fibroblast Activation protein alpha /Seprase are upregulated upon Twist activation. HMEC Twist ER cells and control cells were induced for 15 days with 4-Hydroxy Tamoxifen. During each time point, RNA was collected and a quantitative real time PCR was performed on the samples to determine the levels of the protease. Levels are represented in a logarithmic graph. GAPDH was used as an internal control and the levels were normalized to RNA from time zero.

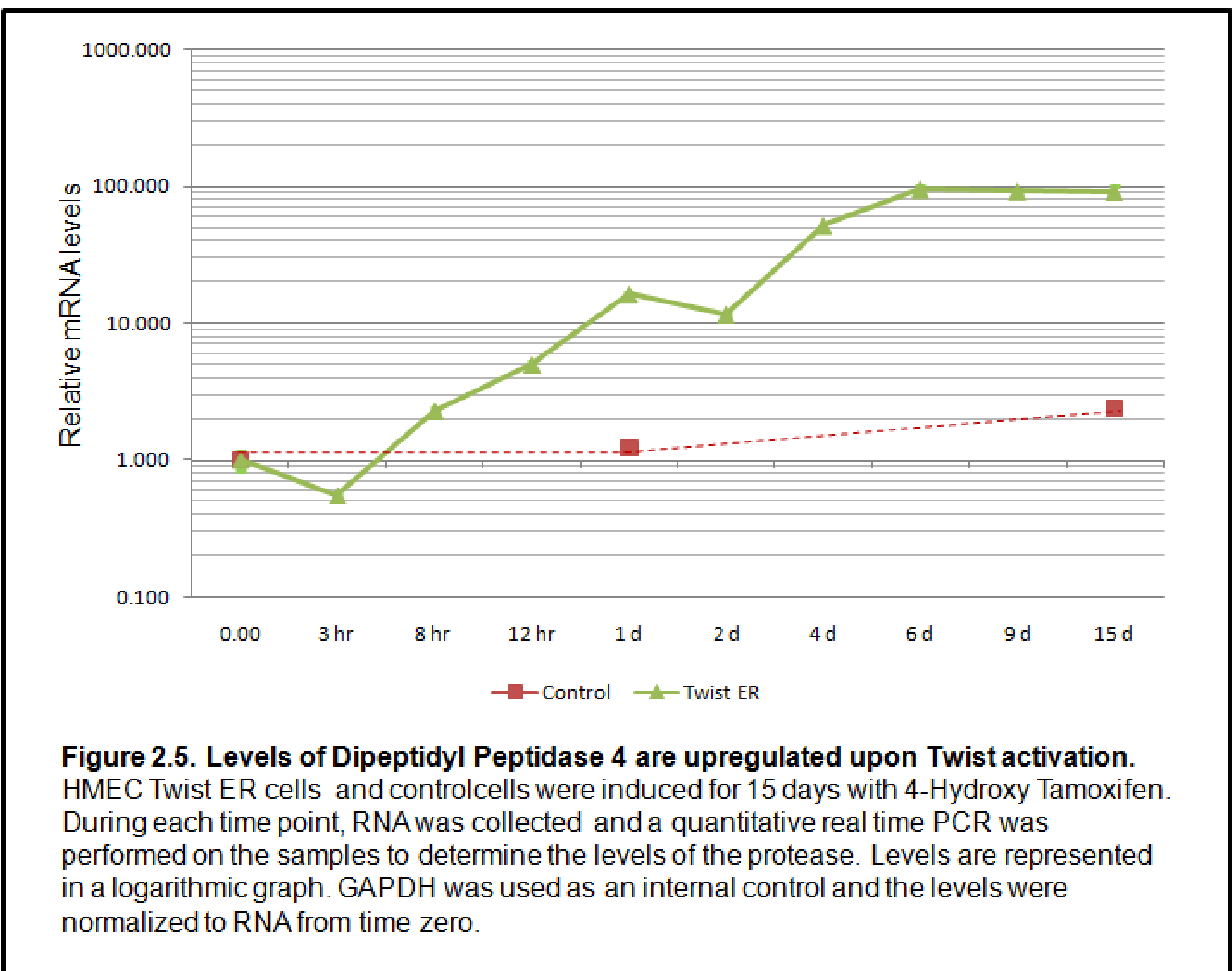


Figure 2.5. Levels of Dipeptidyl Peptidase 4 are upregulated upon Twist activation. HMEC Twist ER cells and controlcells were induced for 15 days with 4-Hydroxy Tamoxifen. During each time point, RNA was collected and a quantitative real time PCR was performed on the samples to determine the levels of the protease. Levels are represented in a logarithmic graph. GAPDH was used as an internal control and the levels were normalized to RNA from time zero.

Twist activation induced an increase in expression of DPPIV starting after just 6 hours (or 0.33 days) of treatment in Twist ER cells (Figure 2.5). Twist ER cells showed steady increases in levels of DPPIV and continued until after day 6 when the increases in levels of DPPIV taper off to a plateau. Control cells show no increase in levels of DPPIV after 6 hours of 4-OHT treatment. The levels of DPPIV in the control cells never reaches the levels of induction reached by Twist. DPPIV levels increased about 100 fold by 15 days in Twist ER cells while DPPIV levels did not increase in the control cells.

Upregulation of proteases is a Twist specific event: Levels of proteases directly correlate with levels of Twist

When Twist was expressed in HMEC cells, levels of ADAM12 increased almost 100 fold (Figure 2.6a). When Snail was introduced into HMEC cells the level of ADAM12 was also highly upregulated. However, when Twist expression was reduced via shRNAin HMEC-Snail cells, the level of ADAM12 expression drastically decreased. Similarly, in HT1080 cells, suppression of endogenous Twist expression showed decreases in ADAM12 expression (Figure 2.6b). These results indicate that Adam12 is transcriptionally regulated by Twist, but not Snail.

When Twist was introduced into HMEC cells, levels of FAPa increased about 11 fold (Figure 2.7a). When Snail was introduced into HMEC cells the levels of FAPa were also upregulated. However, when the level of Twist was

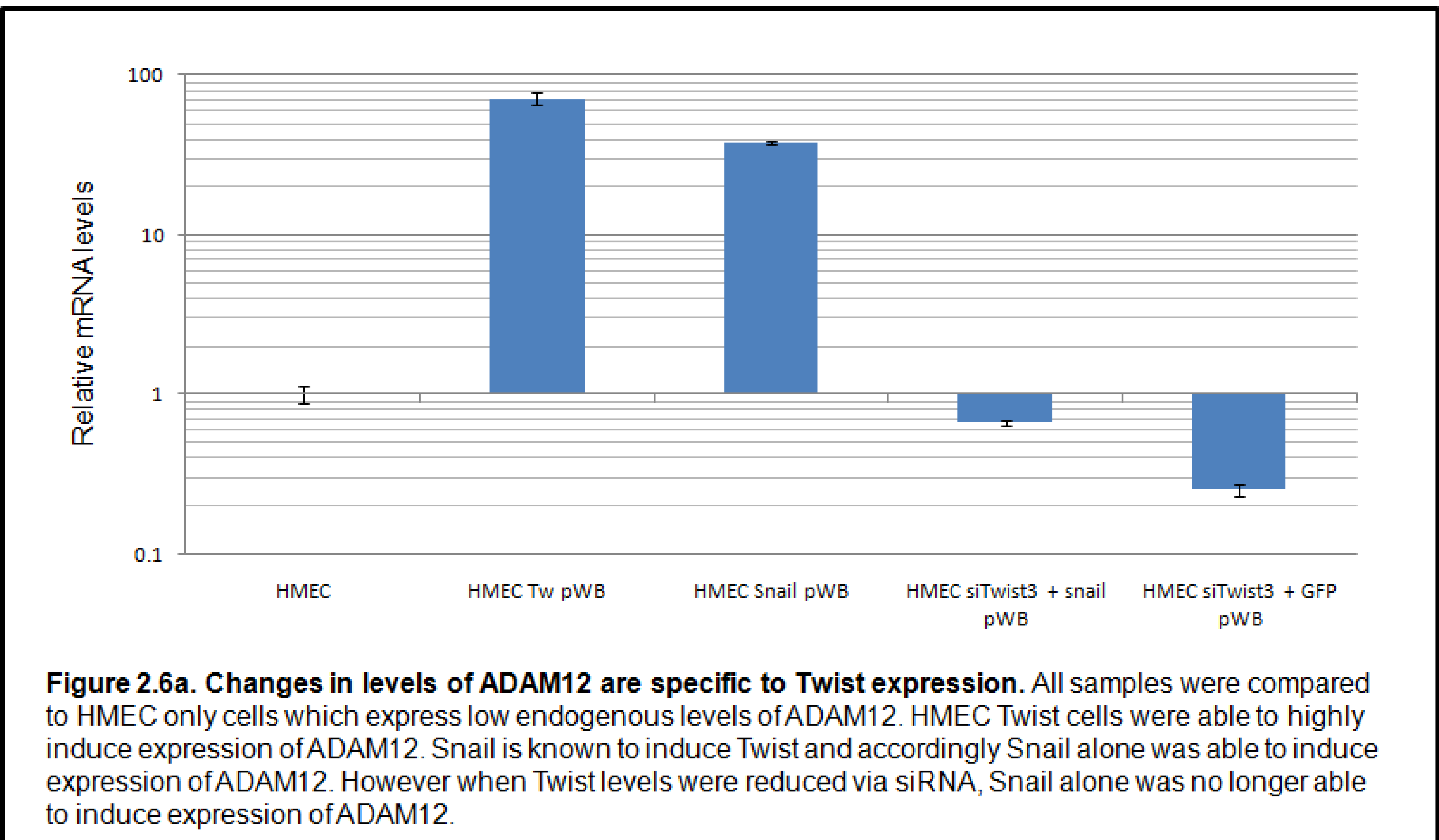


Figure 2.6a. Changes in levels of ADAM12 are specific to Twist expression. All samples were compared to HMEC only cells which express low endogenous levels of ADAM12. HMEC Twist cells were able to highly induce expression of ADAM12. Snail is known to induce Twist and accordingly Snail alone was able to induce expression of ADAM12. However when Twist levels were reduced via siRNA, Snail alone was no longer able to induce expression of ADAM12.

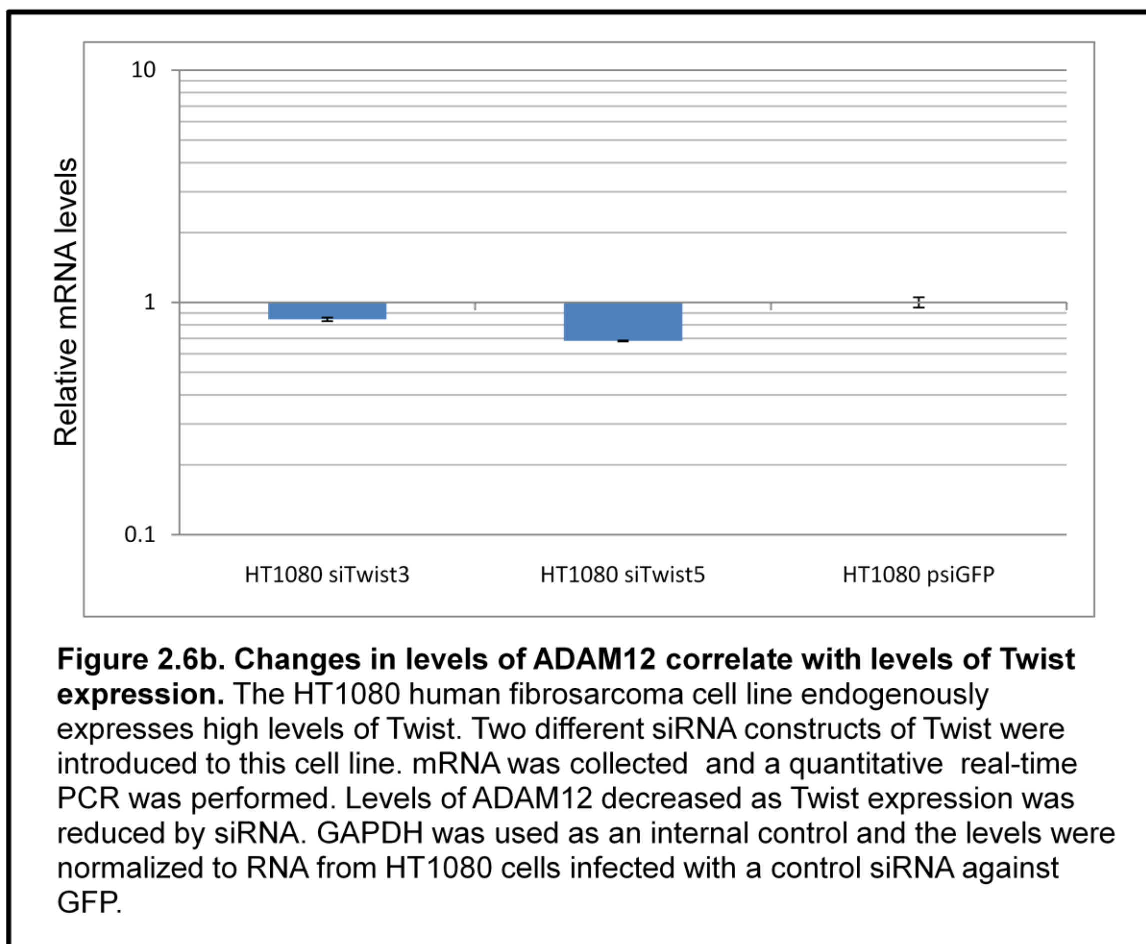


Figure 2.6b. Changes in levels of ADAM12 correlate with levels of Twist expression. The HT1080 human fibrosarcoma cell line endogenously expresses high levels of Twist. Two different siRNA constructs of Twist were introduced to this cell line. mRNA was collected and a quantitative real-time PCR was performed. Levels of ADAM12 decreased as Twist expression was reduced by siRNA. GAPDH was used as an internal control and the levels were normalized to RNA from HT1080 cells infected with a control siRNA against GFP.

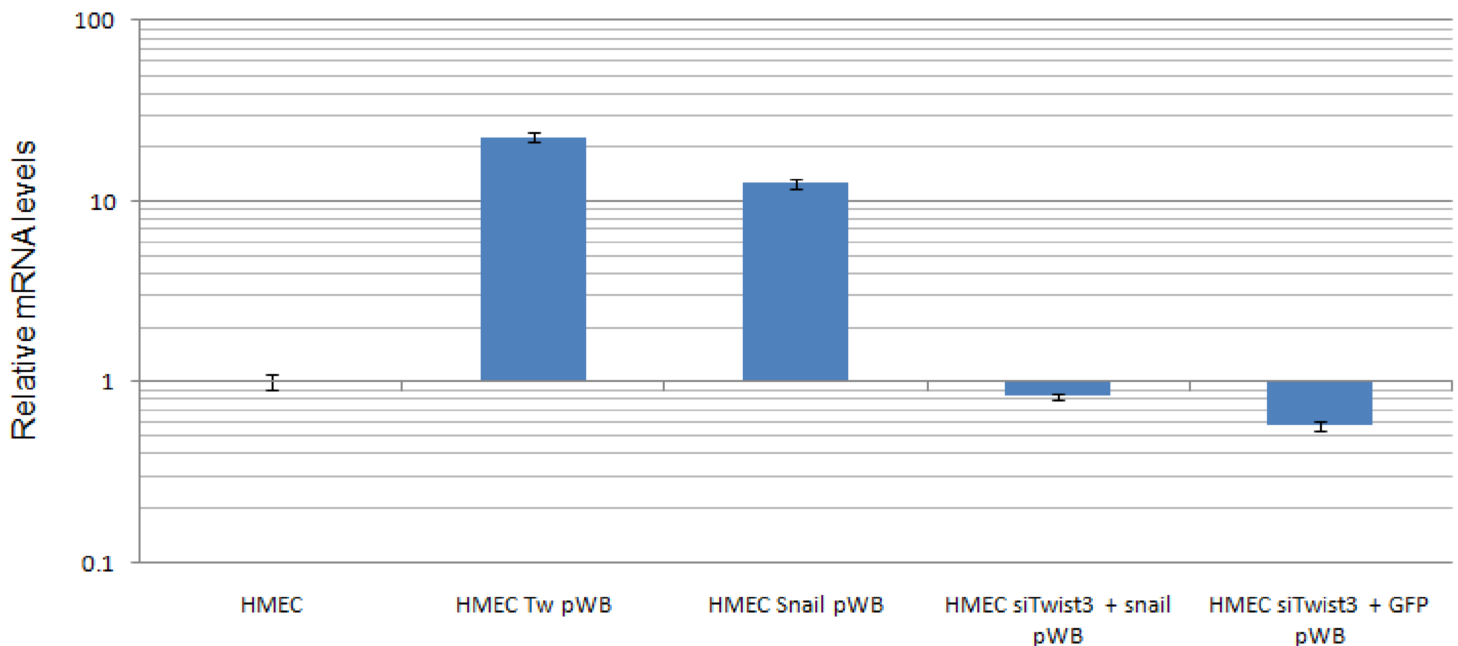


Figure 2.7a. Changes in levels of FAPa are specific to Twist expression All samples were compared to HMEC only cells which express low endogenous levels of FAPa. HMEC Twist cells were able to highly induce expression of FAPa. Snail is known to induce Twist and accordingly Snail alone was able to induce some expression of FAPa. However when Twist levels were reduced via siRNA, Snail alone was no longer able to induce expression of FAPa.

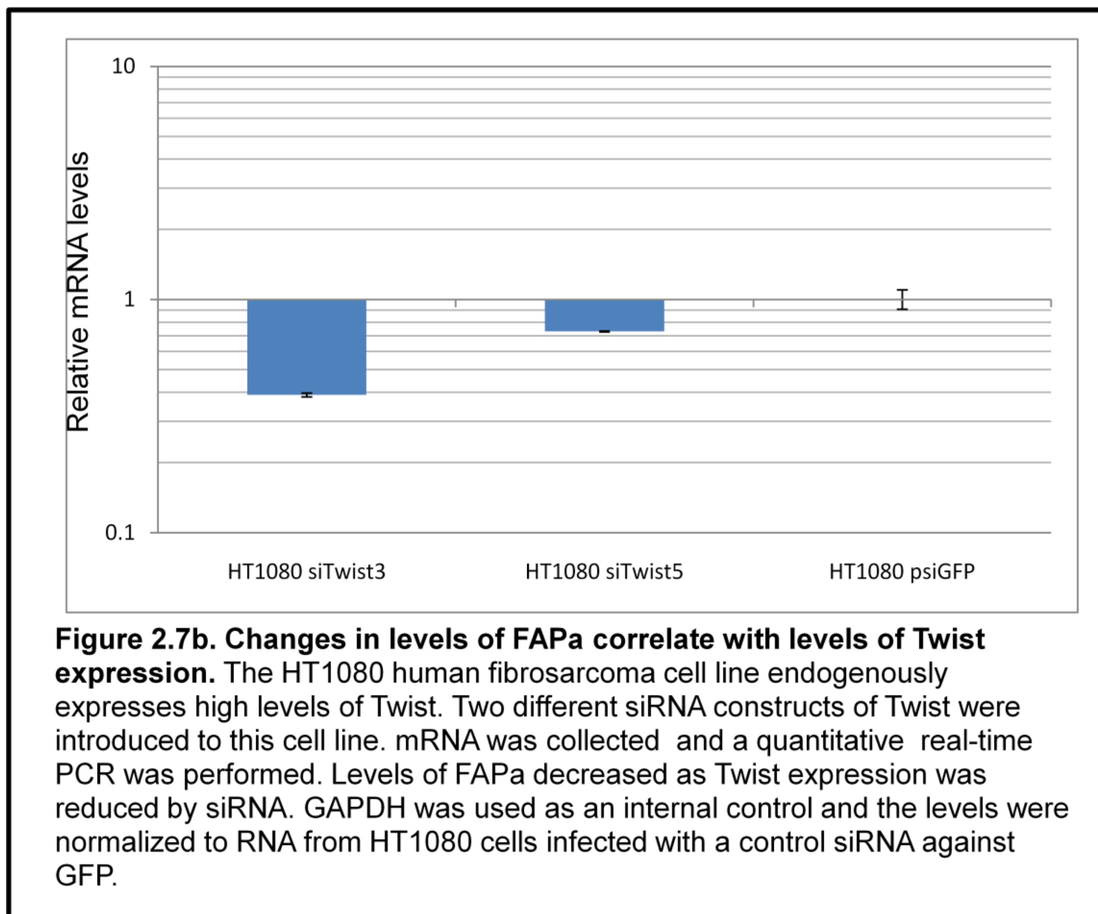


Figure 2.7b. Changes in levels of FAPa correlate with levels of Twist expression. The HT1080 human fibrosarcoma cell line endogenously expresses high levels of Twist. Two different siRNA constructs of Twist were introduced to this cell line. mRNA was collected and a quantitative real-time PCR was performed. Levels of FAPa decreased as Twist expression was reduced by siRNA. GAPDH was used as an internal control and the levels were normalized to RNA from HT1080 cells infected with a control siRNA against GFP.

reduced via shRNAs in HMEC-Snail cells, levels of FAPa were decreased. When levels of FAPa were examined in HT1080 cells, reduction of Twist expression also showed decreases in FAPa expression (Figure 2.7b). Furthermore, the degree of changes in FAPa expression corresponds directly to the degree of changes in Twist expression. siTwist3 was shown to be more effective at reducing Twist expression than siTwist5 (Figure 2.2b). Accordingly, siTwist3 was able to decrease FAPa levels more than siTwist5. These results strongly suggest that Twist regulates the expression of FAPa.

When Twist was introduced into HMEC cells, levels of DPPIV increased about 10 fold (Figure 2.8a). When Snail was introduced into HMEC cells, DPPIV became upregulated. However, unlike Adam12 and FAPa, when levels of Twist were reduced via shRNA in HMEC- Snail cells, levels of DPPIV increased. This result is unexpected and needs further analysis to confirm its accuracy.When levels of DPPIV were examined in HT1080 cells, reduction of Twist expression showed drastic decreases in DPPIV expression (Figure 2.8b).The degree of changes in DDPIV expression corresponded directly to the degree of changes in Twist expression. siTwist3 was shown to be more effective at reducing Twist expression than siTwist5 and accordingly, siTwist3 showed lower levels of DPPIV than siTwist5.

The relative mRNA levels of Tks5 and MT1-MMP, two known key components of invadopodia were also examined. These components did not appear to be regulated by Twist expression in the microarray. Expression of Twist in HMEC cells did not significantly induce expression of Tks5 by quantative

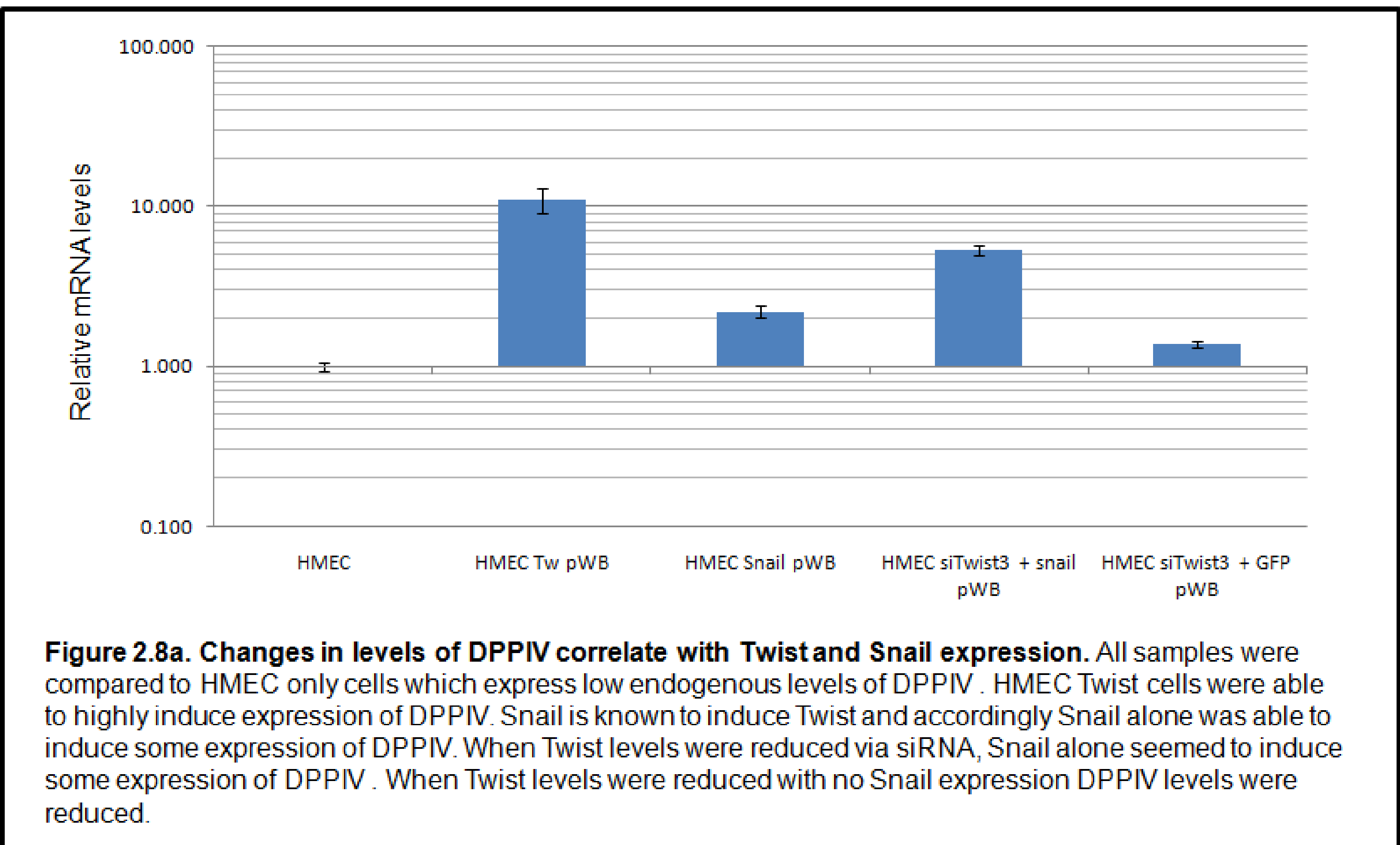


Figure 2.8a. Changes in levels of DPPIV correlate with Twist and Snail expression. All samples were compared to HMEC only cells which express low endogenous levels of DPPIV . HMEC Twist cells were able to highly induce expression of DPPIV. Snail is known to induce Twist and accordingly Snail alone was able to induce some expression of DPPIV. When Twist levels were reduced via siRNA, Snail alone seemed to induce some expression of DPPIV . When Twist levels were reduced with no Snail expression DPPIV levels were reduced.

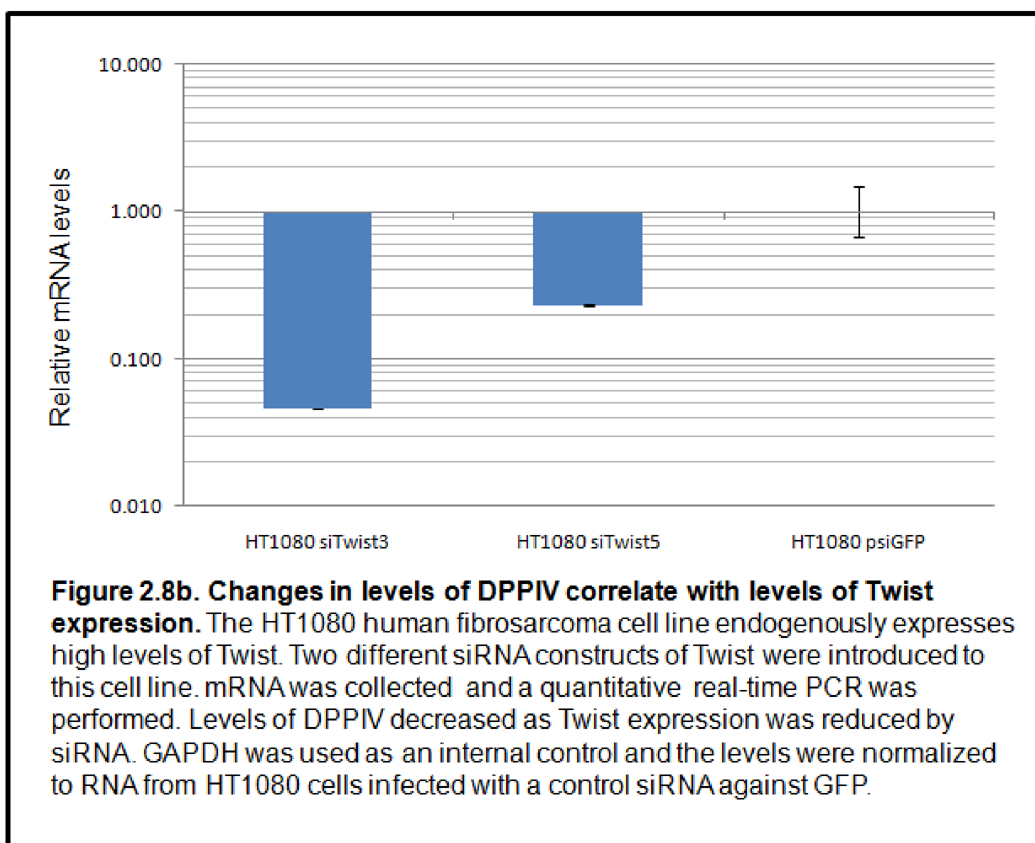


Figure 2.8b. Changes in levels of DPPIV correlate with levels of Twist expression. The HT1080 human fibrosarcoma cell line endogenously expresses high levels of Twist. Two different siRNA constructs of Twist were introduced to this cell line. mRNA was collected and a quantitative real-time PCR was performed. Levels of DPPIV decreased as Twist expression was reduced by siRNA. GAPDH was used as an internal control and the levels were normalized to RNA from HT1080 cells infected with a control siRNA against GFP.

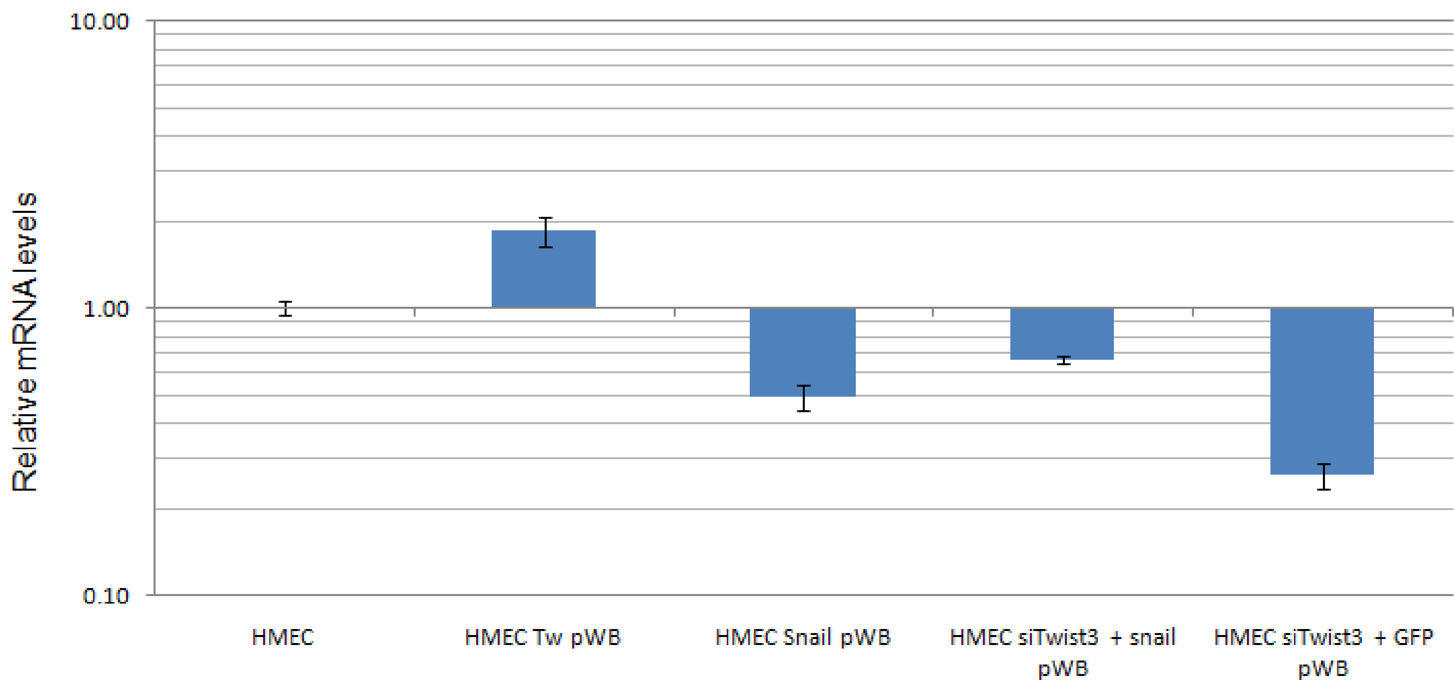


Figure 2.9. Twist does not regulate levels of Tks5. All samples were compared to HMEC only cells which express low endogenous levels of Tks5. HMEC Twist expression does not show significant induction of Tks5. Snail is known to induce Twist but Snail expression was unable to induce expression of Tks5. When Twist levels were reduced via siRNA, Snail alone was also unable to induce expression of DPPIV.

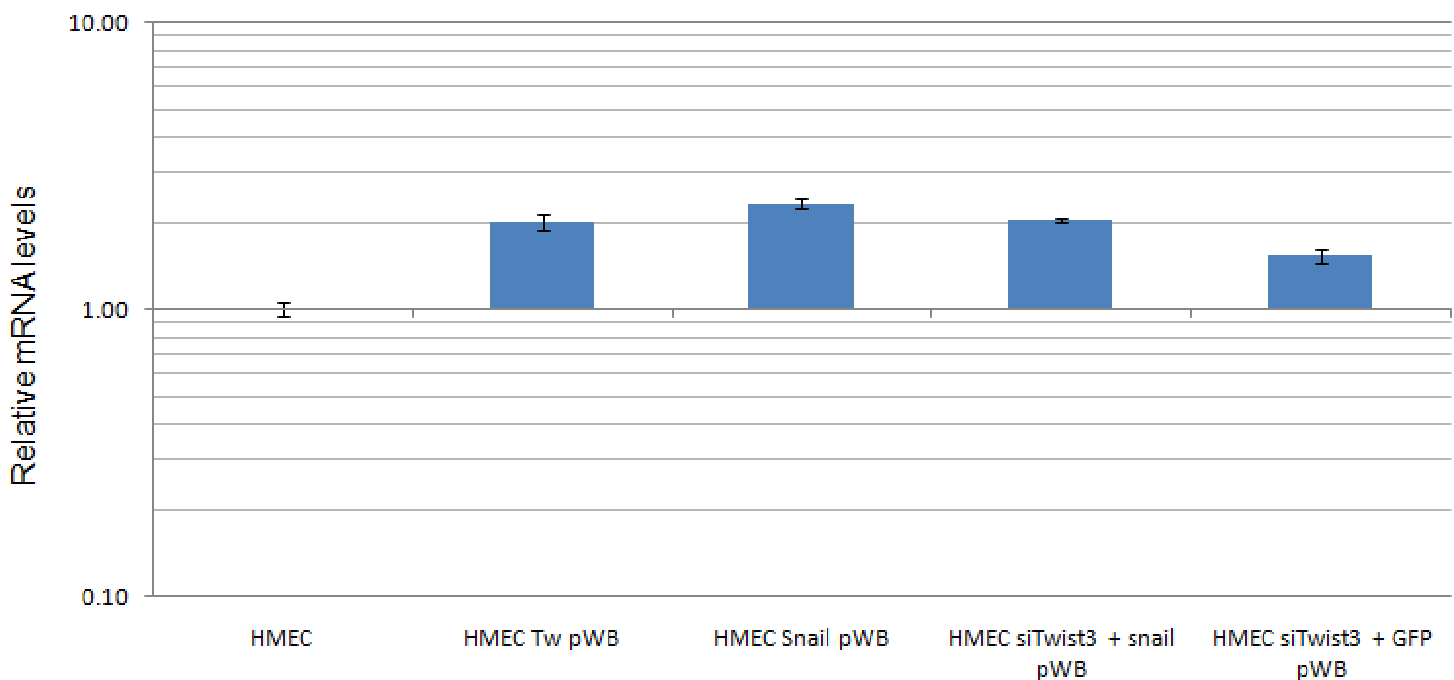


Figure 2.10. Twist does not regulate levels of MT1-MMP. All samples were compared to HMEC only cells which express low endogenous levels of MT1-MMP. HMEC Twist expression does not show significant induction of MT1-MMP. Snail is known to induce Twist and Snail expression was alsp able to induce a small increase in MT1-MMP levels. When Twist levels were reduced via siRNA, there was no corresponding effect to the levels of expressed MT1-MMP.

RT-PCR (Figure 2.9). The expression of Snail actually seemed to decrease the level of Tks5. Examining the levels of MT1-MMP also showed a similar case (Figure 2.10). Twist and Snail expression did not increase its expression significantly. Together, these data suggest that Twist specifically regulates the expression of three membrane-associated proteases associated with invadopodia, Adam12, FAPa and DPP4; in contrast, Twist does not regulate either Tks5 or MT1-MMP, two other components of the invadopodia.

Discussion:

Results from this chapter demonstrate that Twist activation upregulates the expression of three proteases and that this upregulation of proteases is specific to Twist and not Snail, aother inducer of EMT. Levels of ADAM12, FAPa and DPPIV were highly upregulated upon the activation of Twist by 4-OHT in HMEC Twist ER cells. The increase in expression of these proteases happened relatively quickly- within one day. Levels of ADAM12 and DPPIV were increased almost 100 fold by the end of induction and levels of FAPa were increased more than 10 fold by the end of induction.

The upregulation of these proteases was examined to be specific for Twist activity since Twist was able to induce an increase in expression levels of ADAM12, FAPa and DPPIV in HMEC cells. Twist expression was able to induce high levels of ADAM12 (near 100 fold) and FAPa (above 10 fold) expression levels that were comparable to the end point levels of ADAM12 (near 100 fold) and FAPa (above 10 fold) at the end of TwistER induction. Twist expression was not shown to be able to induce comparably high levels of DPPIV. DPPIV levels were induced only about 10 fold in these HMEC Twist cells whereas the HMEC Twist ER cells at the end of induction had DPPIV levels that were increased almost 100 fold.

Snail, another transcription factor in the EMT process was also able to induce an increase in expression levels of ADMA12, FAPa and DPPIV. But this effect is due to the ability of Snail to induce Twist expression in HMEC cells.

When Twist levels were ablated by shRNA in HMEC-Snail cells, the levels of ADAM12 and FAPa decrease drastically. The decreases in levels of ADAM12 and FAPa in HT1080 cells upon suppression of Twist expression also support this conclusion. The expression of ADAM12 and FAPa seem to be specific to levels of Twist being expressed.

The decrease in levels of DPPIV protease in HMEC-Snail cells with shRNA against Twist was not observed. For that sample, the levels of DPPIV surprisingly increased when Twist levels were decreased by shRNA. The changes in levels of DPPIV in HT1080 cells under RNAi of Twist do however support the idea of a Twist dependent induction of DPPIV. The degree of DPPIV reduction corresponds to the degree of Twist reduction with the two different siRNA constructs against Twist. Given the conflicting results, further repeating of the experiments are needed to clarify the link between Twist and DPP4.

After ensuring that the upregulation of these proteases is a true effect and is specific to Twist expression, it is important to identify the exact functionality of the proteases in Twist induced invasion and invadopodia formation. ADAM12, FAPa, and DPPIV are proteases with a wide range of other characteristics. It is critical to determine whether their roles are only proteolytic or whether they can also play a functional role in promoting the formation of the invadopodial structure.

Chapter 3

What is the functionality of ADAM12 in Twist induced invasion?

Microarray data in our lab has found that the three proteases are upregulated downstream of Twist expression as epithelial HMEC cells change morphology into mesenchymal cells. In Chapter 2, we confirm the microarray data and show that the upregulation of these proteases is a Twist specific activity.

Out of the three proteases, ADAM12 was chosen for further functional study. Levels of ADAM12 were consistently induced near 100 fold in both HMEC Twist ER and HMEC Twist expression cell lines. High levels of ADAM12 have been found to be present in independent microarray expression profiles when Twist is found to be upregulated. This consistent co-upregulation of ADAM12 by Twist makes it an appealing target for functional study.

ADAM12 is a member of the ADAMs family, a group of metalloproteinases that consist of multiple domains: a pro-domain, a metalloprotease domain, a disintegrin domain, a cysteine rich EGF-like domain, a transmembrane domain and a cytoplasmic tail (Seals, and Courtneidge, 2003). The pro-domain keeps the metalloprotease site inactive until activated by a cysteine switch. It also functions in the proper folding and transport of ADAM12. The metalloprotease domain allows proteolytic activity with zinc at the catalytic site. The disintegrin domain functions in regulation of cell adhesion and migration processes. There is not much known yet about the EGF like domain. The transmembrane tethers the long, membrane bound form of ADAM12 to the membrane. The cytoplasmic tail is responsible for inside out and outside in signaling as well as cellular

localization of ADAM12, though it is not known how Adam12 signals (Reiss, and Saftig, 2009).

Because of its multiple domains, ADAM12 can function in proteolysis, cellular interactions, signal transduction, and cytoskeletal organization. It can degrade gelatin, type IV collagen, and fibronectin, but not type I collagen or casein (Roy, Wewer, et al, 2004). It can act as a sheddase by cleaving ecto-domains of membrane bound growth factor receptor ligands like IGF, EGF, and Delta for growth factor signaling downstream. ADAM12 can bind α-actinin1, which can cause actin filament bundling into stress fibers connected to the cell membrane (Cao, Kang, and Zolkiewska, 2001). Its binding to beta1 and beta3 integrins cause the formation of focal adhesions (Thodeti, Frohlich, et al, 2005). The ability of ADAM12 to cause proteolysis of ECM molecules, shed growth factor ligands, and alter the actin cytoskeleton can have implications in cancer invasion and invadopodia formation.

Among the members of the ADAMs family, ADAM12 is the most strongly correlated with cancer and implicated in malignancy. In human, the main source of ADAM12 seems to be from the tumor cells themselves and not the stroma. ADAM12 overexpression has been correlated with breast, bladder, and gastric cancers as well as glioblastomas (Carl-McGrath, Lendeckel, et al, 2005; Frohlich, Albrechtsen, et al, 2006; Kodama, Ikeda, et al, 2004; Kveiborg, Frohlich, et al, 2005; Roy, Wewer, et al, 2004). Its expression in liver cancers is associated with matrix remodeling (Le Pabic, Bonnier, et al, 2003).

Tks5 and ADAM12 are shown to be localized at invadopodial structures in

Src transformed fibroblasts (Abram, Seals, et al, 2003b). Recently, it was shown that the adaptor protein Tks5/FISH played a critical role in the formation of invadopodia and matrix degradation in human breast carcinoma cells (Seals, Azucena, et al, 2005). The specific role of ADAM12 at these invadopodial complexes has not yet been reported.

Knowing that Twist can induce formation of invadopodia in human mammary epithelial cells and Twist can specifically induce upregulation of ADAM12 expression, it is necessary to test the role of ADAM12 in matrix degradation and invadopodia formation/function.

To assess the role of ADAM12 in invasion (and invadopodia), different shRNA constructs against ADAM12 were generated. The shRNA constructs were stably infected into HMEC Twist expressing cells. If the expression of ADAM12 is necessary for local matrix degradation, the average area of degradation in ADAM12 knockdown cells should decreased compared to control samples.

ADAM12 has multiple functions: proteolysis, cellular interactions, signal transduction, and cytoskeletal organization. To examine whether ADAM12 is necessary for invadopodia formation), Invadopodial structure was examined by immunofluorescent staining for F-actin, cortactin, Tks5 and phospho-Tyr.

Results

To examine the effect of shRNA against ADAM12 in HMEC Twist expressing cells, a quantitative real-time PCR was performed on the samples. The relative mRNA levels are shown in Figure 3.1. All samples were compared to HMEC cells that express low endogenous levels of ADAM12. All four shRNAs show more than 70% knock-down in HMEC-Twist cells, and ADAM12 sh2 and ADAM12 sh5 were chosen for their ability to cause a change in morphology in HMEC Twist cells (Figure 3.2a). The ADAM12 knock down cells did not change the Twist expression levels in HMEC Twist cells. (Figure 3.2b), therefore the phenotype we observed is due to the loss of Adam12 expression.

HMEC Twist cells normally show an elongated spindle shaped morphology. The ADAM12 knock down cells no longer showed that elongated spindle shape. They were more cuboidal than the HMEC Twist cells, but did not show the distinct cobble-stone epithelial morphology that is characteristic of HMEC cells either. This change in cell morphology occurred relatively rapidly. After complete rounds of stable lentiviral infection, the successful infected cells were selected with puromycin. Within two days of puromycin selection, ADAM12 sh2 and ADAM12 sh5 showed this distinct change in morphology, while the control knockdown cells remained unaffected.

ADAM12 expression is necessary for local matrix degradation

After incubating the ADAM12 knockdown cells on FITC gelatin coated

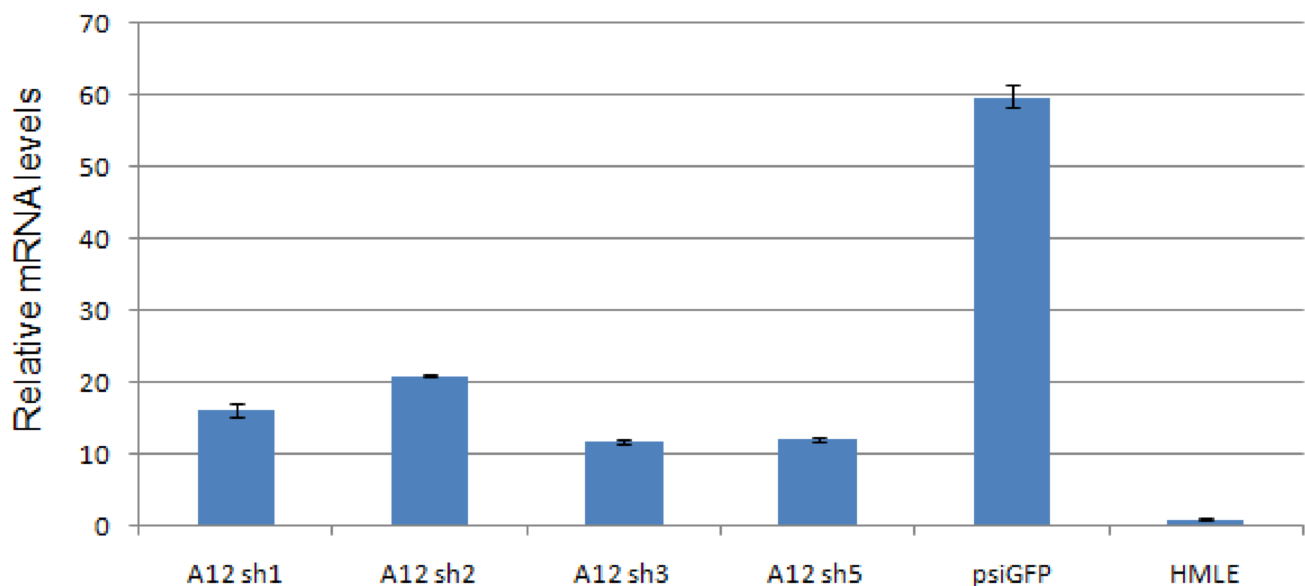
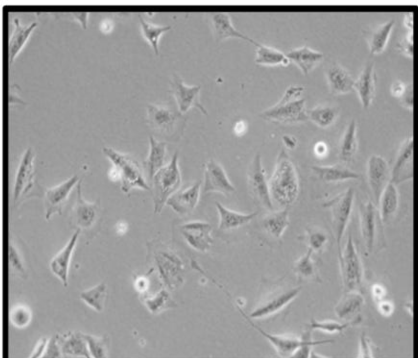
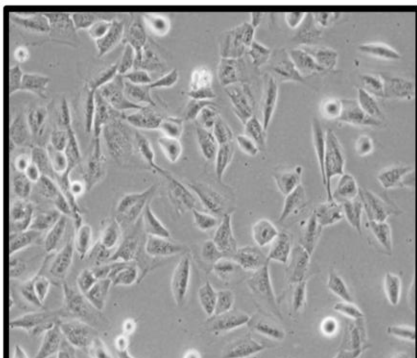


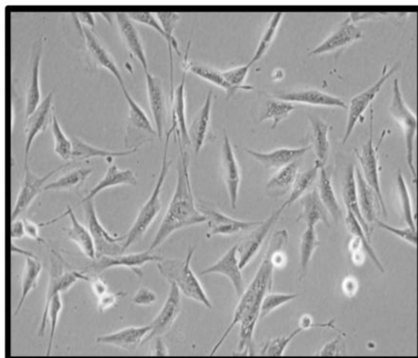
Figure 3.1. Confirmation of ADAM12 RNAi. Relative mRNA levels of ADAM12 in ADAM12 knock down in HMLE Twist cells. All samples were compared to HMEC only cells which express low endogenous levels of ADAM12. ADAM12 sh2 and sh5 demonstrated the most striking changes in cell morphology following table infection and selection of the cells and the those two samples were chosen for further study.



ADAM12 sh2



ADAM12 sh5



shGFP control

Figure 3.2a. ADAM12 knock down cells show a striking change in morphology. siRNA constructs of ADAM12 were introduce to HMEC Twist expressing cell lines. Within two days after infection and selection with puromycin, the knock down cell lines showed a change in morphology.

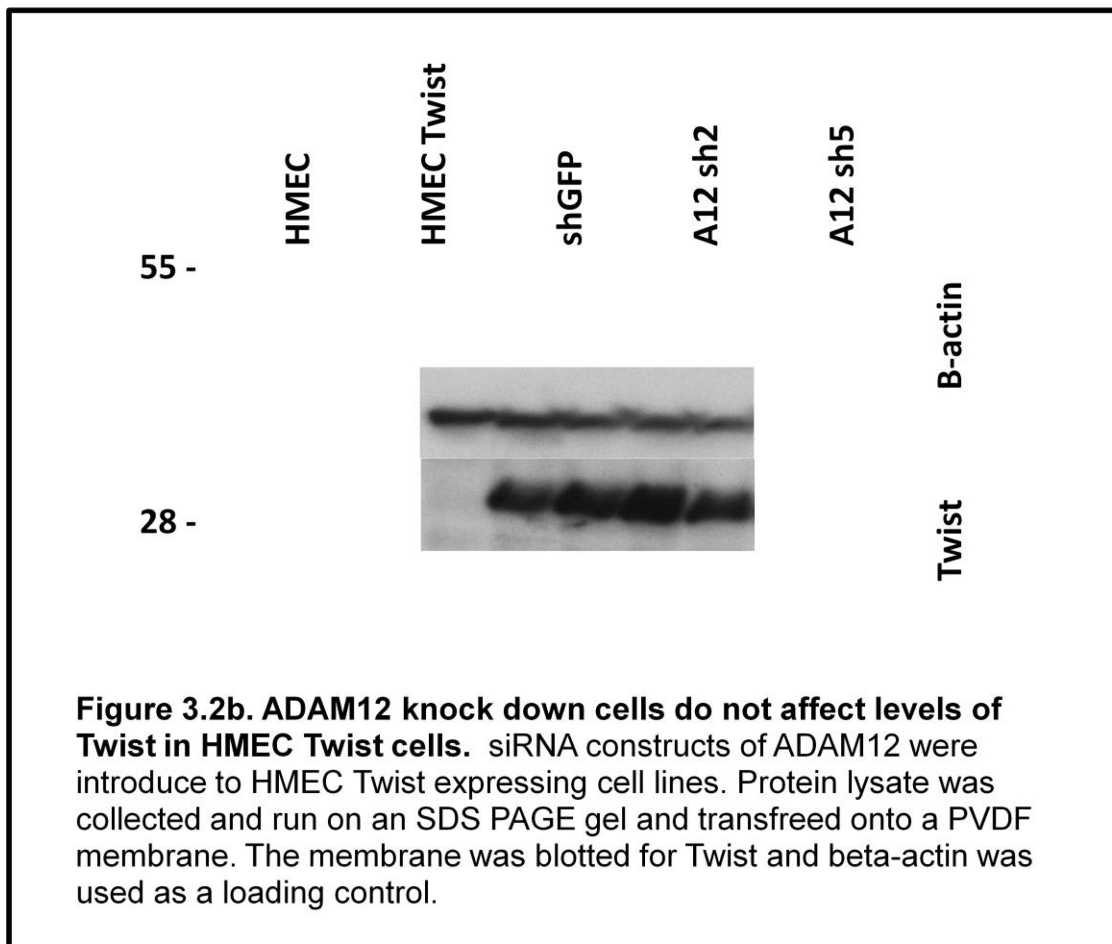


Figure 3.2b. ADAM12 knock down cells do not affect levels of Twist in HMEC Twist cells. siRNA constructs of ADAM12 were introduce to HMEC Twist expressing cell lines. Protein lysate was collected and run on an SDS PAGE gel and transfreed onto a PVDF membrane. The membrane was blotted for Twist and beta-actin was used as a loading control.

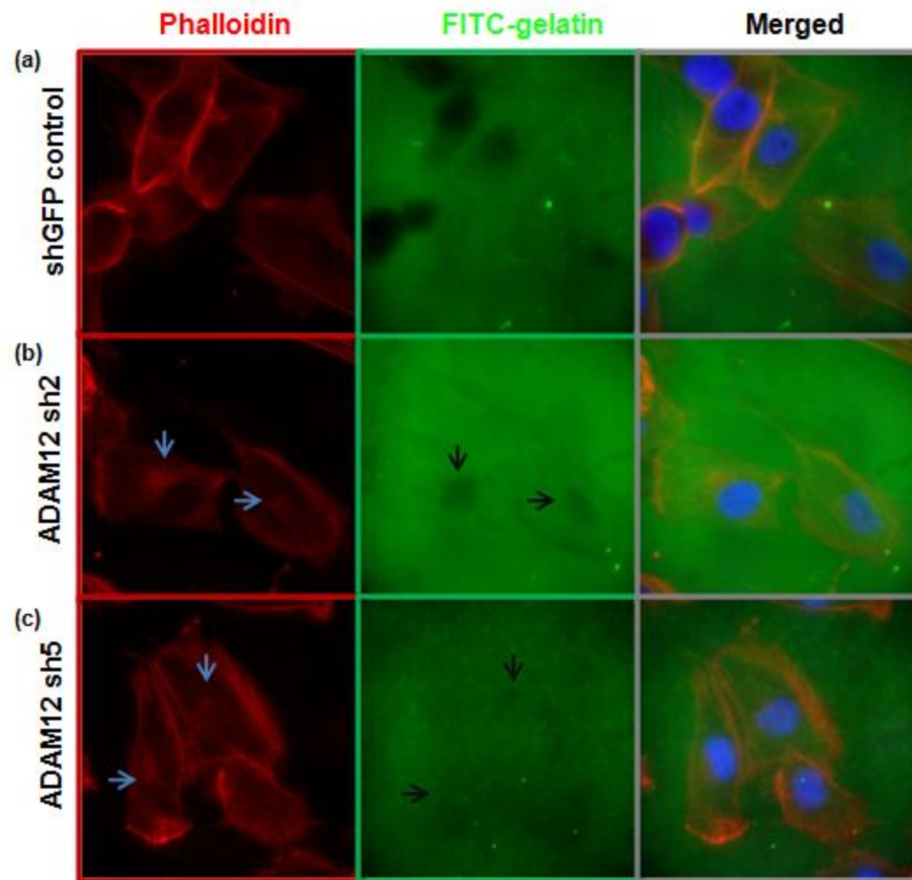
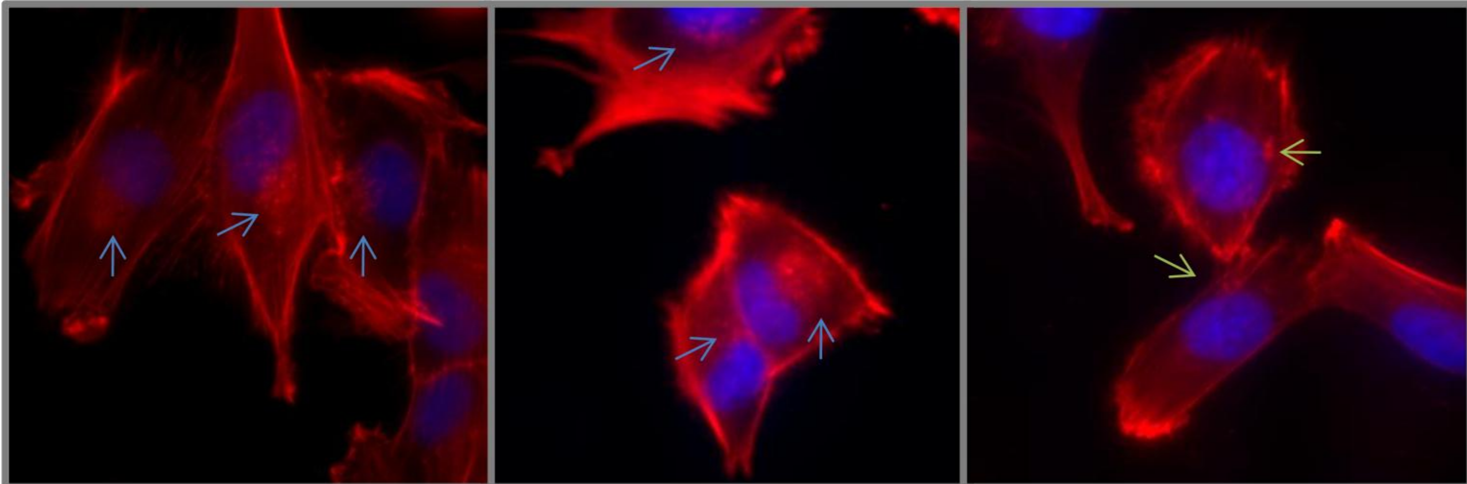


Figure 3.3 (a) (b) (c). ADAM12 expression is necessary for local matrix degradation. FITC gelatin degradation assay with HMEC Twist cells infected with shRNA against ADAM12 or control shRNA against GFP. Cells were incubated on FITC gelatin coverslips for 8 hours. ADAM12 knockdown cell lines had decreased amounts of degradation. Phalloidin showed enlarged F-actin aggregates near the nuclei that seem to co-localize with minimal areas of degradation (arrowheads).



ADAM12 sh5 ADAM12 sh2 shGFP control

Figure 3.3 (d). Loss of ADAM12 expression causes formation of large F-actin aggregates. F-actin was stained with Phalloidin (red) and nuclei were staied with DAPI (blue). The Phalloidin staining showed enlarged F-actin aggregates next to the nuclei of the A12 knockdown cells (blue arrowheads). These enlarged F-actin aggregates are not seen in shGFP control cells that show small and distinct F-actin puncta (green arrowheads).

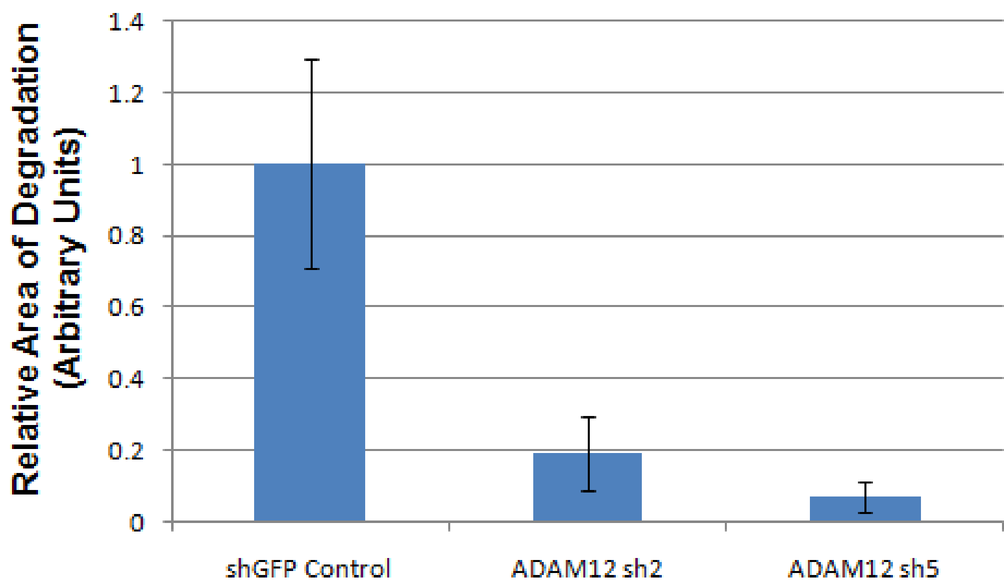


Figure 3.4. ADAM12 expression is necessary for local matrix degradation. Quantification of the FITC gelatin degradation assay. Total area of degradation in each field was measured and normalized by the number of nuclei in each field to give the relative degradation values. The average area of degradation for ten fields are represented for each sample.

coverslips for 8 hours, the control knockdown cells showed extensive areas of degradation underneath the cells (Figure 3.3a). The ADAM12 knockdown cells, on the other hand, showed decreased amounts of gelatin degradation (Figure 3.3b and Figure 3.3c). Furthermore, Phalloidin staining showed enlarged F-actin aggregates near the nuclei in ADAM12 sh2 and ADAM12 sh5 cells (Figure 3.3d).

Quantification of the areas of degradation showed that the average area of degradation by ADAM12 sh2 cells was 5 fold less than the control knock down cells (Figure 3.4). The average area of degradation by ADAM12 sh5 cells was 10 fold less than the control knockdown cells. The values for average area of degradation are compared to the values for the knock down cells which is set as 1. This data indicates that ADAM12 expression is necessary for Twist-induced local matrix degradation.

Proteolytic activity of metalloproteases is necessary for local matrix degradation

After incubating the HMEC Twist cells with the metalloprotease inhibitor GM6001 for 8 hours on FITC gelatin coated coverslips, the control HMEC Twist cells showed extensive areas of degradation underneath the cells (Figure 3.5a). The GM6001 treated cells on the other hand showed decreased amounts of gelatin degradation (Figure 3.5b).

Quantification of the areas of degradation showed that the average area of degradation by GM6001 treated cells was 10 fold less than the average area of degradation by HMEC Twist cells. This data suggest that despite the presence of many other proteases at invadopodia, the proteolytic activity of metalloprotease,

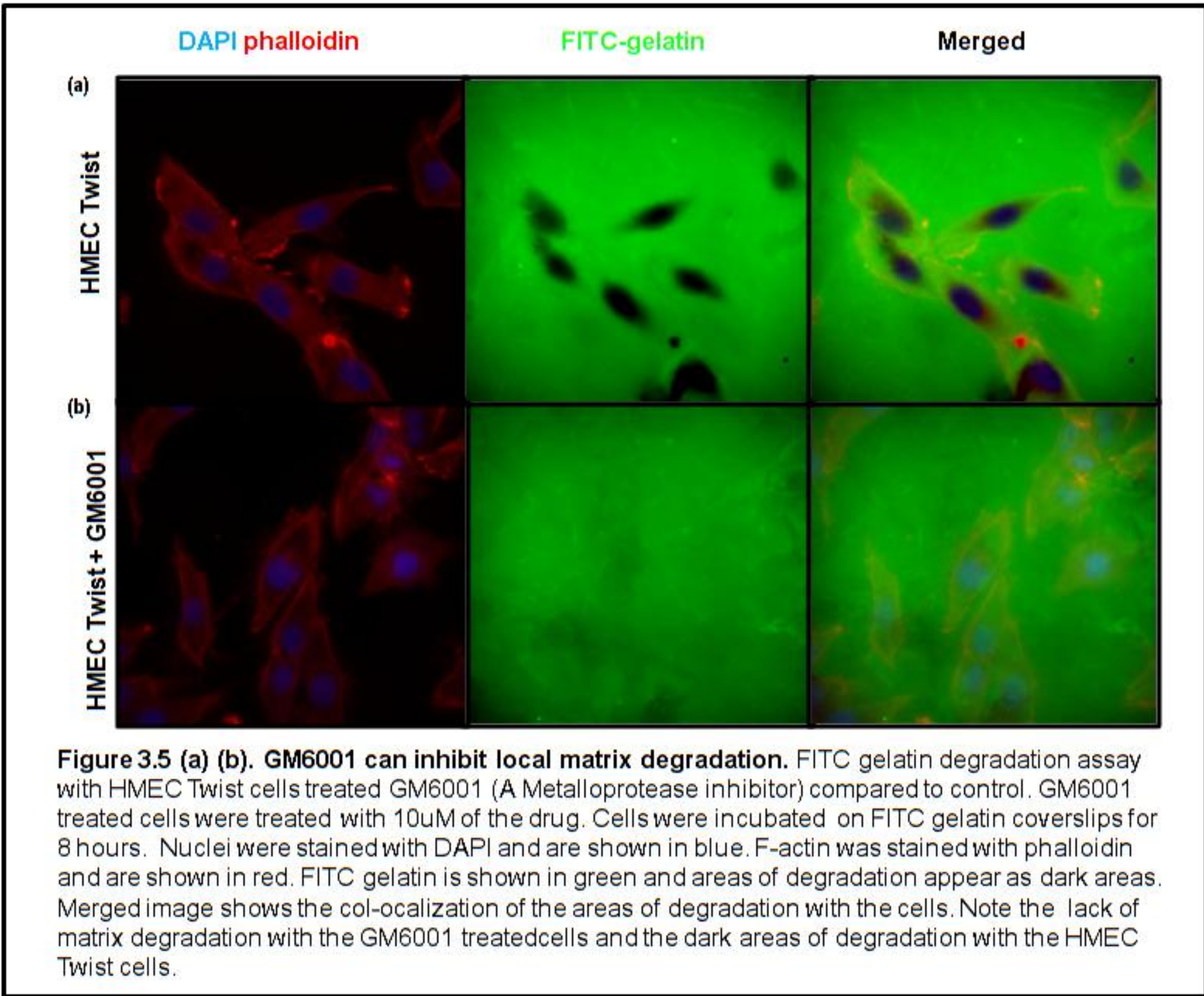


Figure 3.5 (a) (b). GM6001 can inhibit local matrix degradation. FITC gelatin degradation assay with HMEC Twist cells treated GM6001 (A Metalloprotease inhibitor) compared to control. GM6001 treated cells were treated with 10uM of the drug. Cells were incubated on FITC gelatin coverslips for 8 hours. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. FITC gelatin is shown in green and areas of degradation appear as dark areas. Merged image shows the col-ocalization of the areas of degradation with the cells. Note the lack of matrix degradation with the GM6001 treatedcells and the dark areas of degradation with the HMEC Twist cells.

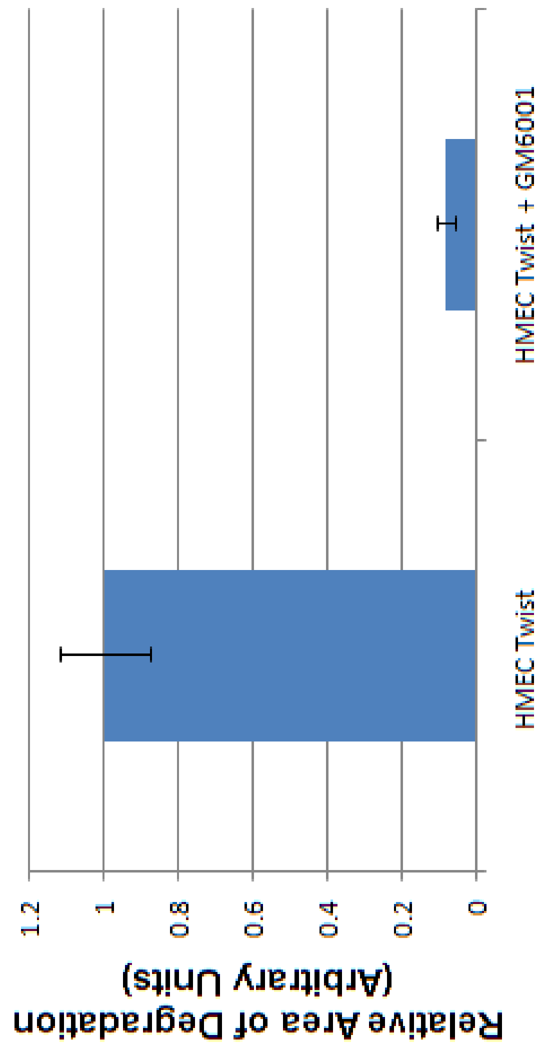


Figure 3.5 (c). GM6001 can inhibit local matrix degradation. Quantification of the FITC gelatin degradation assay. Total area of degradation in each field was measured and normalized by the number of nuclei in each field to give the relative degradation values. The average area of degradation for ten fields are represented for each sample.

including ADAM12, is necessary for proper matrix degradation. However, this

data cannot demonstrate the specific ADAM12 protease activity is required for proper matrix degradation due to the broad spectrum inhibitory effect of GM6001.

Expression of ADAM12 is necessary for proper invadopodia formation

To examine if ADAM12 is necessary for proper invadopodia formation, an immunofluorescence analysis was performed. Here Cortactin and F-actin co-localization as well as Tks5 and F-actin colocalization were used to visualize invadopodia.

After incubating HMEC Twist cells and ADAM12 sh5 in HMEC Twist cells on gelatin coated coverslips, the HMEC cells showed clear F-actin puncta that co-localize with cortactin aggregates (Figure 3.6). The ADAM12 knockdown cells on the other hand showed cortactin and F-actin colocalization, but clear puncta were not seen as shown in HMEC-Twist cells (Figure 3.7). HMEC Twist cells also showed clear Tks5 and F-actin puncta that colocalized (Figure 3.8). The ADAM12 knockdown cells had no punctuate Tks5 staining and no co-localization with F-actin was observed (Figure 3.9). These results indicate that ADAM12 is necessary for the proper formation of invadopodia in HMEC-Twist cells.

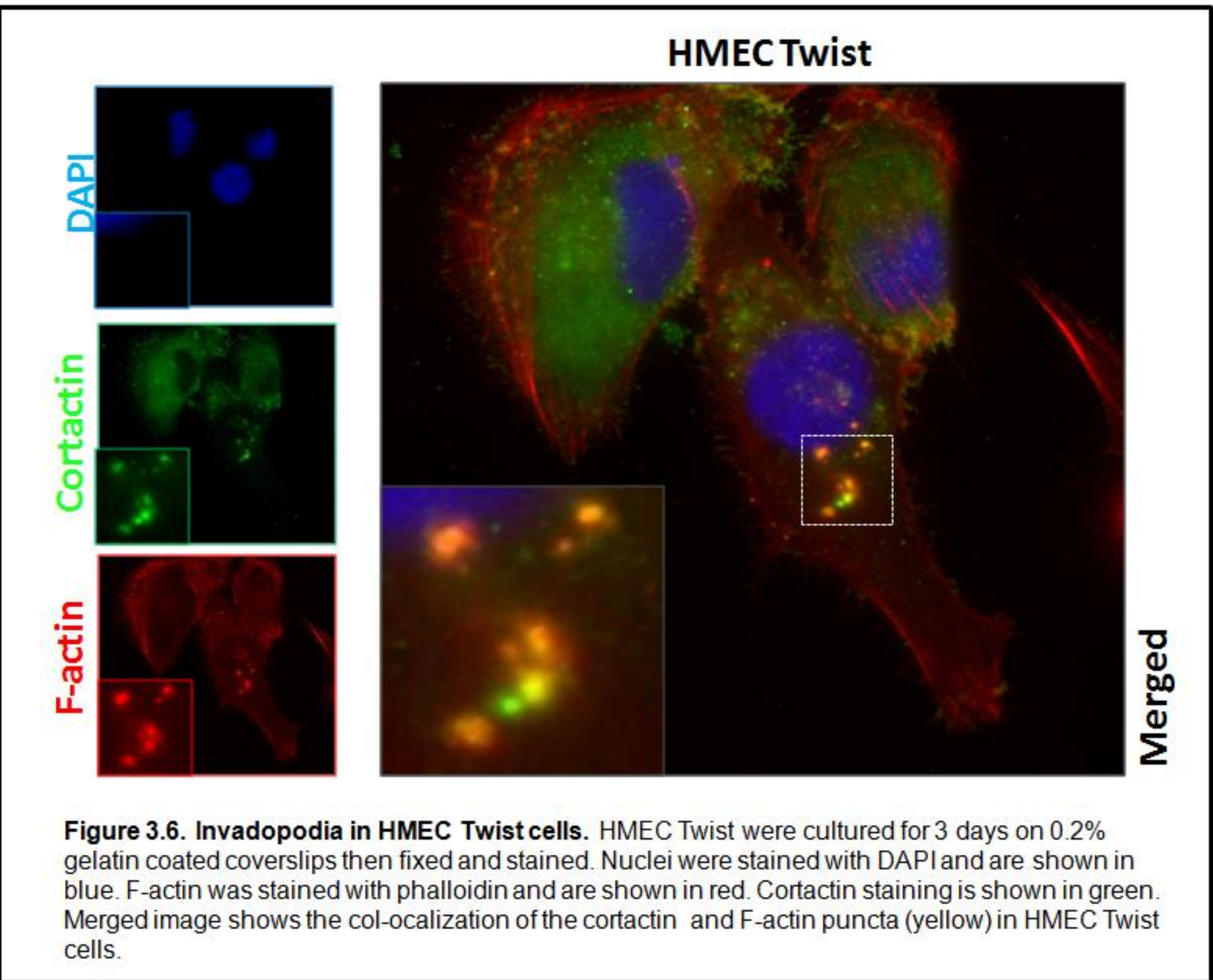


Figure 3.6. Invadopodia in HMEC Twist cells. HMEC Twist were cultured for 3 days on 0.2% gelatin coated coverslips then fixed and stained. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. Cortactin staining is shown in green. Merged image shows the col-ocalization of the cortactin and F-actin puncta (yellow) in HMEC Twist cells.

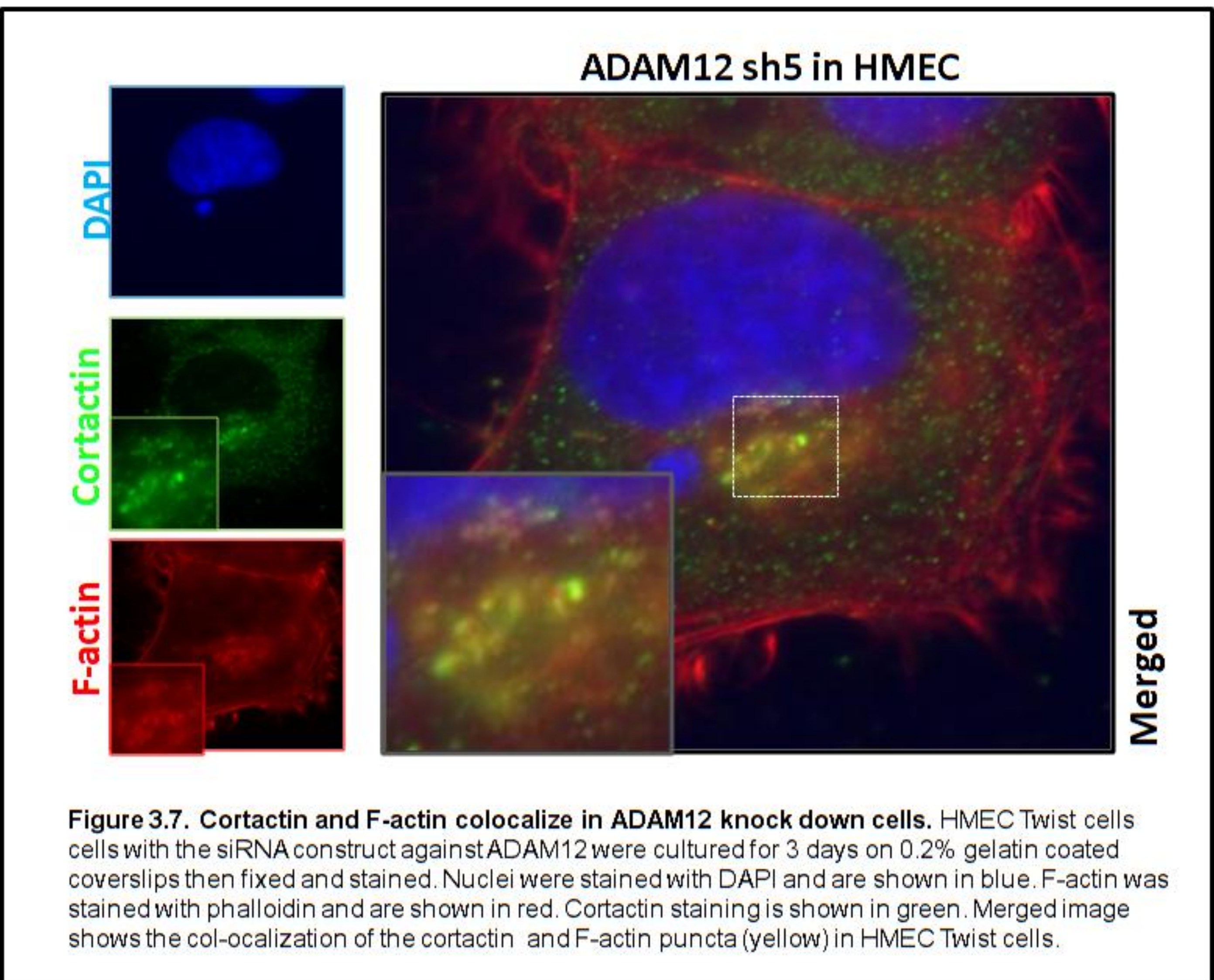


Figure 3.7. Cortactin and F-actin colocalize in ADAM12 knock down cells. HMEC Twist cells cells with the siRNA construct against ADAM12 were cultured for 3 days on 0.2% gelatin coated coverslips then fixed and stained. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. Cortactin staining is shown in green. Merged image shows the col-ocalization of the cortactin and F-actin puncta (yellow) in HMEC Twist cells.

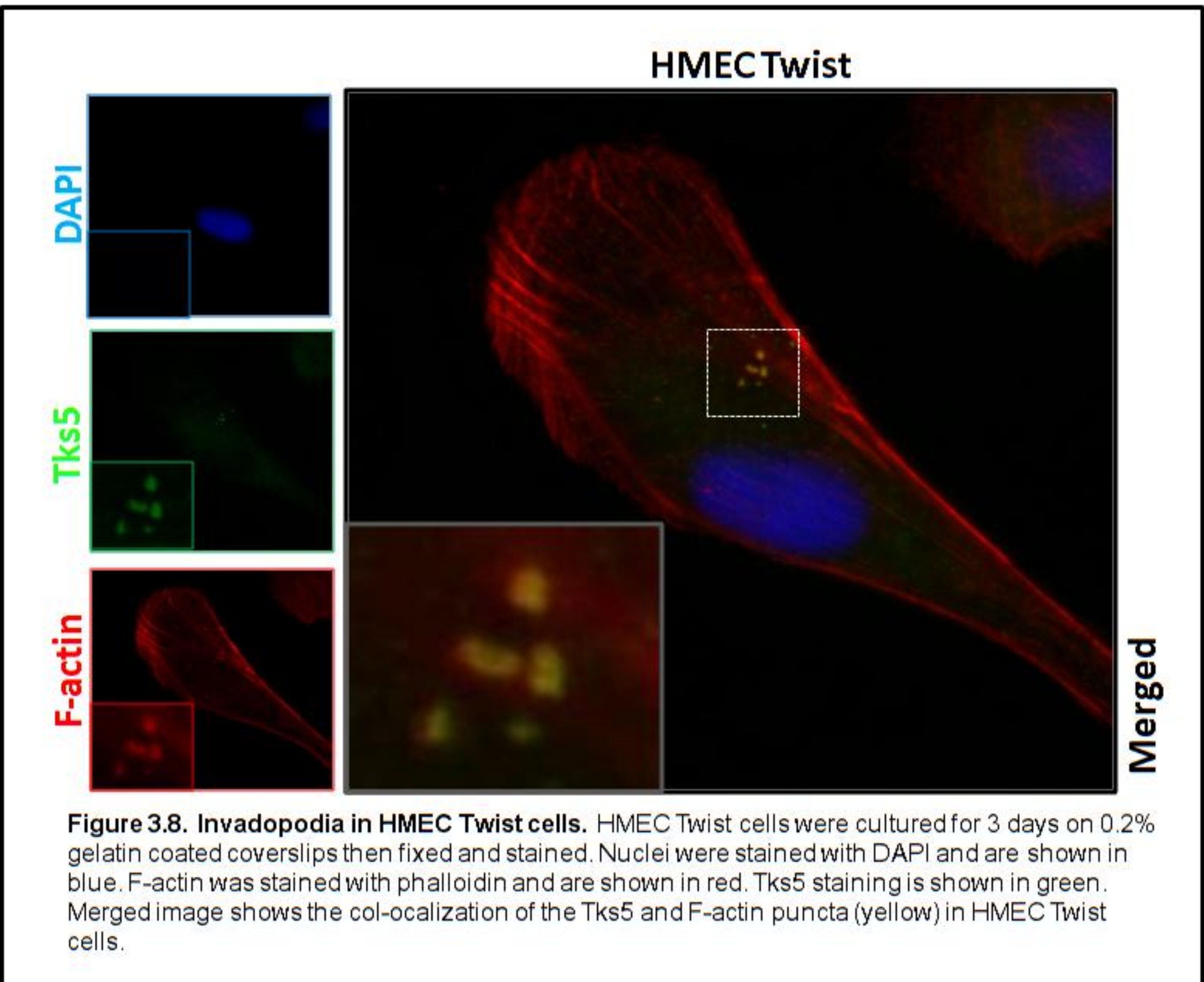


Figure 3.8. Invadopodia in HMEC Twist cells. HMEC Twist cells were cultured for 3 days on 0.2% gelatin coated coverslips then fixed and stained. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. Tks5 staining is shown in green. Merged image shows the col-ocalization of the Tks5 and F-actin puncta (yellow) in HMEC Twist cells.

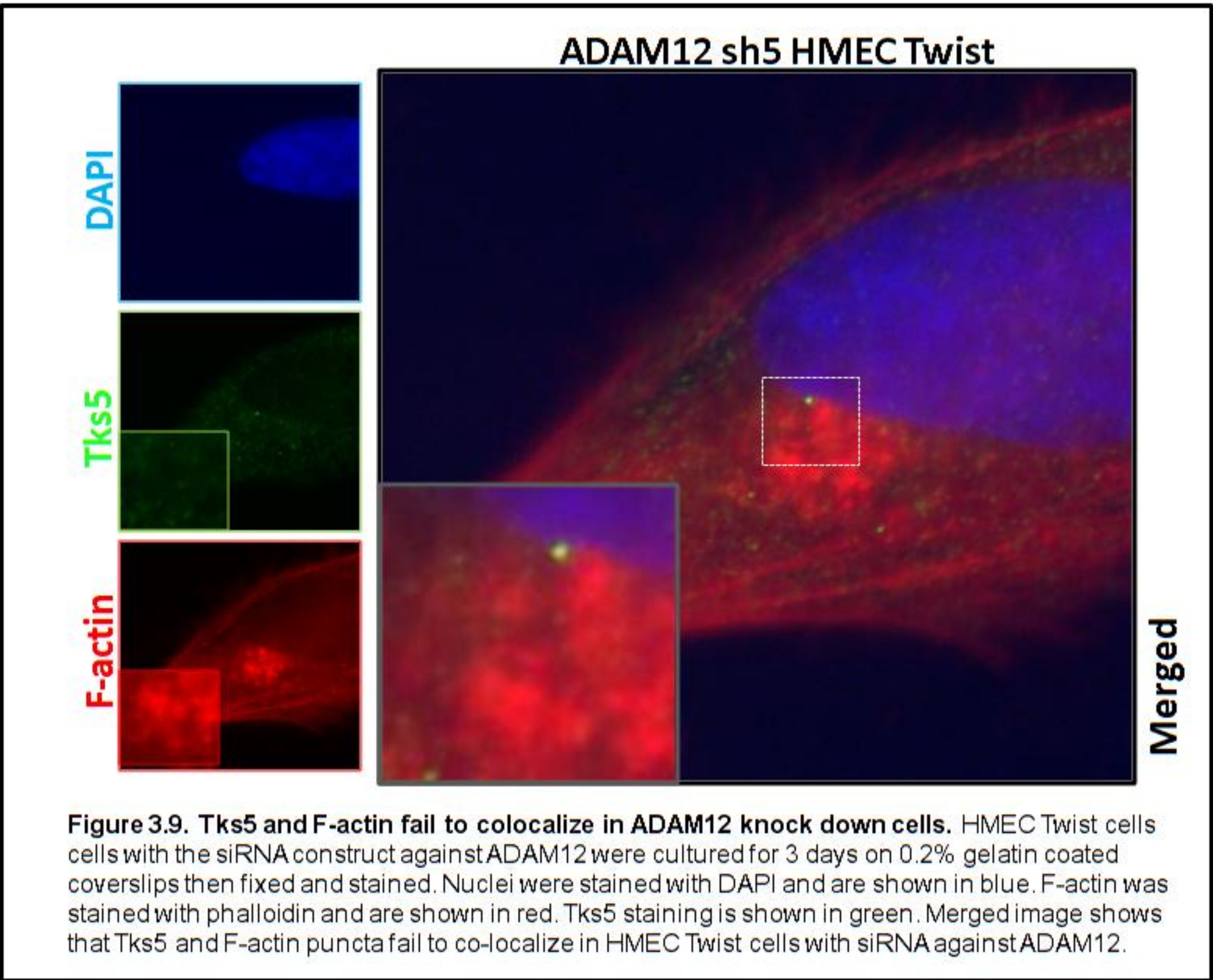


Figure 3.9. Tks5 and F-actin fail to colocalize in ADAM12 knock down cells. HMEC Twist cells cells with the siRNA construct against ADAM12 were cultured for 3 days on 0.2% gelatin coated coverslips then fixed and stained. Nuclei were stained with DAPI and are shown in blue. F-actin was stained with phalloidin and are shown in red. Tks5 staining is shown in green. Merged image shows that Tks5 and F-actin puncta fail to co-localize in HMEC Twist cells with siRNA against ADAM12.

Discussion

ADAM12 is a metalloprotease with multiple functions. To determine if its expression is necessary for local matrix degradation, shRNA constructs against ADAM12 were infected into HMEC Twist expressing cells. The shRNA against ADAM12 caused a dramatic change in HMEC Twist cell morphology which was not seen in the control cells. Given the known function of ADAM12 in actin filament remodeling, ablation of ADAM12 expression may cause changes in the actin cytoskeleton, causing the distinct change in cell morphology.

ADAM12 expression was shown to be necessary for local matrix degradation. shRNA against ADAM12 showed a reduction in the average areas of matrix degradation. This suggests that ADAM12 protease activity could be essential for matrix degradation, and/or that ADAM12 could also play a key role in the formation of functional invadopodia.

To determine whether the proteolytic activity of metalloproteases, including ADAM12, is required for matrix degradation, HMEC cells were treated with a metalloprotease inhibitor. Treatment with GM6001 reduced the average area of degradation by the HMEC Twist cells. This shows that the proteolytic activity of metalloproteases is necessary for proper matrix degradation. However GM6001 is a broad spectrum metalloprotease inhibitor that can inhibit the activity of other proteases that have been shown to be critical in invadopodia function like MT1-MMP and MM9 (Artym, Zhang, et al, 2006). To further confirm this data, HMEC Twist cells will have to be treated with an ADAM12 specific small molecule

inhibitor in the future.

An immunofluorescence analysis was performed to determine whether ablation of ADAM12 expression would affect proper assembly of invadopodial components. While cortactin and F-actin were still able to co-localize, F-actin and cortactin staining showed diffuse large aggregates instead of well defined puncta. Staining for Tks5, a scaffolding protein reported to play a critical role at invadopodia showed that ADM12 knockdown cells do not show punctuate Tks5 staining and does not co-localize with the large diffuse F-actin aggregates. This result indicates that ADAM12 affects the proper formation of punctuate F-actin and cortactin and plays a role in proper localization of Tks5 at invadopodia.

Conclusions & Perspectives

Twist is essential for mouse mammary carcinoma cells to metastasize from the mammary gland to the lung. One known cellular function of Twist in promoting tumor metastasis is to induce the epithelial-to-mesenchymal transition to allow the migration of carcinoma cells Between EMT and the formation of a secondary tumor, there are many other steps in the metastatic cascade that must occur. For tumor cells to intravasate into a nearby vessel for dissemination, they must first invade through the basement membrane and the surrounding extracellular matrix. It has not been shown that Twist plays a critical role in regulating cellular invasion of the basement membrane.

Our group demonstrated that Twist is necessary for matrix degradation and invadopodia formation in both murine and human mammary cell lines. Current studies in the field of invadopodia formation have focused on growth factor and kinase signaling, no transcriptional control of invadopodia has been reported. Our results further support the critical role of Twist in metastasis and specifically examines its role in invasion.

Twist is responsible for matrix degradation via invadopodia formation, but the mechanisms by which Twist can trigger invadopodia formation are still unclear. While research in the field of invadopodia has elucidated steps in invadopodia assembly, there is still little known about the signals that initiate the formation of the structure. We hypothesize that proteases may play a role in the initiation of invadopodia formation by releasing peptides as well as cleaving growth factors and cytokine precursors for downstream signaling.

Three proteases have been identified as being upregulated upon Twist activation. I found that these proteases are specifically upregulated by Twist and not other inducers of EMT like Snail transcription factor. Out of these three proteases, A Disintegrin and Metalloprotease family member ADAM12 was selected for further study. ADAM12 has been correlated with extensive ECM remodel and invasive cancers. Because of its multiple domains, it can function in proteolysis, cellular interactions, signal transduction, and cytoskeletal reorganization. It is important to determine the specific role it plays in promoting matrix degradation and formation of invadopodia.

shRNA against ADAM12 in HMEC-Twist cells showed a striking change in morphology that may come from its role in cytoskeletal reorganization, which will be investigated further. Ablation of its expression showed reduced levels of matrix degradation as well as failure of cortactin and Tks5 staining to co-localize with F-actin staining. Expression of Tks5 has been shown to be required for invadopodia formation in Src transformed cells. Our data suggests that ADAM12 plays multiple roles in local matrix degradation and invadopodia formation.

It remains to be definitively determined whether ADAM12 is indeed the initiating trigger in the formation of invadopodia. While the RNAi data seems promising, further studies need to be carried out in examining its spatial-temporal localization within the cell as invadopodia form. The treatment with GM6001 metalloprotease inhibitor indicates that metalloproteases are necessary for matrix degradation. However this is not a specific small molecule inhibitor for ADAM12, but a broad spectrum inhibitor of metalloproteases. A specific small molecule

inhibitor for ADAM12 will be needed to definitively show if the MMP activity of ADM12 is necessary for local matrix degradation.

While ADAM12 is an appealing target because of its reported associations in invasive cancers, both FAP and DPPIV have also been shown to be active in migration and invasion. It is important to determine the roles of FAP and DPPIV in matrix degradation and invadopodia formation in the future.

Materials & Methods

Cell lines and culture

Cell lines used: 4T1, HEK 293T , HMEC, HT1080

4T1 is a thioguanine resistant variant of cell line 410.4 that was isolated from a single spontaneously arising mammary tumor from a BALB/c mouse{{30 Aslakson,C.J. 1992}}. The 4T1 line spontaneously metastasizes to both the lung and liver, formed visible nodules in these organs. The 4T1 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 5% fetal calf serum, 5% newborn calf serum, 1mM mixed non-essential amino acids, 1mM L-Glutaminutee, 100 units/mL penicillin, and 100 ug/mL streptomycin.

The HEK 293T cell line is a human embryonic kidney cell line that has been transformed by exposing cells to sheared fragments of adenovirus type 5 DNA {{34 Graham,F.L. 1977}}. The HEK 293T also has the SV40 Large T-antigen, which allows for episomal replication of transfected plasmids with the SV40 origin of replication. This allows transfected plasmids to be passed onto daughter cells and extend the temporal expression of the gene. The HEK 293T cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 ug/mL streptomycin.

The human mammary epithelial cell line (HMEC) is a primary cell line that has been transformed into carcinoma cells by the introduction of three genes: the SV40 large-T antigen, the telomerase catalytic subunit and the H-Ras

oncoprotein {{31 Elenbaas,B. 2001}}. The HMEC line results in cells that form tumors when transplanted subcutaneously or into mammary fat pads. The HMEC cells were cultured in MEGM (Mammary Epithelial Growth Medium) mixed 1:1 in DMEM/F12 supplemented with 20ng/mL of EGF (Sigma), 2ug/mL of Hydrocortisone(VWR), 10ug/mL of Insulin (Insulin), 1mM of L-glutaminutee, and 100 units/mL penicillin, and 100 ug/mL streptomycin.

The HT1080 cell line is a tumor cell line derived from human fibrosarcoma tissue. Thse cells grow into large tumors when transplanted into mice and the tumors show invasion of the surrounding tissue and progressive growth {{59 Rasheed,S. 1974}}. The HT1080 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 ug/mL streptomycin.

All cell lines were incubated at 37°C with 5% CO2. To passage cells, plates with cell monolayers were rinsed once with DPBS and incubated with 0.5-1mL of 0.15% Trypsin in EDTA at 37°C for 2 minutes. Trypsin was inactivated by adding serum media at a 1:5 ratio. The cells were spun down at 1500rpm for 3 minutes and resuspended to the necessary concentrations.

Constructs

Small interfering hairpin RNA (shRNA) constructs were created using the Invitrogen Blockit RNAi Designe® and MIT Whitehead’s shRNA designer. Two

different programs are used to get a larger pool of possible shRNA's to choose from. The candidate shRNA's were screened through NCBI BLAST to ensure that the sequence is specific for that protease. The constructs were synthesized by Integrated DNA Technologies (IDT) and were cloned into the psP81 vector via BstBI and BamHI cuts. The U6 lentiviral promoter cassette and the construct were then released with BamHI and SalI and ligated into the psP108 transfer vector. The shRNA construct targeting GFP was used as a control.

Retroviral Infection

HEK 293T cells were seeded at 1x106 onto 6cm dishes in serum media. The next day, the cells were triple transfected with the following plasmids to make lentivirus: 0.9 ug of the pCMV ☐ 8.2R gag/pol expression vector, 0.1ug VSV-G expression vector, and 1 ug of the transfer vector. 2ug total of DNA was transfected using TransIT-LT1 Transfection Reagent (Mirus) and incubated at 37°C overnight. Media was changed the day after transfection. Target cells were seeded at 1x105 or about 20% confluency onto 6cm dishes one day after transfection. Viral supernatants were harvested at 48 and 72 hours after transfection and dripped onto target cells. The viral supernatant was filtered through a 0.45 um filter to get rid of floating 293T cells. 6ug/mL of protaminutee sulfate was added to the viral supernatant to enhance lipid mediated gene transfer {{32 Sorgi,F.L. 1997}}. Cells were selected with the appropriate drugs (Puromycin at 2ug/mL or Blastocidin 10ug/mL) and carried as mentioned above.

Total RNA Isolation

RNA purification was performed using the RNeasy mini kit from Qiagen. Cell monolayers were cultuered for 2 days. Before harvest, cells were rinsed briefly with DPBS (Dulbecco's Phosphate Buffered Saline) before harvesting. A solution of 10uL beta-mercaptoethanol : 1mL of RLT buffer was made and 600uL of this mixture was used to lyse cells on each 10cm plate. The lysate was collected with a rubber scraper and spun through a Qiashredder spin column for homogenization. 1 volume of 70% ethanol was added to the homogenized lysate. The mixture was spun through the RNeasy spin column. The RNA is bound to the silica membrane. The flow through was discarded and the membrane washed with 350 uL of buffer RW1. Traces of DNA that may co-purify are eliminuteiated by subjecting the membrane to a DNase digestion. 70 uL of DNase solution in RDD buffer was added to the spin column and incubated bench top for 30 minutes. The DNase solution is then spun down and the flow through discarded. The membrane is washed a second time with 350 uL of buffer RW1. This is followed by two successive washed with 500uL of buffer RPE. The flow through is discarded each time. The spin column is spun down completely to dry the membrane and the RNA is eluted with 30-50 uL of RNase free water. The RNA concentration is measured using a nano-photometer (Implen). And the RNA is stored at -80°C until use.

Reverse Transcription

cDNA was synthesized from RNA using the Reverse Transcription Kit (Applied Biosystems). 2ug total of RNA input was added to the reaction mix. Reaction mix consists of RT buffer, dNTP, Random Primers, and Reverse Transcriptase from the kit. The reaction was run in a Biorad thermal cycler at the following cycles: cycle1: 25°C 10minutes, 37°C 60minutes, 37°C 60minutes, 85°C 5sec and cycle 2: 25°C 10minutes, 37°C 60minutes, 37°C 60minutes, 85°C 5sec. The 20uL cDNA product was diluted with 180uL of MQ water.

Quantitative Real-Time PCR

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

Total Protein Extraction

Cell monolayers were cultured for two days. Cells were harvested at 70-90% confluence and rinsed briefly with PBS. Boiling 100°C SDS lysis solution (100mM NaCl, 1% SDS, 100mM TrisHCl) was added to the plate. Whole lysate was scraped down with a spatula, collected and boiled at 95°C for 10 minutes. Samples were sonicated 1 sec with 1 sec pause between sonications. This was

repeated 10x for each sample. Samples were snap frozen in liquid nitrogen and stored at -80°C until use.

Determining Protein Concentration

Protein concentration was determined using the DC Protein assay (Bio-Rad). This assay is based on the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. This reaction produces a color change since the proteins cause a reduction of the Folin reagent. The reduced species have a characteristic blue color with absorbances at 750-405nm{{35 Peterson,G.L. 1979}}.

1mL of reagent A, an alkaline copper tartrate solution was mixed with 20uL of reagent S to create a working reagent. 100uL of the working reagent was mixed with 2uL of the protein sample. 800uL of reagent B, the dilute Folin Reagent was added to the sample and incubated at room temperature for 15 minutes.

Protein concentration was measured by using a nanspectrometer (Implen) set to Lowry mode. 900uL of the samples were aliquoted into the cuvette and protein concentrations read at 750nm. Dilutions of 2mg/mL Bovine Serum Albumin were used to create a standard curve.

Immunoblot Assay

Proteins were denatured at 70°C for 10 minutes with 50mM of DTT and 4x

loading buffer. Samples were loaded onto an SDS PAGE gel and run at 150V for about an hour or until loading dye reaches the bottom. Protein was transferred onto a nitrocellulose membrane at 200V for 120 minuteutes. The membrane was stained with Ponceau S Stock (1x) to determinutee total protein transfer.

The membrane was blocked with 5% milk or 2.5% BSA (in PBS or TBS with 0.1% Tween20) for 30 minute. Appropriate amount of primary antibody was added to 5% milk or 2.5% BSA (in PBS or TBS with 0.1% Tween20) and incubated with the membrane for 2hr at room temperature or overnight at 4°C. The membrane was washed 3x10minutes with PBS or TBS with 0.1% Tween20 and incubated with the appropriate secondary HRP conjugated antibody in 5% milk or 2.5% BSA. The membrane was washed 3x10minutes with PBS or TBS with 0.1% Tween20 and 3x10minutes with PBS or TBS.

The membrane was visualized with Amersham ECL Plus™ Western Blotting Detection kit (GE) and exposed onto Kodak Biomax Film.

FITC Degradation Assay

FITC degradation assay was carried out as mentioned in “ECM degradation Assays for Analyzing Local Cell Invasion” by Artym et. Al 2009 with minor changes {{33 Artym,V.V. 2009}}.

Preparation of the FITC gelatin: 2% porcine gelatin was conjugated to fluorescein isothiocyanate in a 0.1M carbonate-bicarbonate buffer (pH 9.3) at room temperature for 18 h. Unreacted dye was removed by extensive dialysis and gel filtration through a G-25 Sephadex column in balanced PBS. Fluorescein isothiocyanate-conjugated gelatin (FITC-gelatin) was stored in aliquots at -20°C.

Preparation of glass coverslips: Round 12mm diameter glass coverslips were etched with 20% Nitric Acid for 30 minutes with gentle swirling. The coverslips were extensively washed with deionized water for 2hrs with frequent changes of deionized water. The coverslips were then rinsed 3x with 70% ethanol, 1x with 100% ethanol and stored submerged in 100% ethanol until use.

Preparation of the FITC gelatin coated coverslips: The acid etched coverslips were dried completely on lens paper and placed in well of the 24-well tissue culture plate. 500uL of 50ug/uL of poly-L-Lysine was added to each well and incubated for 20 minutes. The coverslips were gently washed 3x with PBS. 500uL of 0.5% Gluteraldehyde was added to each well and incubated for 15 minutes. The coverslips were gently washed 3x with PBS. A 1:10 dilution of 0.2% FITC gelatin and 0.2% porcine gelatin was made and warmed in a 37°C water bath until use. 20uL droplets of the diluted FITC gelatin mixture were aliquoted onto sheets of parafilm. The coverslips were gently picked up using a needle and forceps, inverted onto the drops of gelatin matrix, and incubated for 10 minutes. The coverslips were gently washed 3x with PBS. 500uL of 5mg/mL of bubbling

NaBH4 was 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 gently washed 3-5x with PBS or until no more bubbles were seen. 500uL of appropriate media was added to each well and the plate stored in the 37°C 5% CO2 incubator until use. All steps in the preparation of FITC gelatin coated coverslips were carried out in a sterile environment.

Seeding cells: Cells were trypsinized and suspended as mentioned above. The number of cells was counted using a hemocytometer. 5x104 cells/well suspended in 1mL were seeded onto each FITC gelatin coated coverslip in a 24-well plate.

FITC Gelatin degradation assay: The cells were incubated on the FITC gelatin coated coverslips with the appropriate number of cells for 8hrs to allow for matrix degradation.

Fixing and staining: Cells were fixed with 4% warm para-formaldehyde for 30minutes. Fixed cells were gently washed 3x with PBS. Cells were permeabilized with 0.1% TritonX in PBS for 15minutes with shaking. Permeabilized cells were gently washed 3x with PBS. Actin filaments were visualized with 1:200 AlexaFlour594 conjugated phalloidin (Invitrogen) in PBS with 0.1% Tween20 and incubated for 1hr. Nuclei were visualized with DAPI and incubated for 1 hr. Cells were washed 3x with PBS and mounted using hard set

mounting medium (Vector).

Matrigel Transwell Migration & Invasion Assay

The matrigel transwell assay was carried out as mentioned in Hall & Brooks in Methods in Molecular Medicine: Metastasis research protocols with minor changes.

Preparation of matrigel coated transwells: All reagents and equipment that came into contact with matrigel were chilled at -20°C for two hours prior to setting up the experiment. Growth factor reduced matrigel was thawed overnight on ice at 4°C. Thawed matrigel was kept on ice at all times to prevent gelling. GFR Matrigel protein concentration was determined and diluted with serum free media to the appropriate concentrations. Transwell polycarbonate membrane inserts with 8um pore 6.5mm diameter (Corning) were coated with 40uL of dilute 0.258ug/uL of growth factor reduced matrigel (BD). The inserts had a total of 10.35ug GFR matrigel in each well. The matrigel was allowed to set in the 37°C incubator for 2 hours. The matrigel layer was then allowed to dry completely in a laminar flow hood for 1day or in the 37°C incubator for 2 days. The matrigel coated membranes and non-matrigel coated membranes were re-constituted with 80uL of serum free medium in 37°C incubator for 2hours before use.

Seeding cells: Cells were trypsinized and resuspended in serum free medium.

The number of cells was counted using a hemocytometer. 5x104 cells in 100 uL of serum free medium were seeded into the top wells of the chamber.

<u>Invasion & Migration assay:</u> The bottom wells of the chamber were filled with 750uL of full serum medium and the top wells of the chamber were seeded with the appropriate number of cells in serum free medium, creating a 0 => 100% growth factor gradient. All samples were performed in both matrigel coated transwells to assess invasive capabilities and non-matrigel coated transwells to assess migrative capabilities. Cells were incubated in the wells for 48hours.

<u>Fixing and staining:</u> After incubation, media was aspirated from the top and bottom wells and replaced with PBS. Top wells were gently swabbed twice with cotton tipped applicators. Non-swabbed wells are used as controls to determine total number of cells. Cells were fixed with 4% para-formaldehyde for 30minutes. Fixed cells were gently rinsed with MQ H2O and stained with 0.1% crystal violet for 10 minutes. Chambers were rinsed 3x with MQ H2O and allowed to dry at room temperature. Invasion and/or migration was measured by photographing and counting the number of cells that migrated through the transwells or by releasing the dye with 10% acetic acid and quantifying the released dye on a spectrophotomer at 540nm.

<u>ImmunoFluorescence Analysis</u>

Cells were seeded onto 0.2% porcine gelatin coated coverslips to provide a

matrix conducive to invadopodia formation.

Preparation of glass coverslips: Round 12mm diameter glass coverslips were etched with 20% Nitric Acid for 30 minutes with gentle swirling. The coverslips were extensively washed with deionized water for 2hrs with frequent changes of deionized water. The coverslips were then rinsed 3x with 70% ethanol, 1x with 100% ethanol and stored submerged in 100% ethanol until use.

Preparation of 0.2% gelatin coated coverslips: The acid etched coverslips were dried completely on lens paper and placed in well of the 24-well tissue culture plate. 500uL of 50ug/uL of poly-L-Lysine was added to each well and incubated for 20 minutes. The coverslips were gently washed 3x with PBS. 500uL of 0.5% Gluteraldehyde was added to each well and incubated for 15 minutes. The coverslips were gently washed 3x with PBS. 500uL of warmed 0.2% gelatin was added to each well and incubated for 10minutes. The coverslips were gently washed 3x with PBS. 500uL of 5mg/mL of bubbling $NaBH_4$ was 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 gently washed 3-5x with PBS or until no more bubbles were seen. 500uL of appropriate media was added to each well and the plate stored in the 37°C 5% CO_2 incubator until use. All steps in the preparation of 0.2% gelatin coated coverslips were carried out in a sterile environment.

Seeding cells: Cells were trypsinized and suspended as mentioned above. The number of cells was counted using a hemocytometer. 2x104 cells/well suspended in 1mL were seeded onto each 0.2% gelatin coated coverslip in a 24-well plate. The cells were incubated on the 0.2% gelatin coated coverslips with the appropriate number of cells for 3 days or 70% confluency to allow complete formation of invasive structures.

Fixing and staining: Cells were fixed with 4% warm para-formaldehyde supplemented with 0.5mM CaCl2 and 0.5 mM of MgCl2 for 30minutes. Fixed cells were gently washed 3x with PBS+ (PBS+ is Phosphate buffered saline supplemented with 0.5mM CaCl2 and 0.5 mM of MgCl2). Cells were permeabilized with 0.1% TritonX in PBS+ for 15minutes with shaking. Cells were gently washed 3x with PBS+. Permeabilized cells were blocked with 10% Goat Serum in PBS+ for 30minutes-1hour. Cells were gently washed 3x with PBS+. Cells were incubated with the appropriate primary antibody in 10% Goat Serum PBS+ for 1hr at room temperature or overnight at 4°C. Cells were gently washed 3x for 10 minutes each with PBS+. Cells were incubated with the appropriate fluorochrome conjugated secondary antibody in 10% Goat Serum PBS+ for 1hr at room temperature. Cells were gently washed 3x for 10 minutes each with PBS+. Actin filaments were visualized with 1:200 AlexaFlour594 conjugated phalloidin (Invitrogen) in PBS+ and incubated for 1hr. Nuclei were visualized with DAPI and incubated for 1 hr. Cells were washed 3x with PBS and mounted using hard set mounting medium (Vector). The Cortactin mouse primary antibody

(Millipore) was used at 1:1,000 dilution. The Tks5 rabbit primary antibody was a generous gift from the Courtneidge lab and was used at 1:500 dilution. The E-cadherin rabbit primary antibody (Cell Signaling) was used at 1:200 dilution. The Beta-Catenin mouse primary antibody (BD) was used at 1:200 dilution.

Imaging

Cells were imaged using the Zeiss fluorescent microscope and images were acquired using the Metamorph 7.6 imaging software (Molecular Devices). Images were processed using the ImageJ software.

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