UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of MUC1 in Metastasis Dependent on EGFR and Src

A Dissertation submitted in partial satisfaction of the

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Biomedical Sciences

by

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The Dissertation of Steven Kai Ming Lau is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

In recognition of all those who have helped me to reach this day, especially:

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- My parents, for their constant support of my studies

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LIST OF ABBREVIATIONS

CAM – Chorioallantoic membrane

Casp8 – Caspase-8

Ctrl – Control

CTTN – Cortactin

Cyto – Cytoplasmic

ECM – Extracellular matrix protein

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor, also known as ErbB

ERK2 – Extracellular signal-related kinase 2, also known as mitogen-activated protein kinase 1

FACS – Fluorescence activated cell sorting

FG – Fast growing variant human pancreatic carcinoma cell

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GFP – Green fluorescent protein

HER2 – Human epidermal growth factor receptor 2, also known as ErbB2

HRG – Heregulin

HSP90 – Heat shock protein 90

MUC1 – Mucin-1, also known as CA 15-3, CD227, episialin, epithelial membrane antigen, polymorphic epithelial mucin, breast carcinoma-associated antigen DF3, KL-6, peanut-reactive urinary mucin

MUC1.CD – MUC1 cytoplasmic domain

MUC1.CT3 – Cytoplasmic domain-deleted MUC1

MUC1.FL – Full-length MUC1

Nuc – Nuclear

PARP – Poly ADP-ribose polymerase

PBS – Phosphate buffered saline

pY – Phosphorylated tyrosine

Q-PCR – Quantitative polymerase chain reaction

RTK – Receptor tyrosine kinase

SFK – Src family kinase

shRNA – Short hairpin RNA

siRNA – Small interfering RNA

Src – Sarcoma gene

Vil1 – Villin-1

XTT – 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

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ABSTRACT OF THE DISSERTATION

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Tumor metastasis is a complex, multi-step process initially depending on local invasion and followed by intravasation, survival in the circulation, extravasation and colonization of a distant site. Many studies have shown that EGFR and Src signaling and its cooperation with integrins are involved throughout metastasis. However, the mechanisms by which cross-talk between RTK signaling and integrins contribute to metastasis are not well-described. EGFR signaling promotes carcinoma cell metastasis that is dependent on activation of both Src kinase and integrin αvβ5, but little is known about the downstream effectors contributing to EGFR-mediated metastasis. Therefore, the studies in this dissertation proposed to investigate

novel downstream effectors of metastasis dependent on EGFR, Src, and integrin αvβ5.

Lead candidate effector molecules were systematically identified using an *in silico* strategy and validated in a well-described chick CAM model of EGF-induced tumor cell metastasis. This approach revealed a requirement for MUC1 in spontaneous metastasis to the lungs but not primary tumor formation.

MUC1 contributes to the mucous barrier protecting epithelial cells from the environment and participates in intracellular signaling cascades as a substrate for EGFR and Src. Interestingly, both extracellular and intracellular domains of MUC1 have been linked to tumor progression and metastasis. Expression of mutant MUC1 constructs revealed that the cytoplasmic domain is required for EGF-induced cell migration mediated by integrin αvβ5 and promotes metastasis without enhancing primary tumor growth. In biochemical and cell biological approaches, EGFR signaling enhanced MUC1 cleavage, nuclear localization and transcription of genes associated with metastasis. Genetic and pharmacological strategies revealed a requirement for Src in EGF-induced MUC1 cleavage and nuclear translocation, which were associated with sponteanous metastasis in a murine orthotopic pancreatic tumor model. Therefore, the findings described in this dissertation define a

critical role for MUC1 in tumor metastasis regulating by cooperative signaling between EGFR, Src, and integrin αvβ5.

Chapter 1

Introduction

1.1 Tumor metastasis

1.1.1 Overview of tumor biology

Tumor formation and progression is generally considered a sequential, selective and stochastic process involving the acquisition of several cancer "hallmarks." These include unlimited replicative capacity, sustained proliferative signaling, suppression of growth inhibition, evasion of apoptosis, enhanced angiogenesis and invasive and/or metastatic behavior. While each of these processes are tightly controlled in normal tissue, tumor cells have evaded these restrictions.

In the multiple-hit model – first proposed in 1953 [1, 2] – tumors can gradually develop over many years without being detected. Pre-malignant cells undergo a stepwise, selective accumulation of genetic abnormalities leading to the acquisition of successive cancer hallmarks and more aggressive behavior.[3, 4] By the time tumors are clinically detectable and diagnosed, they are generally heterogeneous and contain subpopulations of neoplastic cells with varying biological characteristics.[5, 6, 7] This is significant, since subpopulations of malignant cells from the same tumor may act differently and respond in different ways to the same treatment.

Adding to this complexity, tumors are comprised of a diverse array of components such as endothelial cells, inflammatory cells and fibroblasts in

addition to neoplastic cells. As with malignant cells, the tumor-associated stroma is heterogeneous and can be functionally and spatially diverse.[8]

1.1.2 Tumor metastasis

Of the cancer hallmarks, tumor cell invasion and metastasis may be the most fearful because of its association with morbidity and mortality in cancer patients. Cancer is predicted to cause over 7.6 million deaths worldwide in 2010, with approximately 550,000 deaths in the USA, and tumor metastasis is responsible for most of these cancer deaths.[9, 10] Furthermore, distant metastasis is often resistant to treatment and generally considered incurable.

Epithelial tumor cell metastasis is the culmination of multiple steps including local invasion, intravasation, survival in the vasculature, extravasation, and colonization of a distant site. Each of these steps is complex and could represent a rate-limiting barrier to metastasis. Therefore, it is important to elucidate what enables tumor cells to successfully metastasize. In fact, only a small fraction of tumor cells, even those entering the circulation, develop into clinically-apparent macrometastases.[11, 12] When radio-labeled melanoma cells are injected into the circulation, less than 0.01% of these cells successfully colonize the lung.[12] These observations have led to questions on whether metastasis is a selective or a stochastic process. Additional studies have determined that intrinsic properties of tumor cells can promote

tissue-specific colonization of distant sites.[13, 14, 15, 16] Such findings appear to support tumor cell selection as an important component of metastasis.

The prevailing theory of tumor initiation and progression involves the evolutionary accumulation of genetic abnormalities leading to malignancy.[3, 4] In this model, distant metastasis is usually considered a late event in tumor progression.[17, 18] In contrast, others have reported that tumor cells with stem-like characteristics are responsible for distant metastasis.[19, 20] This implies that distant metastasis could in fact be possible at early stages in tumor formation. Furthermore, if stem-like cancer cells can metastasize, then extensive selection of tumor cells at the primary site cannot universally explain metastasis. While these models of tumor cell metastasis are not mutually exclusive, in this context metastasis appears to be a stochastic process.

While substantial advances have been made in recent years towards the diagnosis and treatment of human cancers, efficient means of treating metastatic disease are missing. This context highlights a critical need for understanding the molecular mechanisms through which tumor cells mobilize and disseminate to secondary sites. Such knowledge could reveal new therapeutic strategies to prevent and/or treat metastatic disease.

1.1.3 Signaling pathways in tumor progression and metastasis

Cell migration is a tightly-controlled process critical for normal embryonic development, the inflammatory response and neural genesis. However, signaling pathways used in normal cell migration are exploited by tumor cells for their dissemination. As an example, the discovery of epidermal growth factor (EGF) and its receptor, EGFR, are instructive. Stanley Cohen first discovered the soluble factor he called EGF in 1962 from salivary gland extracts based on its ability to accelerate tooth and eye development in newborn mice.[21] More than 15 years later, Cohen and colleagues identified the cell surface receptor for EGF, EGFR.[22, 23, 24] Soon after, it became clear that the avian erythroblastosis tumor virus gene, v-*erbB*, was an aberrant form of EGFR.[25, 26, 27] Later studies have demonstrated that both EGF and EGFR are upregulated in various human cancers.[28, 29, 30] These discoveries paved the way for therapies targeting EGFR to be tested and, eventually, approved for the treatment of human tumors.[31, 32, 33, 34]

We now know that EGFR, a receptor tyrosine kinase (RTK), commands a wide array of signaling pathways including those of the phosphoinositol-3-kinases, mitogen-activated protein kinases, and Janus kinases. However, not all EGFR activation is equivalent. The biological outcome of EGFR signaling depends on a combinatorial context of which downstream effectors are available.[35, 36, 37] Importantly, recent studies have demonstrated that activated EGFR recruits and activates Src.[38, 39, 40] We recently observed

that a functional outcome of EGFR-dependent Src activation is the enhancement of tumor cell migration *in vitro* and metastasis *in vivo*.[41]

Presently, Src is among the most well studied molecules in tumor biology. Peyton Rous described the first example of a transmissible tumorigenic virus, the Rous sarcoma virus (RSV), in 1911.[42] Decades later, the viral gene v-*Src*, a homolog of cellular c-*Src*, was identified as the causative agent of RSV-dependent tumorigenesis.[43, 44, 45] Soon after, Src was determined to be a non-receptor tyrosine kinase.[46, 47] We now know that Src, like EGFR, is a key regulatory node of various signaling pathways and controls cellular processes essential for cell migration. For example, Src phosphorylates Crk-associated substrate (p130CAS or CAS) leading to recruitment of Crk and DOCK180 for the coordination of the activity of small GTPases including Rap1.[41, 48, 49] Src also phosphorylates FAK at Y861 and enhances its association with integrin αvβ5 in HUVEC human endothelial cells.[50] Finally, Src phosphorylates the adherens junctions components E-cadherin and β-catenin promoting their internalization and weakening cell-cell adhesion.[51] Additional studies have demonstrated that Src is overexpressed or hyperactivated in human cancers.[52, 53, 54, 55, 56] Together, these discoveries served as the foundation for therapies targeting Src to be tested, and, eventually, approved for the treatment of human cancers.[57, 58, 59, 60, 61, 62] Importantly, several inhibitors of Src activity including dasatinib are now being tested for efficacy in both liquid and solid tumors.

1.1.4 EGFR and Src signaling regulates tumor cell metastasis mediated by integrin αvβ5

Integrins are a family of transmembrane receptors that bind to extracellular matrix (ECM) proteins. In addition, they regulate bidirectional signaling between the extracellular milieu and many intracellular signaling pathways, particularly those regulating adhesion and the cytoskeleton. The critical function of integrins is reflected in their ubiquitous expression.

Mature integrins are noncovalent heterodimers of an α subunit, of which there are 18, and a β subunit, of which there are 8, and these subunits are known to combine in at least 24 unique combinations.[63] Each α-β subunit pairing has varying affinity to different ECM proteins, and a single integrin heterodimer can bind to several ligands. Ligand specificity can be modified by the extracellular divalent cations (Ca^{+2}, Mg^{+2} or Mn^{+2}) [64, 65] or by restricting the available ligands in a tissue-specific manner. Upon ligation of integrins to their cognate ligands in the ECM, β-subunit integrins form focal adhesions by clustering, anchoring the actin cytoskeleton to the ECM and recruit and activate a number of signaling proteins including Src [66, 67] and FAK [68, 69] to form focal adhesions.

A growing body of literature has revealed cooperative signaling between growth factor and cytokine receptors and integrins to regulate cell

adhesion [70, 71], migration [72, 73, 74, 75], invasion [73], and survival.[76, 77] Interestingly, while integrins promote cell death in the absence of ligation [78], integrin signaling in tumor cells deficient for this integrin-mediated death actually enhances metastasis.[79] In many tumor types, including pancreatic cancer, members of the ErbB family contribute to tumor formation and metastasis.[28, 29, 30, 80] Could cross-talk between RTKs such as EGFR and integrins participate in tumor progression? We previously observed that integrin αvβ5, in the absence of growth factor stimulation, is unable to form focal adhesions and initiate FG human pancreatic carcinoma cell migration/invasion.[81] However, following EGF stimulation, cells expressing integrin αvβ5 as their only vitronectin receptor gain the ability to invade *in vitro* and metastasize *in vivo*.[73, 82] Interestingly, cell invasion mediated by β1 integrins is independent of EGF.[73, 82] Furthermore, cell migration and metastasis mediated by integrin αvβ5 requires EGF-dependent activation of Src, whereas migration mediated by β1 integrins is independent of Src activity [41]. These studies describe a signaling pathway that enhances tumor cell metastasis and thereby help distinguish between tumor cells with greater or lesser propensity for metastasis. The molecular mechanism by which EGFR and Src signaling cooperate to enhance metastasis is not well-understood.

An understanding of integrin-mediated cell-matrix adhesion has led to targeting of integrins as a therapeutic approach to human cancers. Interestingly, integrins expressed by both tumor cells and host cells are useful

targets of such therapies. For example, the vitronectin receptor integrin αvβ3 is overexpressed in several human cancers, including those of the pancreas, and is correlated with metastasis.[83, 84] Furthermore, host cell integrin αvβ3 plays an essential role in tumor angiogenesis.[85, 86] These and other findings led to the development of the peptide inhibitor cilengitide, which antagonizes integrins αvβ3 and αvβ5 and is currently in phase 3 clinical trials for the treatment of glioblastoma multiforme.[87]

1.2 Mucin Glycoproteins

1.2.1 Mucin family members in normal and tumor epithelium

The mucin family of proteins is expressed by ductal epithelial cells and is composed of 20 members, each of which share the common feature of a tandem repeat domain of variable length rich in proline, threonine and serine residues (PTS domain) (**Figure 1.1**).[8] The PTS domain is subject to extensive glycosylation via GalNAc *O*-linkages at threonine and serine residues.[8] Mucin family members are classified as either secreted or transmembrane. While both types contribute to the mucous gel protecting epithelial cells from extracellular stresses such as bacteria, toxins and acidic pH, the transmembrane forms, as their name implies, possess short cytoplasmic tails which activate intracellular signaling cascades.[8]

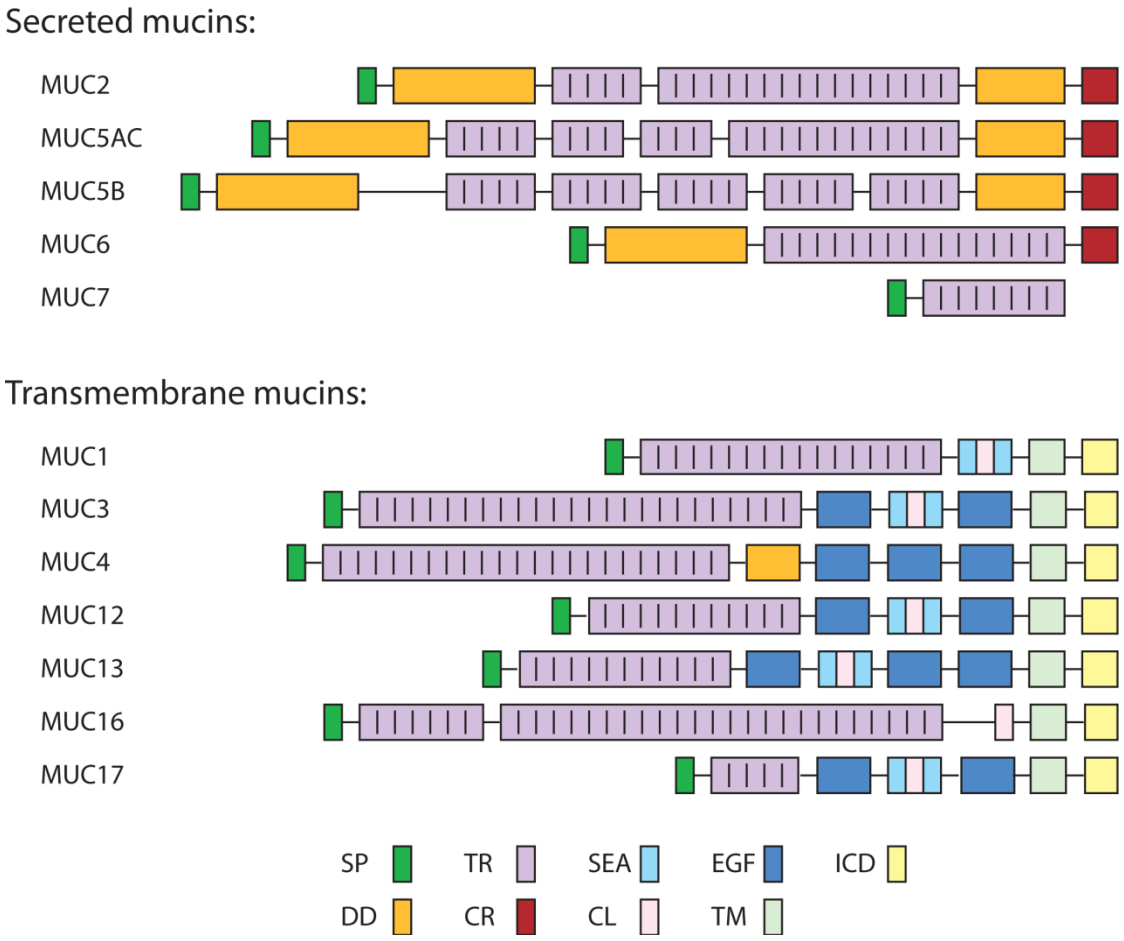

Figure 1.1: Schematic of mucin family members, of which there are 20. The mucin family is characterized by a large tandem repeat domain (TR) of variable length and rich in serine and threonine targets of GalNAc *O*-glycosylation. Secreted mucins may have D domains (DD) with homology to the dimerization domains of von Willebrand factor, and D domains mediate mucin oligomerization. Transmembrane mucins generally have EGF-like domains (EGF) thought to mediate mucin association with ErbB family members. The sperm protein, enterokinase and agrin domain (SEA) may participate in mucin association with carbohydrates and frequently contains an extracellular cleavage site (CL). Notably, transmembrane mucins possess short cytoplasmic tails (ICD) which participate in intracellular signaling. Signal peptide (SP), transmembrane domain (TM).

Transmembrane mucins are overexpressed in human cancers. Mucin-1 (MUC1, also known as CA15-3) is the most-studied mucin family member and is overexpressed in a wide range of epithelial carcinomas, including those of the pancreas, breast, prostate and lung.[88, 89, 90] In fact, serum-associated MUC1 is clinically-approved for monitoring disease progression in cancers of the breast.[91] Interestingly, seroconversion against MUC1 independently predicts better outcomes in pancreatic and breast cancer patients.[92, 93] Transmembrane MUC4 is also elevated in pancreatic cancer and predicts poor prognosis.[94, 95] Another transmembrane mucin, MUC16 (also known as CA125) is upregulated in ovarian cancer, and serum-associated MUC16 is clinically-approved for detecting and monitoring disease.[96, 97] In contrast, the secreted mucin MUC2 has been reported to function as a tumor suppressor in colorectal carcinomas by controlling inflammation induced by microbial flora.[98]

1.2.2 How do transmembrane mucins contribute to tumor formation and progression?

The normal epithelium is polarized, and MUC1 and RTKs such as EGFR are restricted to the apical and basolateral surfaces, respectively.[88, 99, 100] In contrast, polarity is irreversibly lost in carcinoma cells, and MUC1 and EGFR freely interact throughout the cell surface.[101, 102, 103] This association enhances spontaneous tumor formation and progression. For

example, whereas wild-type mice expressing transgenic TGFα uniformly develop mammary tumors which spontaneously metastasize, tumor formation in a *Muc1*$^{-/-}$ background was delayed and metastasis was abolished.[104] In this model, MUC1 may enhance tumor formation and metastasis by preventing ligand-induced EGFR internalization and degradation to stabilize signaling.[101, 105, 106] MUC1-EGFR interaction is also thought to enhance binding of Src and β-catenin to MUC1 to facilitate tumor cell invasion and metastasis.[101, 107, 108] In addition, association of MUC1 and EGFR activates mitogenic MAPK signaling.[102, 109] Importantly, the short cytoplasmic tail of MUC1 is subject to tyrosine [101, 107], serine [110] and threonine [111] phosphorylation, which regulates its function in intracellular signaling.

MUC4 also interacts with the ErbB family member HER2 in carcinoma cells.[112] Unlike MUC1, which is thought to interact with EGFR via extracellular Galectin-3 [106], MUC4 has extracellular EGF-like domains through which it binds HER2 and enhances its signaling to promote tumor cell survival.[112, 113, 114] Thus, unlike MUC1, there is currently no evidence for phosphorylation of the MUC4 cytoplasmic tail or a role for it in signaling.

Less is known about the role of MUC16 in tumor formation and progression. While MUC16 has not been reported to interact with ErbB family members, the MUC16 ectodomain binds to mesothelin, which is thought to

mediate peritoneal seeding of ovarian carcinomas.[115, 116] Like MUC4, there is no currently no evidence for phosphorylation or signaling of the MUC16 cytoplasmic tail.

1.2.3 Both extracellular and intracellular domains of MUC1 promote tumor progression

MUC1 is synthesized as a single polypeptide chain, but is thought to be co-translationally processed into a stable heterodimer by conformational stress at its extracellular SEA domain.[117, 118] As a result, MUC1 is expressed at the cell surface as a stable heterodimer. The extensively glycosylated MUC1 ectodomain extends over 100 nm from the cell surface, well beyond the 10 nm glycocalyx.[119] As such, it has been reported to contribute to tumor cell invasion and metastasis by inhibiting E-cadherin-mediated cell-cell adhesion [120] and β1 integrin-mediated cell-matrix adhesion.[121] In contrast, the MUC1 ectodomain has also been demonstrated to promote transendothelial migration mediated by ICAM-1 [122, 123] and E-selectin [124] and perineural invasion mediated by Siglec-4a.[125] The MUC1 ectodomain is also proteolytically cleaved from the cell surface by TACE/ADAM17 [126] and MT1-MMP [127] and shed into the circulation, where it is clinically used to follow tumor progression.[91] Therefore, the MUC1 ectodomain facilitates tumor cell invasion and metastasis by modulating cell-cell and cell-matrix interactions.

Recent studies have focused on the role of the smaller transmembrane MUC1 subunit, particularly the short 72-amino acid intracellular domain, as an effector molecule downstream of RTKs including ErbB family members [101, 102, 105, 128, 129], *c*-Met [130], FGFR [131] and PDGFR [132]. While the precise mechanism of MUC1 cleavage and/or internalization is not well understood, cytokine stimulation initiates translocation of the MUC1 cytoplasmic domain to various intracellular locations (**Figure 1.2**). For example, whereas EGF stimulation promotes nuclear translocation in association with β-catenin [101, 107], the EGF family member HRG induces nucleolar localization in association with γ-catenin.[129] Within the nucleus and lacking its own DNA-binding domain, MUC1 acts as a co-activator for expression of genes associated with tumor cell invasion and metastasis including *TWIST1*, *SNAI1* and *SNAI2*.[130, 131, 133, 134] HRG stimulation is also reported to enhance MUC1 mitochondrial localization, where MUC1 promotes tumor cell survival in the presence of genotoxic agents.[135, 136] Interestingly, expression of transgenic MUC1 under control of the MMTV promoter results in spontaneous mammary tumor formation, and the MUC1 cytoplasmic domain is required.[137] These studies demonstrate that the MUC1 cytoplasmic domain functions as a component of signaling pathways to promote tumor formation and metastasis.

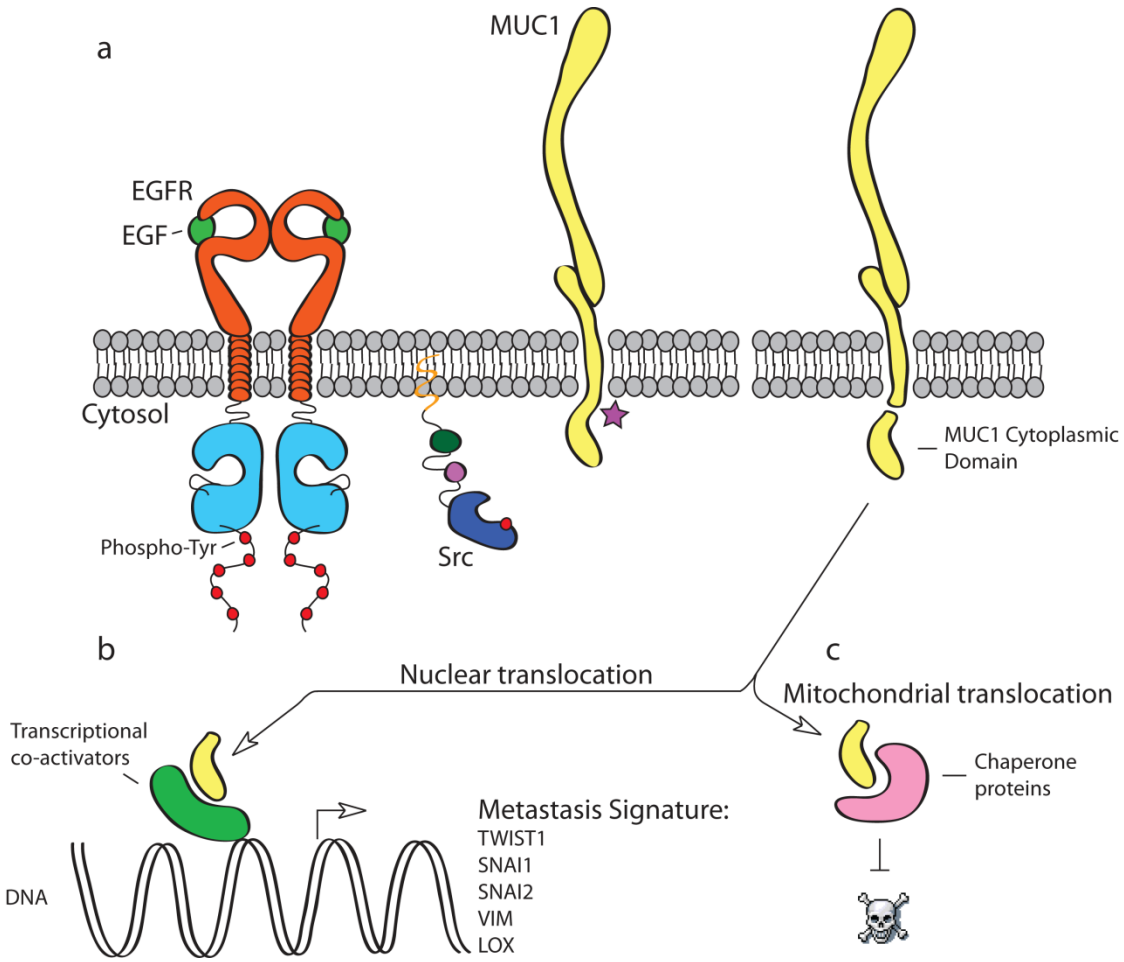

Figure 1.2: Cartoon of MUC1 processing. (**a**) EGFR and Src phosphorylate tyrosine residues on the MUC1 intracellular domain. This has been proposed to result in proteolytic cleavage (indicated by the star) on the intracellular portion of MUC1 and release from the plasma membrane. (**b**) The MUC1 cytoplasmic domain translocates to the nucleus in association with other transcriptional co-activators including β-catenin, resulting in the transcription of target genes associated with metastasis. (**c**) The MUC1 cytoplasmic domain also localizes to the mitochondrial outer membrane in association with chaperone proteins including HSP90, resulting in suppression of apoptosis.

Interaction with Src, a key node of many signaling pathways, mediates many tumor-promoting functions of MUC1. For example, spontaneous tumor formation, growth and metastasis are suppressed in *Muc1*$^{-/-}$ mice compared to wild-type mice in a well-established murine breast tumor model driven by polyoma middle T antigen.[138] Tumor development in this model is dependent on Src kinase activity [139], and tumors developing in *Muc1*$^{-/-}$ mice exhibit decreased Src activity.[140] Src-dependent phosphorylation of the MUC1 cytoplasmic domain at Y46 enhances its interaction with HSP90 in human 293 fibroblasts and HCT116 colorectal carcinoma cells.[136] Other SFKs including Lck [141] and Lyn [142] have also been demonstrated to phosphorylate the MUC1 cytoplasmic domain at Y46, which leads enhances its interaction with β-catenin in 293 cells.[142] In addition, using the pharmacological Src inhibitor PP2, an unidentified SFK or SFKs was shown to phosphorylate the MUC1 cytoplasmic domain and enhance its interaction with CrkL in T-47D human breast carcinoma cells.[123] Therefore, while Src regulates MUC1 function in tumor progression and metastasis, the precise effect of Src on MUC1 is not well-understood.

1.2.4 Therapeutic implications of MUC1 in tumor progression and metastasis

Substantial advances have been made in our understanding of tumor progression and metastasis. Therapies targeting pharmacological inhibitors of

EGFR [33, 34] and Src [58, 59, 60, 61, 62] have had clinical success, with several more in development. In addition, an integrin antagonist is in phase 3 clinical trials.[87] While these therapies have incrementally improved the outlook for cancer patients, efficient methods for the prevention and treatment of tumor metastasis are still lacking. Therefore, further characterization of the molecular mechanisms coordinating the carcinoma cell metastasis is critical.

Recently, MUC1 has become a candidate of targeted therapies. Several MUC1 vaccines are currently in phase 3 clinical trials in patients with breast or lung cancer. These vaccines are designed to stimulate an inflammatory response to MUC1 as a tumor antigen, which has already been shown to correlate with positive outcomes in breast and pancreatic cancers.[92, 93] However, given that the MUC1 cytoplasmic domain interacts with RTKs and Src to promote tumor progression and metastasis, such vaccines may have limited benefit in controlling cancers dependent on EGFR and Src signaling. Interestingly, elevated serum MUC1 is predictive of *de novo* resistance to EGFR inhibitors.[143, 144] To address these concerns, recent studies have identified peptide antagonists and small molecule inhibitors of the MUC1 cytoplasmic domain and found them effective in preclinical mouse models of breast and prostate cancer.[145, 146, 147, 148] These therapies may be useful in preventing and treating metastatic disease in a wide variety of tumors known to express MUC1.

Our findings describe a pathway in which cross-talk between EGFR and integrin αvβ5 promotes tumor cell invasion and metastasis via Src-dependent MUC1 cleavage. These studies may explain in part how inhibitors of EGFR and its downstream effectors including Src and integrin αvβ5 suppress the metastatic spread of tumor cells. An improved understanding of the molecular mechanisms leading to metastasis could reveal novel therapeutic strategies for the treatment of malignant disease.

1.3 Hypotheses

This study investigates several hypotheses related to the differences between tumor cells that successfully metastasize and those that do not. It seeks:

1. To identify and validate novel effectors essential for tumor cell migration and metastasis dependent on EGFR and Src.
2. To examine the molecular basis by which MUC1 is required for EGF-induced tumor cell metastasis.
3. To assess the impact of Src on MUC1 in tumor cell migration.

Chapter 2

Methods

2.1 *In silico* strategy

Substrates of tyrosine phosphorylation following EGF treatment and Src substrates and interactants were identified in open-access databases (**Appendix**). Hits found in both groups were considered candidates for further analysis. Each candidate was systematically evaluated using MEDLINE, and the following filters were applied to candidates:

1) Experimental evidence for role in cell migration
2) Evidence for role in tumor cell metastasis as demonstrated either in experimental models or through clinical correlation
3) Inverse correlation between expression in human tumors and overall survival of cancer patients
4) Linked to human pancreatic ductal adenocarcinoma.

2.2 Cell culture

Mycoplasma-negative FG human pancreatic carcinoma cells [149] were grown in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc.) supplemented with 10% fetal bovine serum (Omega Scientific, Inc.), L-glutamine (Mediatech, Inc.), sodium pyruvate (Mediatech, Inc.) and non-essential amino acids (Mediatech, Inc.).

2.3 Short hairpin RNA knockdown

Expression of lead candidates was suppressed by shRNA in FG human pancreatic carcinoma cells. Lentiviral shRNA constructs in pLKO.1 expressing

system were purchased from Open Biosystems. Lentiviruses were produced in 293FT cells using FuGENE6 transfection reagent (Roche). Cells were selected 48 hours after infection with 1 ug/mL puromycin (Cellgro / Mediatech, Inc.) and single-cell clones were isolated, propagated and screened by immunoblot. Three or more clones were pooled for further experiments to avoid non-specific clonal effects.

2.4 Expression constructs

Full-length and cytoplasmic domain-deleted MUC1 were generously provided by Michael Hollingsworth.[150] The 3'UTR was removed from these constructs before transfection into cells. MUC1 cytoplasmic domain was cloned by PCR and sequenced. MUC1 knockdown cells were transfected with rescue constructs in pcDNA3.1 using Lipofectamine2000 (Life Technologies) and serum starved overnight, and migration assays were performed at 48 h after transfection. For some experiments, the stop codon was removed by site-directed mutagenesis (Agilent Technologies) and the construct was sequentially cloned into pGFP (Clontech Laboratories) and then pCDH lentivirus expressing system (System Biosciences). Cells were sorted twice by flow cytometry for cells highly expressing GFP-labeled constructs.

2.4 Immunoblotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors. Lysate was

incubated with gentle agitation for 20 minutes at 4 °C prior to centrifugation at 14,000 rpm for 15 minutes at 4 °C to remove insoluble particles. Protein was quantified with BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. 50 ug of each sample was loaded into a polyacrylamide gel for separation by SDS-PAGE gel electrophoresis. Proteins were transferred to PVDF membranes (Bio-Rad) and blocked with 5% BSA (Sigma) in TBST prior to incubation with primary antibody followed by secondary antibody. Antibodies were purchased from Abcam (GFP), BD Biosciences (PARP), Cell Signaling Technologies (MUC1 VU4H5, Src pY416), Millipore (GAPDH, Src GD11), Santa Cruz (ERK2, HSP90), Sigma (β-actin), and Thermo Fisher Scientific (MUC1 CT-2). For some experiments, cells were pre-treated with or without Src inhibitor (bosutinib [151], 500 nM, ChemieTek) for 30 minutes.

2.5 Flow cytometry

FACS analysis and cell sorting was performed at the Moores UCSD Cancer Center Shared Resource. Expression of integrins was assessed with mouse anti-human αvβ5 (P1F6) [152] and β1 (P4C10) [152] followed by incubation in Alexa Fluor 647 nm-conjugated secondary antibodies (Life Technologies).

2.6 Adhesion assays

5 x 10^5 cells were allowed to adhere to 96-well plates coated with vitronectin (10 ug/mL) or fibronectin (5 ug/mL) for 16 hours at 4 °C and

blocked with 5% BSA (Sigma) in PBS for 2 hours at 37 °C. Cell adhesion (30, 60, 90 minutes) was terminated with 0.01% crystal violet (Sigma) in 20% methanol PBS for 10 minutes. Excess staining solution was washed away with PBS prior to elution with 100% methanol and reading in a 96-well plate reader (PowerWave XS2, BioTek) at optical density 570 nm. Similar results were found in two independent experiments with three replicates per cell line.

2.7 Viability assays

5 x 10^4 cells were plated in a 96-well plate and incubated for 24, 48, or 72 hours. Cell viability was assessed with XTT (1 mg/mL, Sigma) activated with N-methyl dibenzopyrazine methyl sulfate (PMS, 1 mg/mL, Sigma). Color was read in a 96-well plate reader (PowerWave XS2, BioTek) at optical density 450 nm. Similar results were found in three independent experiments with six replicates per cell line.

2.8 Migration assays

Cells were serum-starved for 16 hours before treatment with or without EGF (50 ng/mL, Millipore) for 15 minutes. Cells were washed with PBS to remove EGF prior to inoculation (2 x 10^6 cells) on Boyden chambers in triplicate. The bottoms of 0.8 μm pore Boyden chambers (Corning) were pre-coated with vitronectin (10 ug/mL) or type I collagen (5 ug/mL, BD Biosciences) for 1 hour at 37 °C before washing with PBS to remove excess protein. Cell migration on vitronectin (16 hours) or collagen (3 hours) was

terminated with 0.01% crystal violet (Sigma) in 20% methanol PBS for 15 minutes. The tops of the Boyden chambers were cleaned with cotton swabs, and wells were washed thoroughly with water prior to counting the number of cells that had migrated through the chamber. For some experiments, cells were transfected with nonsilencing or MUC1 small interfering RNAs (Qiagen) using Lipofectamine2000 (Life Technologies), and migration assays were performed 48 hours after transfection. For some experiments, cells were treated with or without Src inhibitor (bosutinib, 500 nM, ChemieTek) for 30 minutes prior to migration assays. Results are expressed as mean ± s.e.m. of three replicates. Similar findings were observed in 3 independent experiments.

2.9 Metastasis assays

Cells were serum-starved for 16 hours before treatment with or without EGF (50 ng/mL) for 15 minutes. Cells were washed with PBS to remove EGF prior to inoculation (2×10^7 cells) on the chorioallantoic membrane of 10 day-old embryonated chicken eggs (McIntyre Poultry & Fertile Eggs, Lakeside, California). After 10 days, primary tumors were collected and weighed to determine tumor mass and lungs were harvested to assess spontaneous pulmonary metastasis. Genomic DNA was extracted from the chick lungs with phenol:chloroform (Amresco) and the number of cells that had metastasized was quantified by Q-PCR for human *Alu* sequence and chicken GAPDH normalized to a standard curve generated from genomic DNA extracted from

chicken lung homogenates containing a serial dilution of a known quantity of FG cells. Primer sequences are as follows:

Alu: 5'-ACGCCTGTAATCCCAGCACTT-3'

5'-TCGCCCAGGCTGGAGTGC-3' [153]

GAPDH: 5'-GAGGAAAGGTCGCCTGGTGGATCG-3'

5'-GGTGAGGACAAGCAGTGAGGAACG-3' [153]

Each point represents a separate egg, n ≥ 6 eggs per group. Similar findings were observed in 3 independent experiments.

2.10 Subcellular fractionation

Cells were lysed in 0.05% NP-40 (Sigma) supplemented with protease inhibitors. Lysate was incubated with gentle agitation for 15 minutes at 4 °C prior to centrifugation at 3,000 rpm for 5 minutes at 4 °C to pellet nuclei. The supernatant was collected and centrifuged 14,000 rpm for 15 minutes at 4 °C to remove insoluble particles. The resulting supernatant was collected as the cytoplasmic fraction. The nuclear pellet was washed 10 times with cytoplasmic buffer prior to lysing with RIPA buffer supplemented with protease inhibitors. Nuclear lysate was also centrifuged 14,000 rpm for 15 minutes at 4 °C to remove insoluble particles, and the resulting supernatant was collected as the nuclear fraction.

2.11 Immunofluorescence

5×10^5 cells were seeded directly on 22mm coverslips (Fisher Scientific) in 6-well plates for 24 hours at 37 °C. Cells were serum-starved for 16 hours before treatment with or without EGF (50 ng/mL) for 15 minutes. Cells were fixed with 4% paraformaldehyde (Ted Pella, Inc.) in PBS for 15 minutes at 25 °C. Following permeabilization with 0.1% Triton X-100 (Bio-Rad) in PBS for 2 minutes, cells were blocked with 3% BSA (Sigma) in PBS for 1 hour at 25 °C. Nuclei were visualized with TO-PRO-3 (Life Technologies). Coverslips were mounted onto slides with VECTASHIELD Hardset (Vector Labs) and images were acquired using laser scanning confocal microscopy under a 60x/1.4 NA oil objective (Nikon C1si, Nikon Instruments). Nuclear Mucin-1 was quantified using MetaMorph (Molecular Devices). Data is expressed as a percentage of total detectable Mucin-1 ± s.e.m. of $n \geq 20$ images per group acquired during two independent experiments with three replicates each.

2.12 Quantitative PCR

Cells were serum-starved for 16 hours prior to lysis with Trizol (Life Technologies) according to the manufacturer's instructions. cDNA was generated using the Superscript III First-strand Synthesis Kit (Life Technologies). Reactions containing 200ng cDNA were prepared in QuantiTect SYBR Green Master Mix (Qiagen) and subjected to quantitative RT-PCR analysis using a Smart Cycler (Cepheid). Values were obtained for the threshold cycle (Ct) for each gene and normalized to β-actin. For some

experiments, cells were treated with EGF (50 ng/mL) for 15 minutes prior to washing with PBS and incubation for 1, 3, 8, or 24 h before RNA extraction. Primer sequences are as follows:

TWIST: 5'-AAGAGGTCGTGCCAATCAG-3'

5'-GGCCAGTTTGATCCCAGTAT-3'

Designed using Primer Express 3.0 (Applied Biosystems)

SNAI1: 5'-AATCGGAAGCCTAACTACAGCG-3'

5'-GTCCCAGATGAGCATTGGCA-3'

PrimerBank ID# 18765741a1

SNAI2: 5'-ATATTCGGACCCACACATTACCT-3'

5'-GCAAATGCTCTGTTGCAGTGA-3'

PrimerBank ID# 11276067a3

VIM: 5'-AGAACTTTGCCGTTGAAGCTG-3'

5'-CCACAGGGAGTGAATCCAGATTA-3'

PrimerBank ID# 4507895a2

LOX: 5'-ATGAGTTTAGCCACTTGTACCTGCTT-3'

5'-AAACTTGCTTTGTGGCCTTCA-3' [154]

Values were obtained for the threshold cycle (Ct) for each gene and normalized to β-actin. Values are provided as fold change. For some experiments, cells were treated with EGF (50 ng/mL) for 1, 3, 8, or 24 hours before RNA extraction.

2.13 Mouse tumor experiments

Tumor lysates from nude mice bearing orthotopic pancreatic tumors of FG cells were generously provided by Jay S. Desgrosellier, a senior member of the Cheresh lab. The mice were treated with vehicle or Src inhibitor (dasatinib [57], 30 mg/kg, ChemieTek) twice daily by oral gavage for four weeks as previously described.[79]

2.14 Statistical analysis

Data presented represent mean ± s.e.m. Statistical analyses were performed with Excel (Microsoft) or Prism (GraphPad). Statistical differences for one factor between two groups or more than two groups were determined with an unpaired Student's *t*-test or an analysis of variance (ANOVA) with *post-hoc* testing, respectively. Statistical significance was defined as $P < 0.05$.

Chapter 3

Identification of novel candidate effectors of tumor cell metastasis mediated by EGFR and Src

3.1 Introduction

EGFR and Src signaling have been demonstrated to play essential roles in tumor cell migration and metastasis. Therefore, it is important to characterize the molecular mechanisms by which EGFR and Src signaling enhance cell motility.

We previously demonstrated two distinct pathways of tumor cell migration and metastasis that differ based on their dependence of EGF-mediated Src kinase activity and the activation of integrin αvβ5.[41, 73] While integrin αvβ5 – the only vitronectin receptor in these cells – is unable to initiate FG human pancreatic carcinoma cell migration/invasion in the absence of growth factor stimulation [81], EGF stimulation enhances the ability of cells expressing integrin αvβ5 to invade *in vitro* and metastasize *in vivo*.[41, 73] Furthermore, cell migration and metastasis mediated by integrin αvβ5 requires EGF-dependent activation of Src, and activated Src is sufficient for carcinoma cell migration and metastasis.[41] In contrast, tumor cell migration mediated by β1 integrins is independent of EGFR and Src activity.[41] Similarly, an EGF pre-treatment enhances spontaneous tumor cell metastasis without affecting primary tumor growth in the well-characterized embryonic chicken chorioallantoic membrane (CAM) model [153, 155] by activating Src kinase and integrin αvβ5.[41] Thus, EGF-induced tumor cell migration mediated by integrin αvβ5 serves as a surrogate assay for metastasis *in vivo*. Together,

these findings suggest that an EGFR/Src/αvβ5 signaling axis promotes the metastatic spread of human carcinomas.

Here, we systematically evaluate EGFR and Src substrates taking an *in silico* approach to identify novel effector molecules essential for tumor cell metastasis mediated by EGFR and Src. Taking advantage of shRNA technology, we assessed the functional requirement of lead candidates in EGF-induced cell migration *in vitro*. We also evaluated the biological requirement of lead candidates in EGF-induced tumor cell metastasis using the chick CAM model. We show that the transmembrane glycoprotein MUC1 is required for EGF-induced pulmonary metastasis, but not primary tumor growth, of FG human pancreatic carcinoma cells.

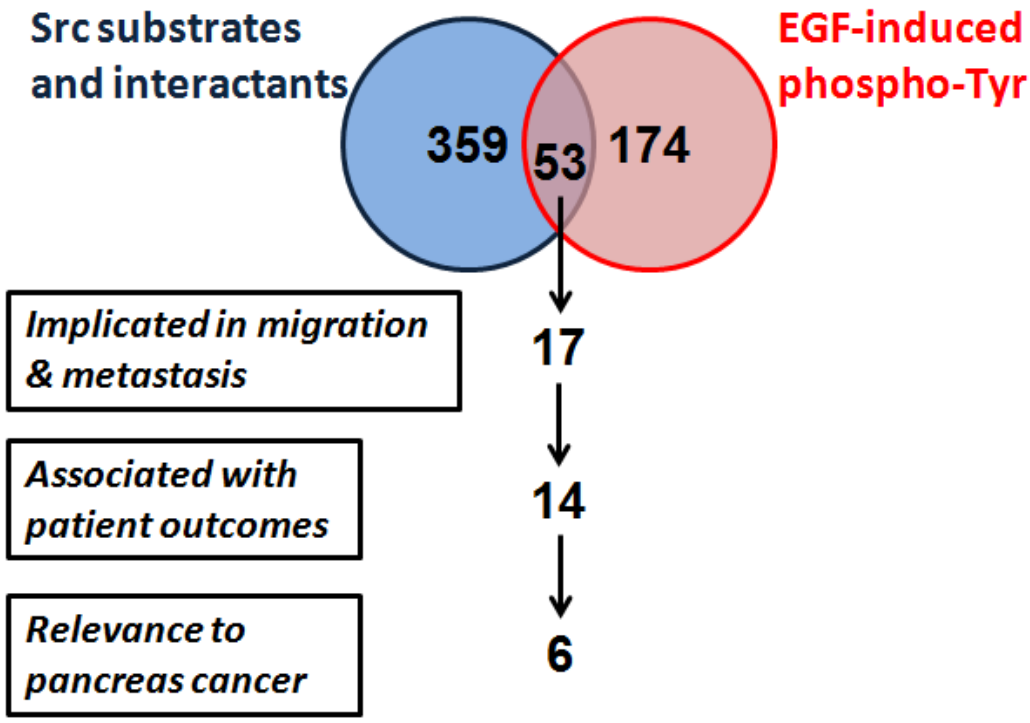

Figure 3.1: Venn diagram depicting *in silico* approach to identifying novel effectors of tumor cell metastasis mediated by EGFR and Src. Known Src substrates and interactants and substrates of tyrosine phosphorylation following treatment with EGF were cross-referenced to identify common candidate effectors. Candidates were systematically evaluated for their association with cell migration and metastasis, overall survival and pancreatic ductal adenocarcinoma.

3.2 Results

3.2.1 *In silico* screening approach identifies candidate effectors of tumor metastasis dependent on EGFR and Src

EGFR and Src signaling promote tumor cell metastasis.[14, 16, 41, 156, 157] EGFR controls various signaling pathways including those of the phosphoinositol-3-kinases, mitogen-activated protein kinases, and Janus kinases. In addition, activated EGFR recruits and activates Src, another key node in signaling pathways.[38, 39, 40] Importantly, we recently determined that EGF-induced metastasis requires activation of Src.[41] Therefore, we sought to identify novel effectors essential for tumor cell metastasis dependent on EGFR and Src activity using an *in silico* approach (**Figure 3.1**). Known Src substrates and interactants (n = 359) were cross-referenced with substrates of tyrosine phosphorylation following treatment with EGF (n = 174). Hits found in both categories (n = 53) were filtered for their association with cell migration, tumor cell metastasis, and cancer patient survival. Six lead candidates (*c*-Abl, Caspase-8, Cortactin, Mucin-1, Stat3 and Villin-1) were selected for initial follow-up.

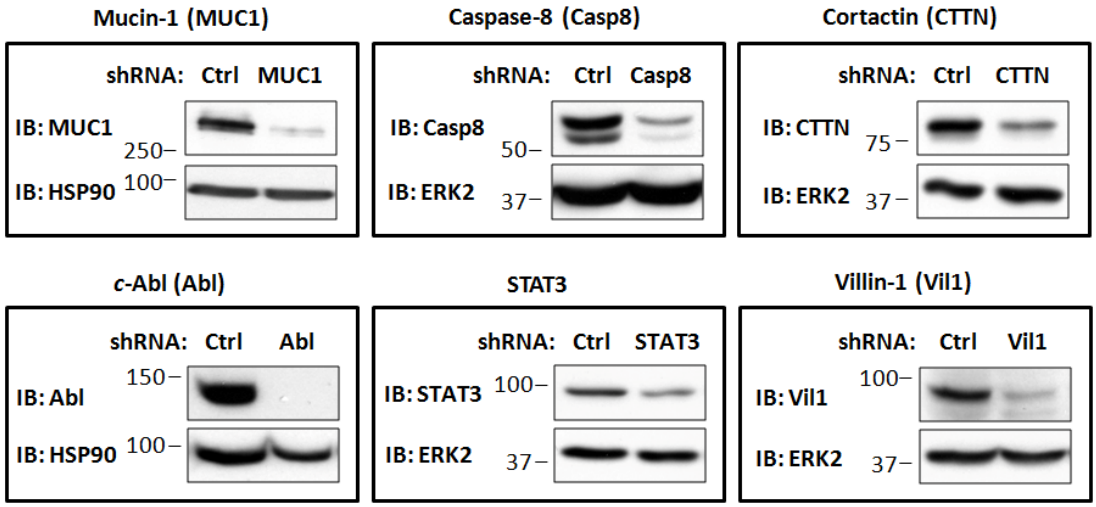

Figure 3.2: Stable knockdown of six lead candidates in FG cells. Immunoblot of whole cell lysates from cells expressing a shRNA targeting each lead candidate or a control shRNA. Three or more clones were pooled to avoid non-specific clonal effects. Similar findings were observed in two independent experiments.

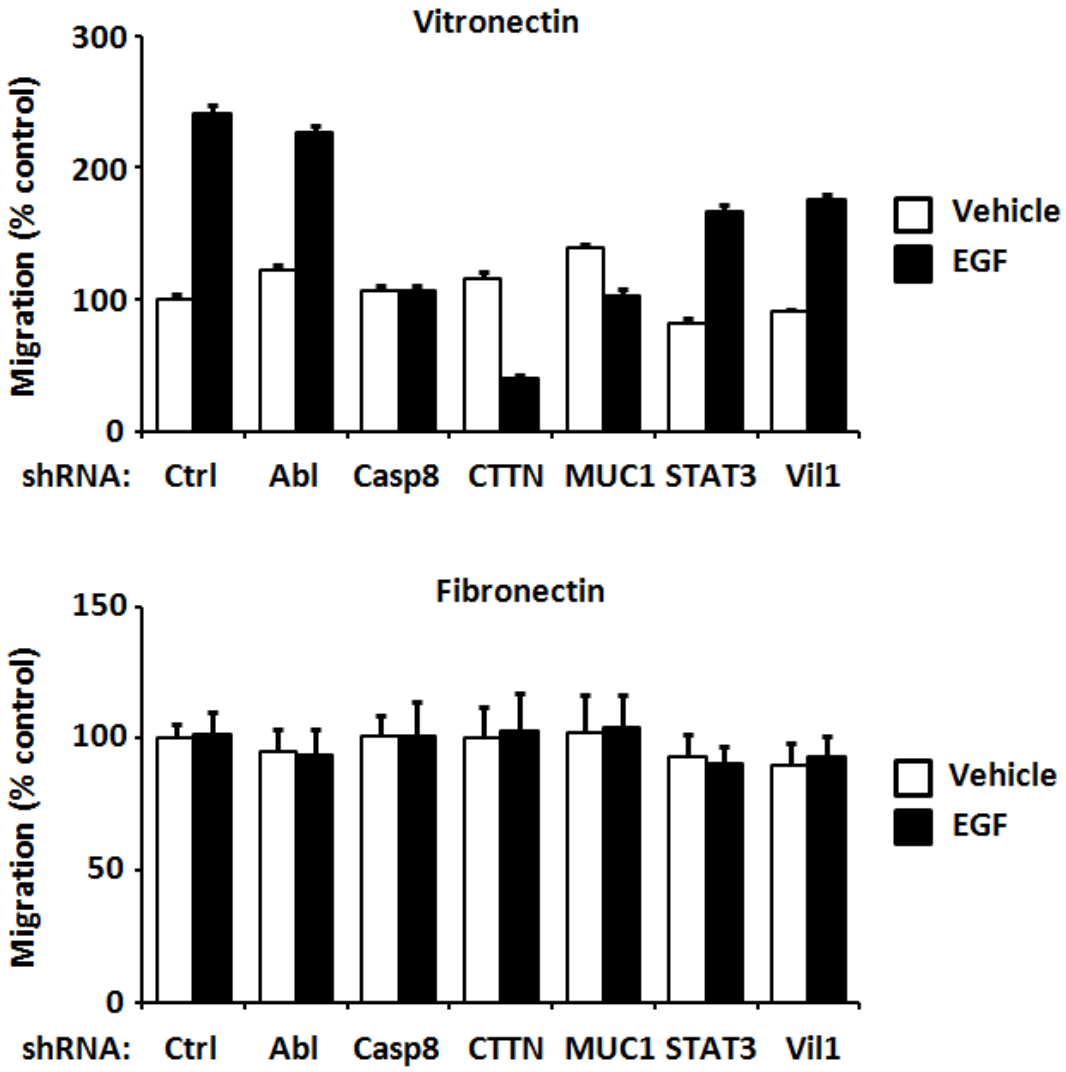

Figure 3.3: MUC1, Casp8 and CTTN are required for EGF-induced cell migration. Migration assays on a vitronectin (top) or a fibronectin (bottom) substrate comparing FG cells expressing a shRNA targeting each lead candidate or a control shRNA with or without a 15 minute pre-treatment of EGF. Cells were washed with PBS prior to inoculation on Boyden chambers to remove EGF. Results are expressed as mean ± s.e.m. of three replicates. Similar findings were observed in three independent experiments.

3.2.2 Functional validation of lead candidates reveals a requirement for MUC1, Caspase-8 and Cortactin in EGF-induced cell migration

To determine the functional relevance of lead candidates derived from our *in silico* screen in EGF-induced tumor metastasis, we first assessed their roles in EGF-induced cell migration as a surrogate assay for metastasis. Expression of each lead candidate was silenced by shRNA in FG human pancreatic carcinoma cells (**Figure 3.2**), which were then allowed to migrate through a Boyden chamber coated with a vitronectin or a fibronectin substrate following a 15 minute pre-treatment with vehicle control or EGF. As expected, EGF enhanced the migration on vitronectin for cells expressing a control shRNA (**Figure 3.3**). While knockdown of *c*-Abl, Stat3, or Villin-1 had no effect on EGF-induced cell migration, knockdown of MUC1, Caspase-8, or Cortactin abolished EGF-induced cell migration (**Figure 3.3**). This finding supports a role for MUC1, Caspase-8 and Cortactin in the EGF-dependent cell migratory response on vitronectin mediated by integrin αvβ5, these cell's sole vitronectin receptor. In contrast, tumor cell migration on fibronectin mediated by β1 integrins was independent of EGF stimulation and each lead candidate since knockdown of the lead candidates had no effect on this migration response (**Figure 3.3**).

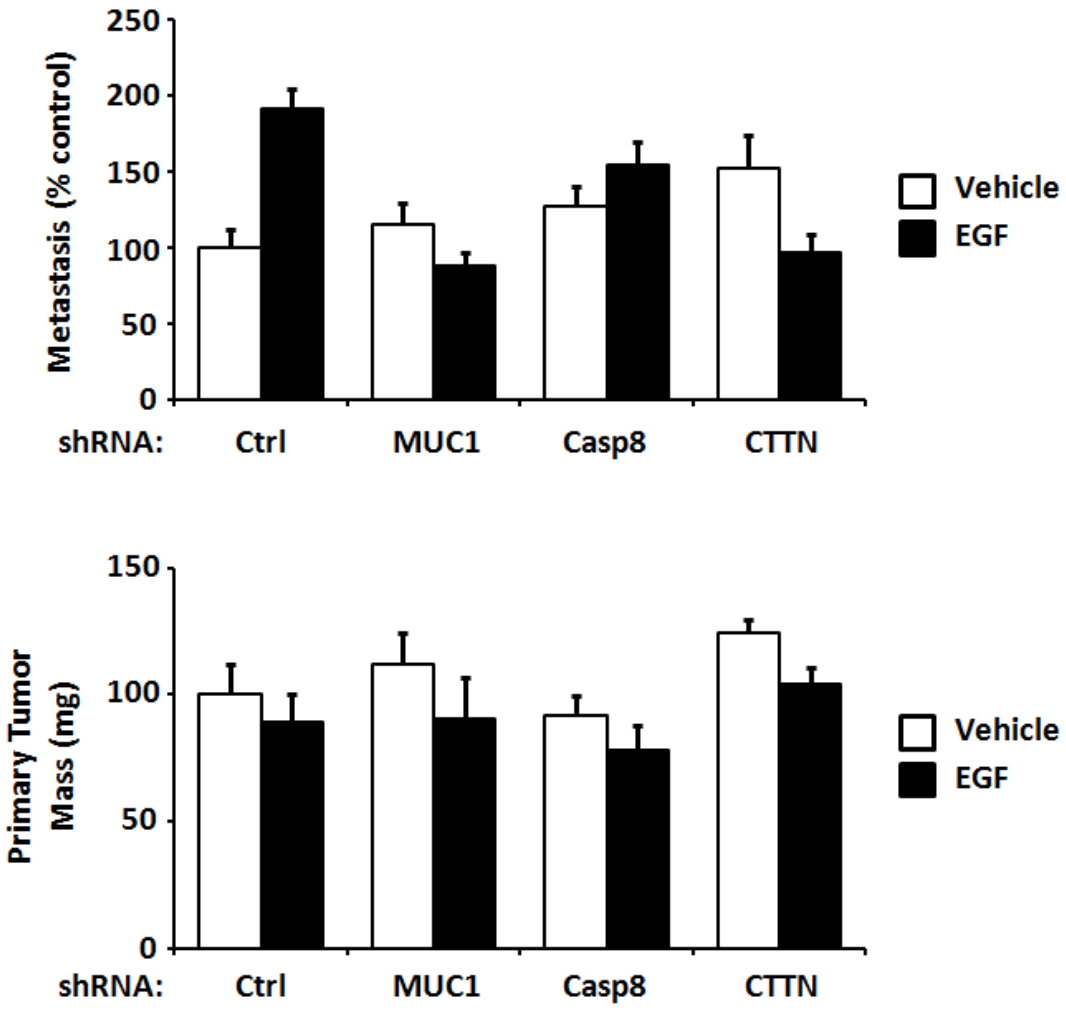

Figure 3.4: MUC1 is required for EGF-induced tumor cell metastasis without affecting primary tumor growth. FG cells expressing a shRNA targeting each lead candidate or a control shRNA with or without a 15 minute pre-treatment of EGF were inoculated on to the chorioallantoic membrane of 10 day-old embryonated chicken eggs and assessed for spontaneous pulmonary metastasis (top) and primary tumor formation (bottom) after 10 days. Cells were washed with PBS prior to inoculation to remove EGF. Metastasis was quantified by Q-PCR for human *Alu* sequence and chicken *GAPDH* normalized to a standard curve. Results are expressed as mean ± s.e.m. of at least 18 eggs pooled from three independent experiments.

3.2.3 MUC1 is required for EGF-induced tumor cell metastasis but not primary tumor growth

We next assessed the biological relevance of MUC1, Caspase-8, and Cortactin in EGF-induced tumor metastasis *in vivo* using the well-characterized embryonic chick CAM model.[153, 155] FG cells stimulated with a 15 minute treatment of vehicle control or EGF were implanted on the CAM of 10 day-old chick embryos and allowed to spontaneously metastasize to the lungs. As expected, EGF enhanced the pulmonary metastasis of cells expressing a control shRNA (**Figure 3.4**). With knockdown of Caspase-8, we observed a trend for enhanced metastasis with EGF stimulation (**Figure 3.4**). Knockdown of Cortactin eliminated EGF-induced metastasis but enhanced pulmonary metastasis in the absence of EGF when compared to cells expressing a control shRNA (**Figure 3.4**). Importantly, knockdown of MUC1 abolished EGF-induced tumor cell metastasis without preventing primary tumor growth (**Figure 3.4**). Altogether then, these data support an essential role for MUC1 in EGF-induced metastasis of FG cells.

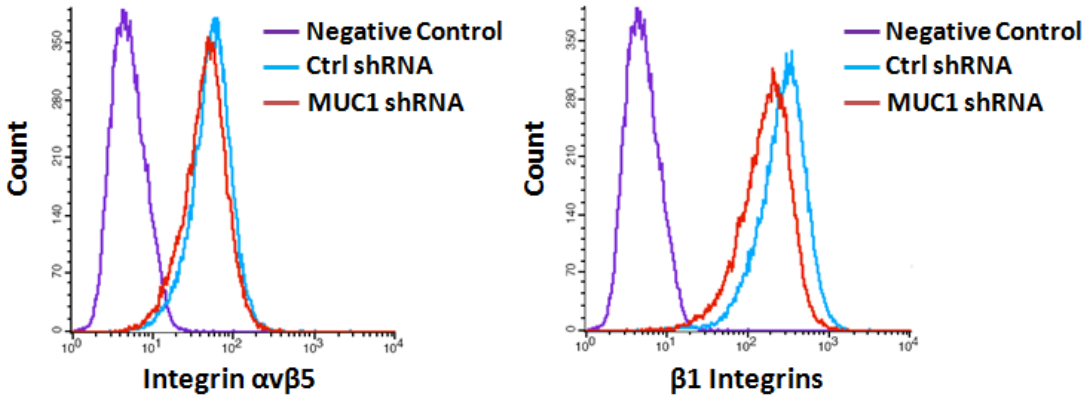

Figure 3.5: Effect of MUC1 knockdown on integrin expression. Cell surface expression of integrin αvβ5 (left) and β1 integrins (right) were assessed by FACS in FG cells expressing a control shRNA or MUC1 shRNA. Integrins were detected with mouse anti-human αvβ5 (P1F6) or β1 (P4C10). MUC1 knockdown slightly reduces surface expression of both αvβ5 and β1 integrins. Similar findings were observed in three independent experiments.

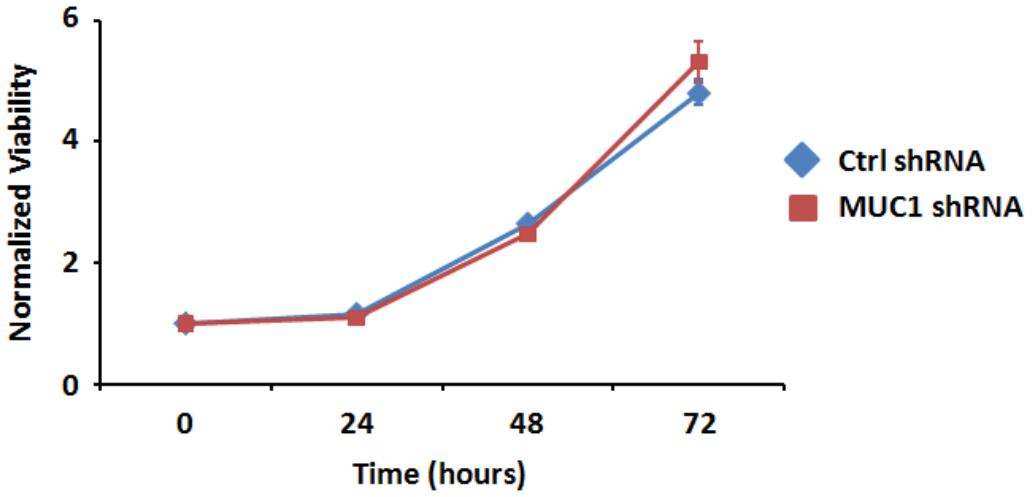

Figure 3.6: MUC1 knockdown has no significant effect on cell viability. FG cells expressing a control shRNA or MUC1 shRNA were incubated for varying amounts of time and cell viability was assessed by XTT assay. Results are expressed as mean ± s.e.m. of six replicates. Similar findings were observed in three independent experiments.

3.2.4 Effect of MUC1 knockdown on integrin expression

The heavily glycosylated ectodomain of MUC1 extends over 100 nm from the cell surface, well beyond the 10 nm glycocalyx.[119] As such, MUC1 has been demonstrated to both enhance and disrupt cell-matrix adhesion mediated by β1 integrins.[121, 158] Furthermore, knockdown of MUC1 has been shown to suppress expression of both αv and β1 integrins.[159] Therefore, we considered whether knockdown of MUC1 in FG human pancreatic carcinoma cells inhibited integrin expression. Cell surface expression of both integrin αvβ5 and β1 integrins was slightly suppressed by knockdown of MUC1 (**Figure 3.5**).

3.2.5 Knockdown of MUC1 does not inhibit tumor cell proliferation

MUC1 expression has been demonstrated to regulate tumor cell proliferation under two-dimensional [160, 161] and anchorage-independent [161, 162, 163] conditions. Likewise, MUC1 expression regulates tumor growth in subcutaneous [162] and orthotopic pancreatic mouse models.[158] Thus, we considered whether knockdown of MUC1 in FG cells inhibited cell proliferation. Cell viability as measured by XTT assay was not significantly affected by knockdown of MUC1 (**Figure 3.6**). This is consistent with our observation that primary tumor mass in the chick CAM model was not significantly changed by knockdown of MUC1 (**Figure 3.4**). These findings

support a role for MUC1 in EGF-induced tumor cell migration and metastasis that is independent of cell viability *in vitro* and *in vivo*.

3.2.6 MUC1 knockdown does not impair EGFR signaling

MUC1 interacts with EGFR, and this interaction inhibits ligand-induced EGFR internalization and degradation.[101, 105, 106] We asked whether MUC1-mediated stabilization of EGF signaling might promote EGF-induced FG cell migration and metastasis. We observed that knockdown of MUC1 did not significantly suppress either basal surface expression of EGFR (**Figure 3.7**) or EGFR signaling in cells following treatment with EGF as measured by phospho-Tyrosine, EGFR pY1173 and Src pY416 (**Figure 3.7**). Likewise, MUC1 knockdown did not lead to enhanced ligand-induced EGFR degradation (**Figure 3.7**). These findings support a critical role for MUC1 in EGF-induced tumor cell migration and metastasis that is independent of EGFR recycling and general signaling.

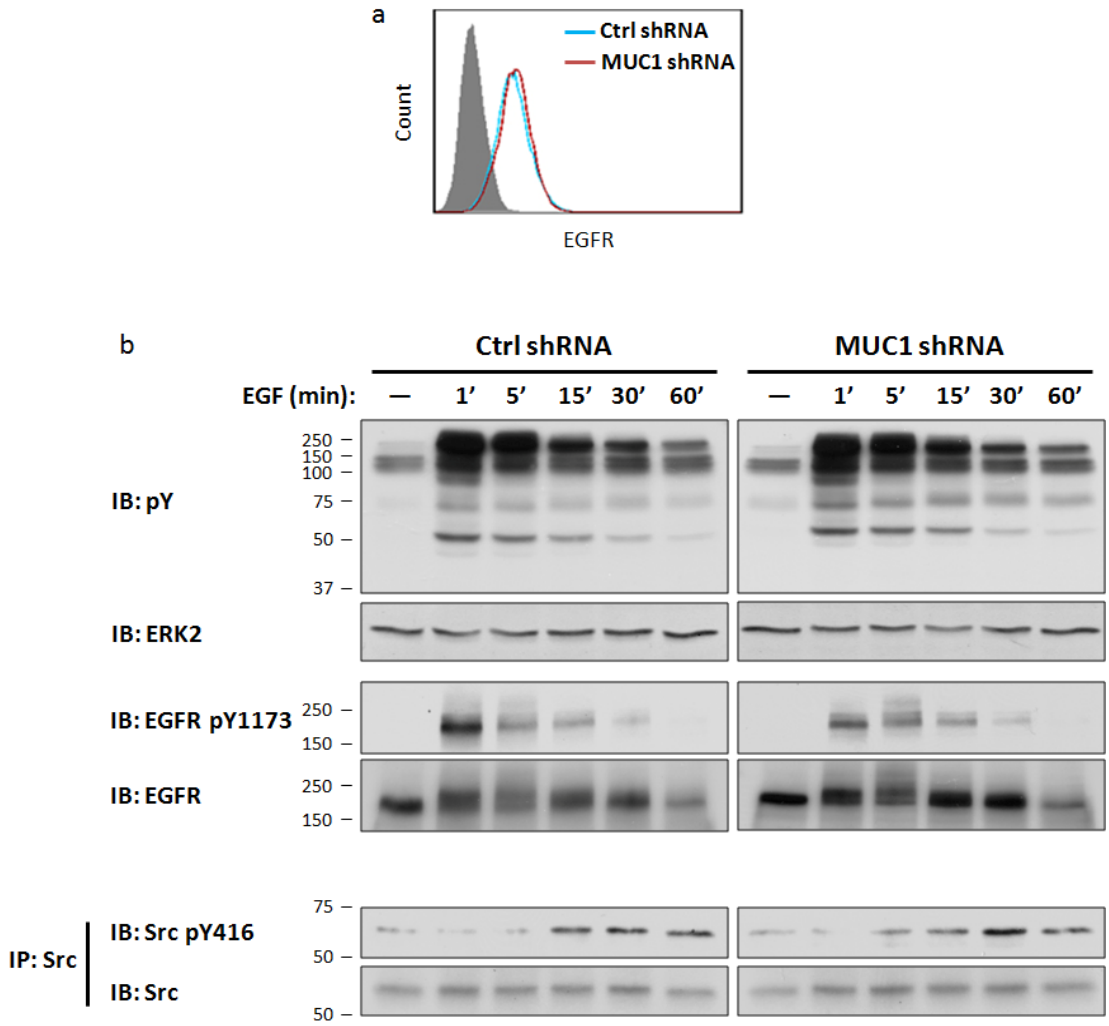

Figure 3.7: MUC1 knockdown does not impair EGFR signaling. (**a**) Cell surface expression of EGFR was assessed by FACS in FG cells expressing a control shRNA or MUC1 shRNA (histogram). (**b**) Ligand-induced EGFR signaling was also assessed by immunoblot in cells treated with EGF (50 ng/mL) for varying amounts of time. Similar findings were observed in two independent experiments.

3.3 Conclusions

Both EGFR and Src are key nodes of multiple signaling pathways, and both are associated with tumor progression and metastasis. We previously reported that EGF-induced tumor cell metastasis requires Src activity.[41] However, the precise molecular mechanisms by which EGF-induced Src activity promotes metastasis mediated by integrin αvβ5 are unknown. Here we systematically evaluate substrates of EGFR and Src signaling to identify novel effector molecules essential for EGF-induced tumor cell metastasis.

Among the lead candidate effectors evaluated in our study, MUC1 was found to be required for EGF-induced FG cell metastasis without affecting primary tumor formation. MUC1 has been linked to expression of EGFR and integrins and to cell proliferation, but we did not find evidence that knockdown of MUC1 affected these characteristics in FG cells. Therefore, the molecular mechanism by which MUC1 promotes EGF-induced FG cell metastasis will be explored in the subsequent work described in this dissertation.

Chapter 4

MUC1 cleavage by EGFR and Src signaling promotes tumor cell metastasis

4.1 Abstract

Tumor metastasis is a primary contributor to morbidity and mortality in cancer. We recently reported that EGF and other cytokines induce human carcinoma cell invasion and metastasis that is prevented by antagonists of Src or integrin αvβ5.[41, 82] In chapter 3, we demonstrated a requirement for MUC1 in EGF-induced FG cell metastasis in the chick CAM model without affecting primary tumor formation.

This chapter describes the finding that EGF stimulation promotes MUC1 cleavage and nuclear localization, resulting in the expression of genes linked to metastasis. The MUC1 cytoplasmic domain enhances FG cell migration on vitronectin *in vitro* and metastasis in the chick CAM model without enhancing primary tumor growth. Furthermore, pharmacological inhibition of Src kinase activity blocks both MUC1 cleavage and metastasis in mice. These findings establish MUC1 as a downstream effector of EGFR and Src signaling to promote FG cell invasion and metastasis.

4.2 Introduction

Tumor cell metastasis is a complex multi-step process involving local invasion, survival and colonization. Recent studies have revealed that cross-talk between growth factor receptors and integrins promotes carcinoma cell invasion and metastasis. Chapter 3 of this dissertation describes the finding that MUC1 is required for EGF-induced FG carcinoma cell migration on vitronectin and metastasis in the chick CAM model mediated by integrin αvβ5, the only vitronectin receptor expressed by these cells.

The MUC1 transmembrane glycoprotein is expressed by normal epithelial cells, but it is sharply upregulated and aberrantly glycosylated in human neoplasms, where it is associated with metastasis.[88, 89, 90] MUC1 interacts with ErbB family members including EGFR and is a substrate for Src.[101, 102, 107, 136] Importantly, the role of MUC1 in metastasis is associated with its intracellular domain, which enters the nucleus and initiates the transcription of genes associated with tumor metastasis such as *TWIST1*.[130, 131, 133, 134]

In this chapter of the dissertation, it is proposed that EGF-induced Src kinase activity promotes tumor cell metastasis mediated by integrin αvβ5 by inducing MUC1 cleavage. We demonstrate that EGF stimulation of FG cells promotes MUC1 cleavage and nuclear translocation of an intracellular fragment. Furthermore, the MUC1 cytoplasmic domain is required for EGF-

induced FG cell migration on vitronectin *in vitro*, and expression of the MUC1 cytoplasmic domain promotes spontaneous cell migration on vitronectin *in vitro* and pulmonary metastasis in the chick CAM model without enhancing primary tumor formation. In addition, we show that Src kinase activity is required for MUC1 cleavage using pharmacological and genetic approaches. Finally, pharmacological inhibition of Src in an orthotopic pancreatic mouse tumor model blocks both MUC1 cleavage and FG cell metastasis. These findings identify a pathway of EGF-induced tumor cell metastasis that is mediated by Src-dependent cleavage of MUC1 and the activation of a specific integrin to promote cell invasion.

4.3 Results

4.3.1 EGF treatment induces MUC1 tyrosine phosphorylation

The MUC1 intracellular domain has been demonstrated to be a substrate of tyrosine phosphorylation in carcinoma cells following treatment with EGF [101, 131], FGF1 [131], HGF [130], and PDGF-BB [132]. Therefore, we asked whether EGF treatment induced tyrosine phosphorylation of MUC1 in FG cells. As expected, we observed that the MUC1 intracellular domain was tyrosine phosphorylated in cells following treatment with EGF (**Figure 4.1**).

4.3.2 EGF treatment induces MUC1 cleavage

Recent studies have demonstrated an important role for the MUC1 cytoplasmic domain in tumor cell invasion.[132, 150] Therefore, we considered whether EGF stimulation of FG cells could lead to cleavage of MUC1. To determine whether EGFR signaling induces cleavage of MUC1, whole cell lysates from FG cells treated with or without EGF were probed for the MUC1 cytoplasmic domain by immunoblotting. Within 5 minutes of EGF treatment, we observed increased levels of MUC1 cytoplasmic domain (**Figure 4.2**).

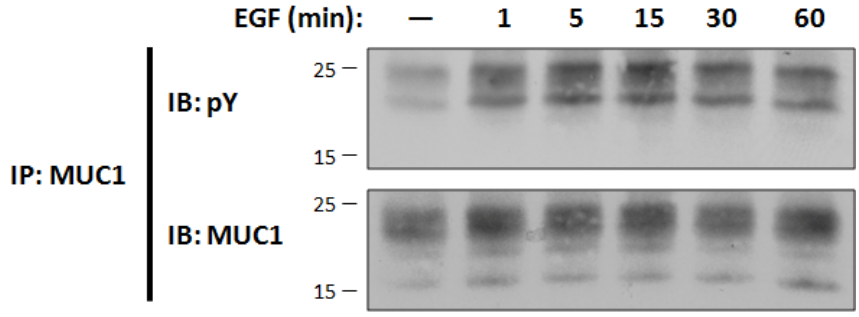

Figure 4.1: EGF treatment enhances MUC1 tyrosine phosphorylation. Immunoblot detecting tyrosine phosphorylation of the MUC1 transmembrane subunit with EGF treatment. MUC1 was immunoprecipitated from whole cell lysates of FG cells treated with EGF (50 ng/mL) for varying amounts of time and probed for phosphorylation on tyrosine. Membrane was stripped and reprobed for MUC1 as a loading control. Similar findings were observed in two independent experiments.

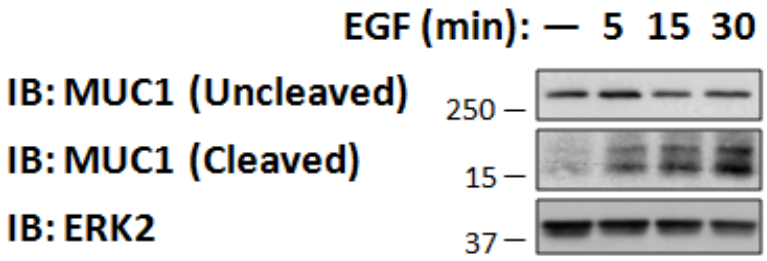

Figure 4.2: EGF treatment promotes cleavage of MUC1. Whole cell lysates from FG cells treated with EGF (50 ng/mL) were probed for MUC1 cleavage by immunoblot. Similar findings were observed in three independent experiments.

4.3.3 MUC1 expression correlates with aggressiveness in pancreatic tumor cell lines

MUC1 is overexpressed and aberrantly glycosylated in human neoplasms, and its expression correlates with metastasis.[88, 89, 90, 103] Therefore, we asked whether MUC1 expression correlates to metastatic propensity of human pancreatic carcinoma cell lines. We found that whereas only one of four cell lines derived from primary tumors expressed MUC1, all four cell lines derived from liver metastases or ascites fluid expressed MUC1 (**Figure 4.3**). Importantly, we detected cleaved MUC1 cytoplasmic domain in these aggressive cell lines (**Figure 4.3**). These data suggest that expression of MUC1 is associated with more aggressive behavior.

4.3.4 The MUC1 cytoplasmic domain is required for EGF-dependent cell migration

We next asked whether this EGF-dependent MUC1 cleavage product might play a role in EGF-dependent migration. To determine whether the MUC1 cytoplasmic domain was necessary for EGF-dependent migration, FG cells stably expressing MUC1 shRNA were transfected with shRNA-resistant full length MUC1 (MUC1.FL) or cytoplasmic domain-deleted MUC1 (MUC1.CT3) and allowed to migrate with a 15 minute pre-treatment of vehicle control or EGF. Whereas knockdown of MUC1 suppressed EGF-mediated cell migration on a vitronectin substrate, this response was reversed with

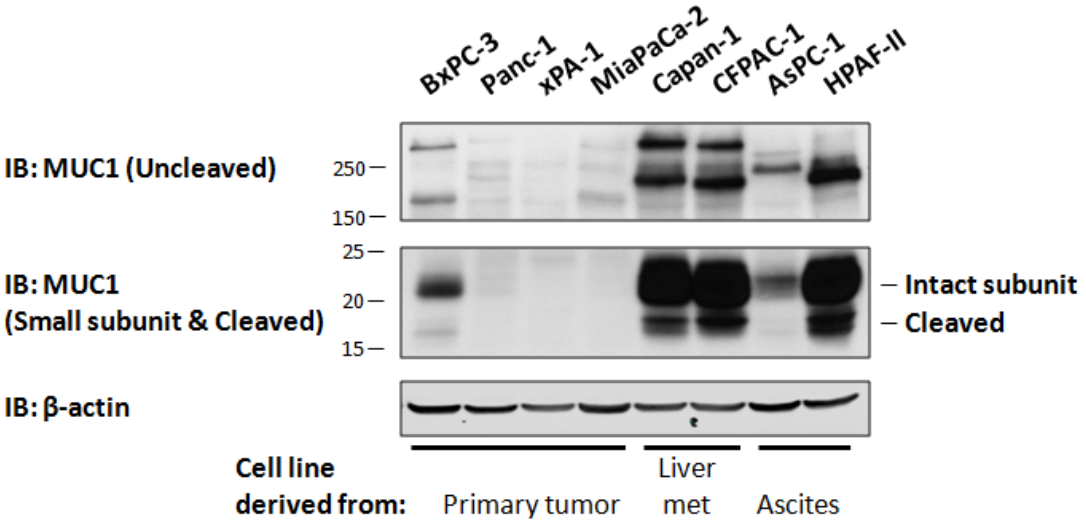

Figure 4.3: MUC1 expression correlates with tumor aggressiveness. Whole cell lysates from human pancreatic carcinoma cell lines were probed for MUC1 by immunoblot.

MUC1.FL but not MUC1.CT3 (**Figure 4.4**). In contrast, expression of either MUC1.FL or MUC1.CT3 did not significantly affect cell migration on a collagen substrate, consistent with our findings that MUC1 is not required for EGF-independent cell migration mediated by β1 integrins (**Figure 4.4**).

4.3.5 The MUC1 cytoplasmic domain is sufficient for carcinoma cell migration and metastasis

We next asked whether the MUC1 cytoplasmic domain (MUC1.CD) was sufficient to induce migration of FG cells in the absence of EGF. Interestingly, expression of MUC1.CD in FG cells was sufficient to promote spontaneous migration on a vitronectin substrate without EGF treatment but did not significantly affect migration on a collagen substrate (**Figure 4.5**).

Given that MUC1 is required for both EGF-dependent and Src-dependent tumor cell migration and that both EGF treatment and active Src are promote MUC1 cleavage, we considered whether the MUC1 cytoplasmic domain might enhance tumor cell metastasis. We tested whether expression of MUC1.CD or MUC1.CT3 in FG cells could drive spontaneous pulmonary metastasis in the chick CAM model. Interestingly, expression of MUC1.CT3 significantly enhanced spontaneous pulmonary metastasis compared to control cells (**Figure 4.6**). However, MUC1.CD enhanced spontaneous pulmonary metastasis to an even greater degree (**Figure 4.6**). Importantly,

expression of MUC1.CT3 enhanced primary tumor formation compared to MUC1.CD (**Figure 4.6**). These data indicate the MUC1 cytoplasmic domain promotes the spontaneous pulmonary metastasis of FG tumor cells without enhancing primary tumor growth in the chick CAM model.

4.3.6 Expression of the MUC1 cytoplasmic domain enhances cell proliferation

MUC1 expression regulates tumor cell proliferation *in vitro* [160, 161, 162, 163] and *in vivo* [158, 162]. Therefore, we asked whether expression of MUC1.FL, MUC1.CT3 or MUC1.CD altered the growth characteristics of FG cells. Interestingly, all three MUC1 constructs enhanced cell proliferation in two-dimensional conditions as measured by XTT assay (**Figure 4.7**). Surprisingly, while MUC1.FL stimulated cell proliferation relative to cells expressing a vector control, MUC1.CT3 did so to a greater degree (**Figure 4.7**). Furthermore, expression of MUC1.CD enhanced cell proliferation even more than MUC1.CT3 (**Figure 4.7**).

4.3.7 EGF treatment induces nuclear translocation of the MUC1 intracellular domain

The MUC1 cytoplasmic domain has been demonstrated to translocate to the nucleus, where it promotes the transcription of various genes linked to

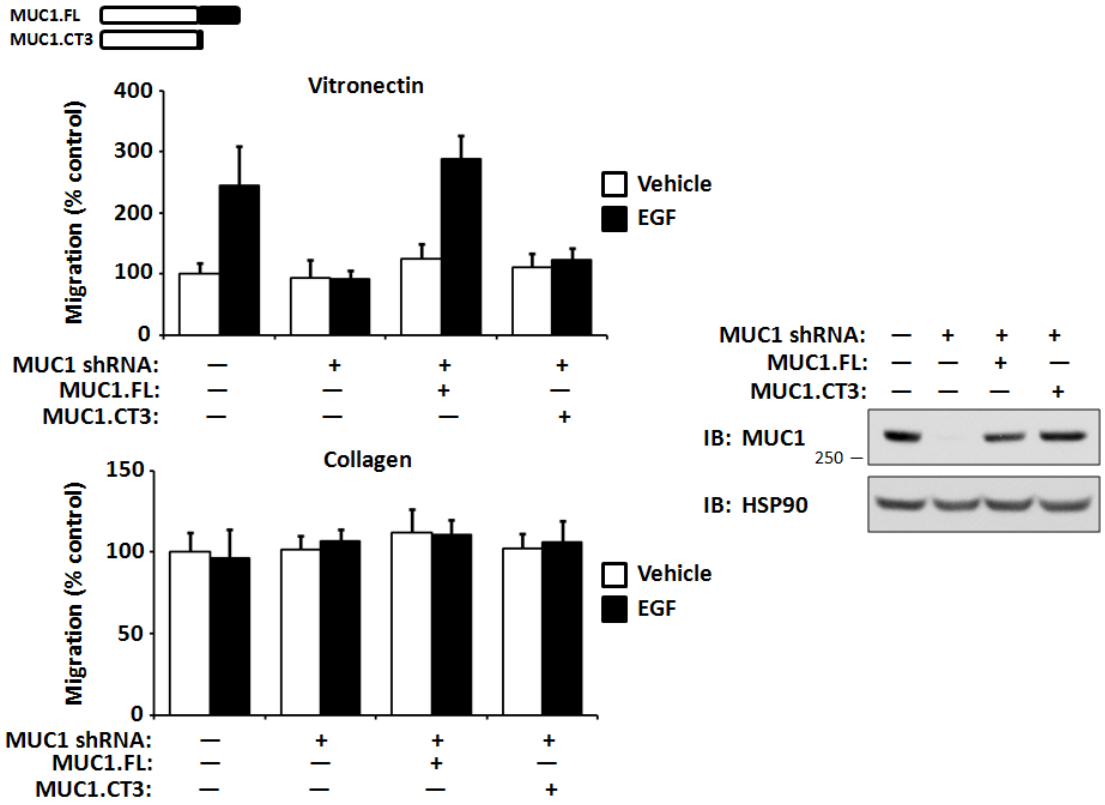

Figure 4.4: MUC1 cytoplasmic domain is required for EGF-induced cell migration. Migration assays on a vitronectin (top) or a collagen (bottom) substrate comparing FG cells co-expressing MUC1 shRNA and shRNA-resistant full-legnth MUC1 (MUC1.FL) or cytoplasmic domain-deleted MUC1 (MUC1.CT3) with or without a 15 minute pre-treatment of EGF. Cells were washed with PBS prior to inoculation on Boyden chambers to remove EGF. Results are expressed as mean ± s.e.m. of three replicates. Schematic illustration (top) of MUC1.FL and MUC1.CT3 protein products including ectodomain (white) and cytoplasmic domain (black). Immunoblot detecting MUC1 expression (right). Similar findings were observed in three independent experiments. $P < 0.0001$ comparing cells expressing MUC1.FL treated with vehicle control or EGF, $P = 0.3$ comparing cells expressing MUC1.CT3 treated with vehicle control or EGF, $P = 0.3$ (ANOVA) for migration on collagen.

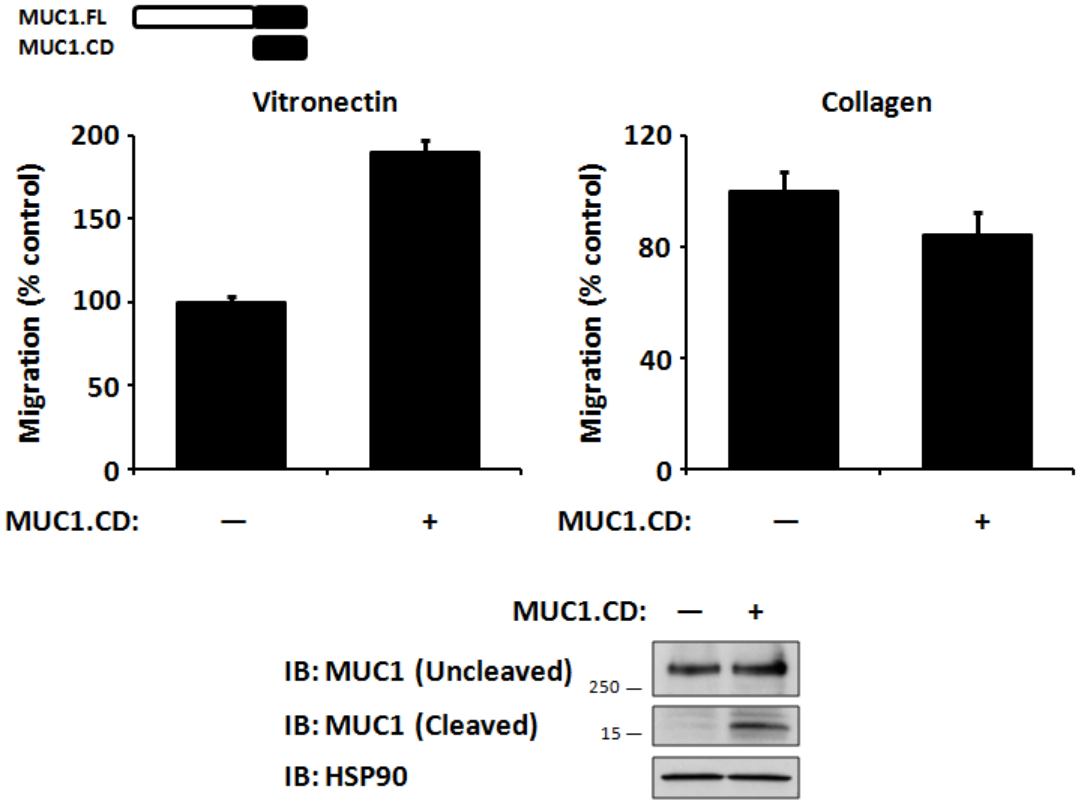

Figure 4.5: MUC1 cytoplasmic domain promotes cell migration mediated by integrin αvβ5. Migration assays on a vitronectin (left) or a collagen (right) substrate comparing FG cells expressing vector control or MUC1 cytoplasmic domain (MUC1.CD). Results are expressed as mean ± s.e.m. of three replicates. Schematic illustration (top) of full-length MUC1 (MUC1.FL) and MUC1.CD protein products including ectodomain (white) and cytoplasmic domain (black). Immunoblot detecting MUC1 expression (bottom). Similar findings were observed in three independent experiments. $P = 0.0003$ for migration on vitronectin, $P = 0.2$ for migration on collagen.

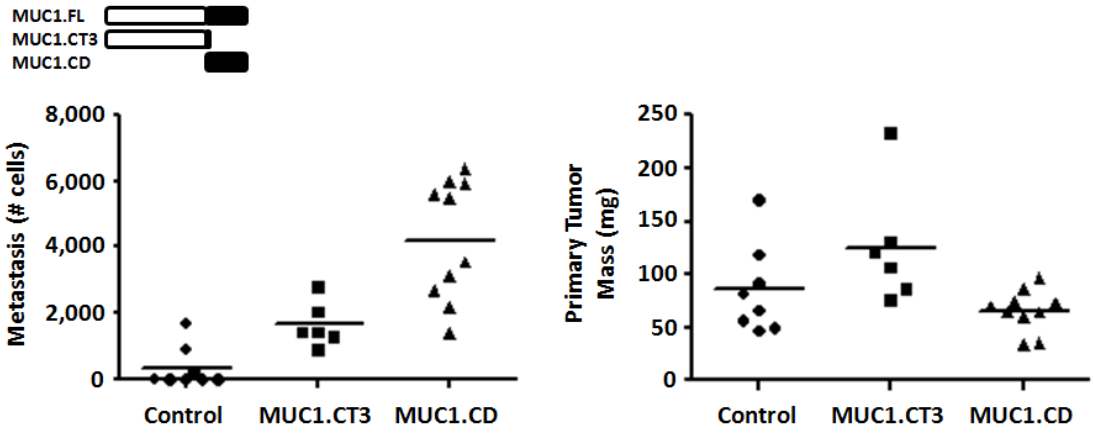

Figure 4.6: MUC1 cytoplasmic domain promotes spontaneous pulmonary tumor cell metastasis without enhancing primary tumor growth. FG cells expressing vector control, cytoplasmic domain-deleted MUC1 (MUC1.CT3), or MUC1 cytoplasmic domain (MUC1.CD) were inoculated on to the chorioallantoic membrane of 10 day-old embryonated chicken eggs and assessed for spontaneous pulmonary metastasis (left) and primary tumor formation (right) after 10 days. Metastasis was quantified by Q-PCR for human *Alu* sequence and chicken *GAPDH* normalized to a standard curve. Each point represents a separate egg, n ≥ 6 eggs per group. Schematic illustration (top) of full-length MUC1 (MUC1.FL), MUC1.CT3 and MUC1.CD protein products including ectodomain (white) and cytoplasmic domain (black). $P = 0.004$ for cells expressing vector control or MUC1.CT3, $P < 0.0001$ for cells expressing vector control or MUC1.CD, $P < 0.05$ for primary tumor mass of MUC1.CT3 compared to MUC1.CD.

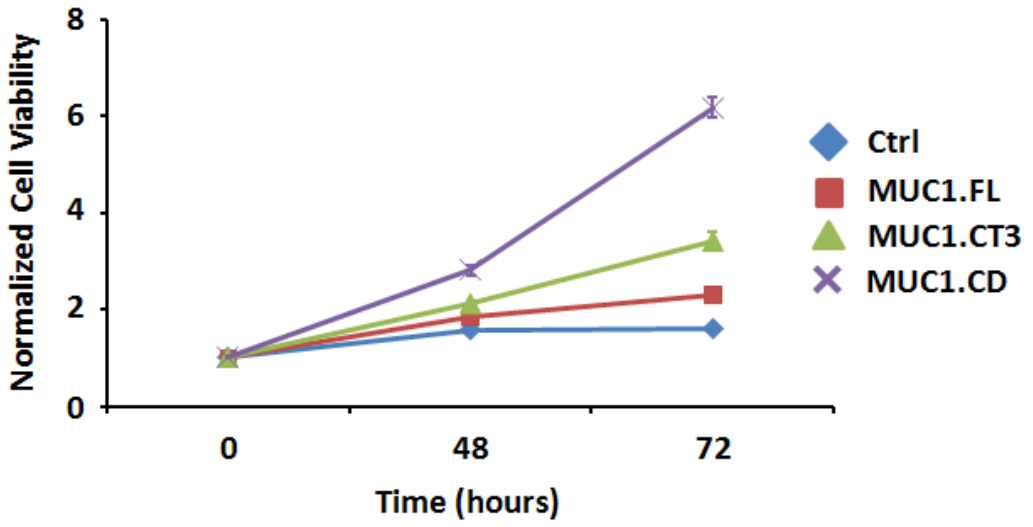

Figure 4.7: Effect of MUC1 expression on cell viability. FG cells expressing vector control, full-length MUC1 (MUC1.FL), cytoplasmic domain-deleted MUC1 (MUC1.CT3) or MUC1 cytoplasmic domain (MUC1.CD) were incubated for varying amounts of time and cell viability was assessed by XTT assay. Results are expressed as mean ± s.e.m. of four replicates. Similar findings were observed in three independent experiments.

tumor cell invasion and metastasis [128, 132, 133, 134, 164]. Therefore, we considered whether EGF stimulation of FG cells could lead to nuclear translocation of MUC1. To determine whether EGFR signaling induces nuclear translocation of MUC1, FG cells stimulated with a 15 minute treatment of vehicle control or EGF were probed for the MUC1 cytoplasmic domain by immunoblotting. EGF treatment increased the level of MUC1 cytoplasmic domain in the nuclei of these cells (**Figure 4.8**).

To further assess the role of the MUC1 cytoplasmic domain in the nuclei of tumor cells, we expressed MUC1.FL or MUC1.CD fused to GFP and monitored the cells for MUC1 localization. In the absence of EGF, MUC1.FL localized to the plasma membrane (**Figure 4.9**). However, a 15 minute treatment with EGF significantly enhanced nuclear localization of MUC1 (**Figure 4.9**). Interestingly, we observed that MUC1.CD spontaneously localized to the nucleus in the absence of EGF (**Figure 4.10**). Importantly, following EGF treatment, we detected enhanced transcription of several MUC1 target genes linked to tumor cell invasion and metastasis including *TWIST1*, *SNAI1*, and *SNAI2* (**Figure 4.11**). We observed that MUC1.CD spontaneously enhanced transcription of the same MUC1 target genes to a similar degree as EGF treatment (**Figure 4.11**). Together, these results indicate that EGFR signaling promotes translocation of the MUC1 cleavage product to the nucleus, where it regulates transcription of genes linked to invasion and metastasis.

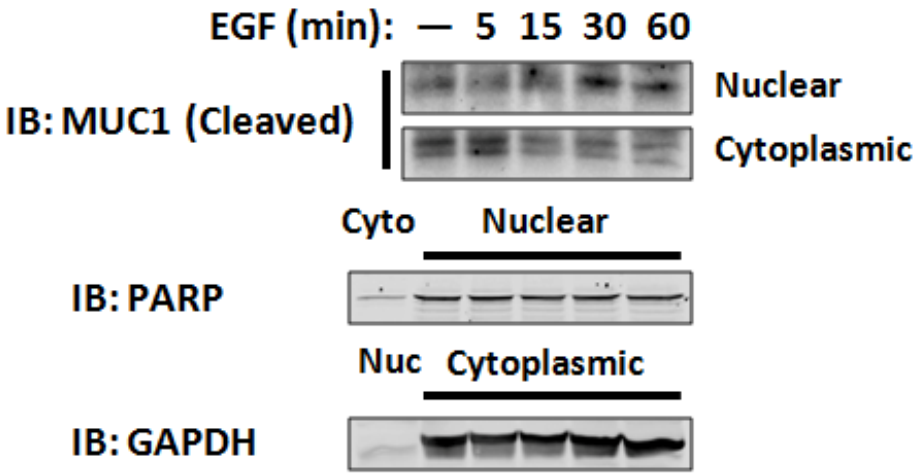

Figure 4.8: EGF treatment induces MUC1 nuclear localization. Immunoblot detected MUC1 cytoplasmic domain showing enrichment of MUC1 cytoplasmic domain with EGF treatment (50 ng/mL) in the nuclear fraction of FG cells. Fraction purity and loading were determined by immunoblot for PARP (Nuclear, Nuc) and GAPDH (Cytoplasmic, Cyto). Similar findings were observed in three independent experiments.

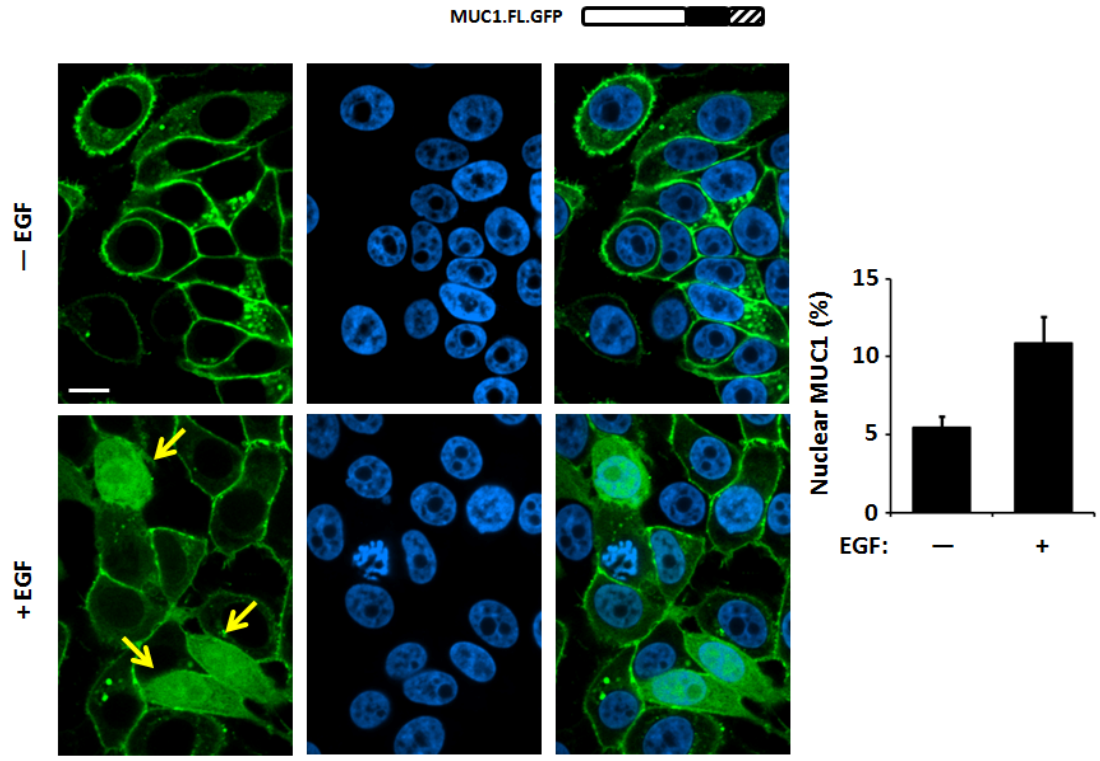

Figure 4.9: EGF treatment induces MUC1 nuclear localization. Representative images of immunofluorescence of MUC1.FL fused to GFP (MUC1.FL.GFP, green) with a 15 minute treatment with vehicle control or EGF. Nuclei are counter-stained with TO-PRO-3 (blue). Schematic illustration (top) of MUC1.FL.GFP including ectodomain (white), cytoplasmic domain (black) and GFP (hatched). Yellow arrows indicate cells with high levels of nuclear MUC1. Quantification of nuclear MUC1 (bar graph) is expressed as a percentage of total detectable MUC1 with n ≥ 20 images per group from three independent experiments. Scale bar represents 10 μm. $P < 0.0001$ for nuclear MUC1 with vehicle control or EGF.

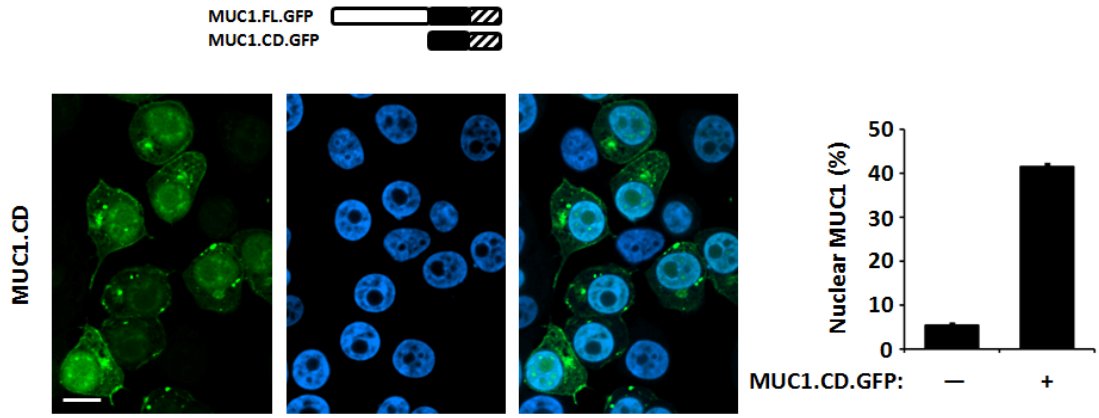

Figure 4.10: MUC1 cytoplasmic domain spontaneously localizes to the nucleus. Representative images of immunofluorescence of MUC1.CD fused to GFP (MUC1.CD.GFP, green). Nuclei are counter-stained with TO-PRO-3 (blue). Schematic illustration (top) of MUC1.FL.GFP and MUC1.CD.GFP including ectodomain (white), cytoplasmic domain (black) and GFP (hatched). Quantification of nuclear MUC1 (bar graph) is expressed as a percentage of total detectable MUC1 with n ≥ 20 images per group from three independent experiments. Scale bar represents 10 μm. $P < 0.0001$ for nuclear MUC1 with MUC1.CD.GFP compared to MUC1.FL.GFP.

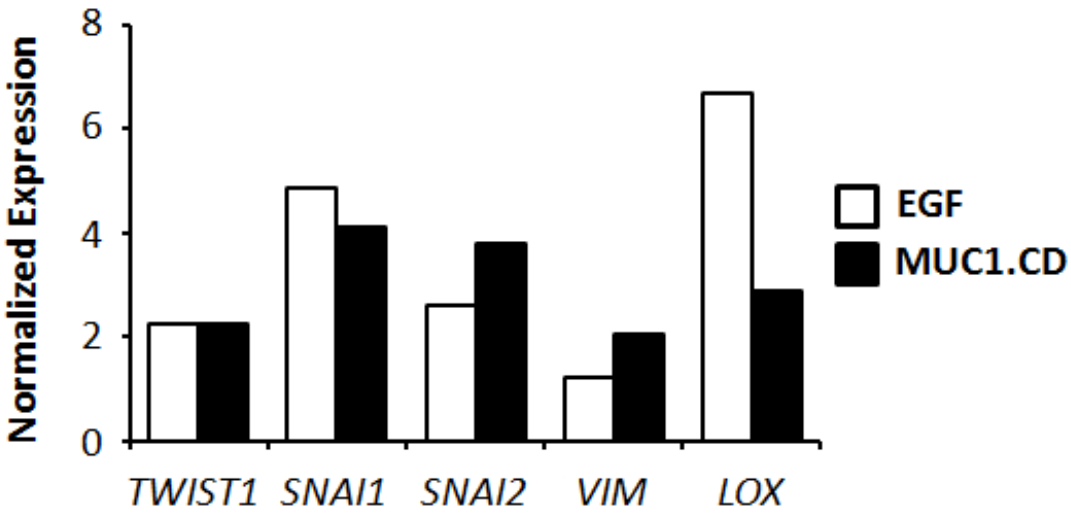

Figure 4.11: EGF treatment and MUC1 cytoplasmic domain promote expression of a metastasis signature. Quantitative RT-PCR of FG cells treated for 15 minutes with EGF (white) or expressing MUC1.CD (black) compared to either untreated or vector controls, respectively. For cells treated with EGF, peak expression over a 24 hour period are reported. Values have been normalized to β-actin.

4.3.8 Src is required for EGF-induced MUC1 tyrosine phosphorylation

Given that MUC1 is a substrate for Src [107], we considered whether EGF-mediated MUC1 tyrosine phosphorylation was Src-dependent. FG human pancreatic carcinoma cells were stimulated with a 15 minute treatment of vehicle control or EGF in the presence or absence of the Src inhibitor bosutinib and analyzed for the presence of MUC1 tyrosine phosphorylation. As shown above, EGF stimulation led to MUC1 tyrosine phosphorylation, and this was sensitive to Src inhibition (**Figure 4.12**). We next asked whether Src was sufficient to drive MUC1 tyrosine phosphorylation in FG cells. Expression of constitutively active Src readily promoted MUC1 tyrosine phosphorylation (**Figure 4.13**). Thus, both pharmacological and genetic approaches indicate that Src kinase activity is required for MUC1 tyrosine phosphorylation.

4.3.9 Src is required for EGF-induced MUC1 cleavage and nuclear localization

Given that MUC1 is a substrate for Src kinase (**Figure 4.12**, **Figure 4.13**) [107], we considered whether EGF-mediated cleavage of MUC1 required Src kinase activity. FG cells were stimulated with a 15 minute treatment of vehicle control or EGF in the presence or absence of the Src inhibitor bosutinib and analyzed for the presence of intact and cleaved MUC1. As shown above, EGF stimulation enhanced MUC1 cleavage (**Figure 4.14**). However, pre-treatment with the Src inhibitor abolished EGF-induced MUC1

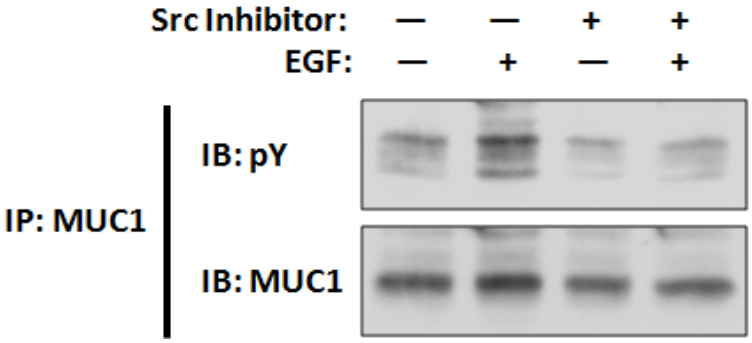

Figure 4.12: Src kinase activity is required for EGF-induced MUC1 tyrosine phosphorylation. Immunoblot detecting tyrosine phosphorylation of the MUC1 transmembrane subunit. MUC1 was immunoprecipitated from whole cell lysates of FG cells treated with EGF (50 ng/mL) in the presence or absence of Src inhibitor (bosutinib, 500 nM) and probed for phosphorylation on tyrosine. Membrane was stripped and reprobed for MUC1 as a loading control. Similar findings were observed in two independent experiments.

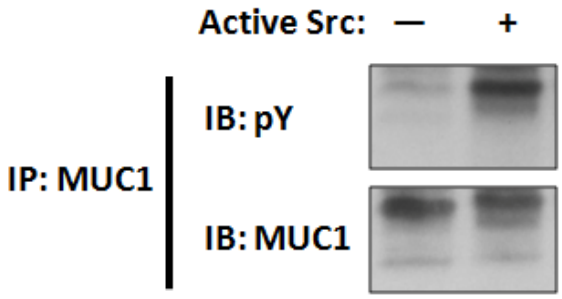

Figure 4.13: Src promotes MUC1 tyrosine phosphorylation. Immunoblot detecting tyrosine phosphorylation of the MUC1 transmembrane subunit. MUC1 was immunoprecipitated from whole cell lysates of FG cells transfected with either vector control or constitutively active Src and probed for phosphorylation on tyrosine. Membrane was stripped and reprobed for MUC1 as a loading control. Similar findings were observed in two independent experiments.

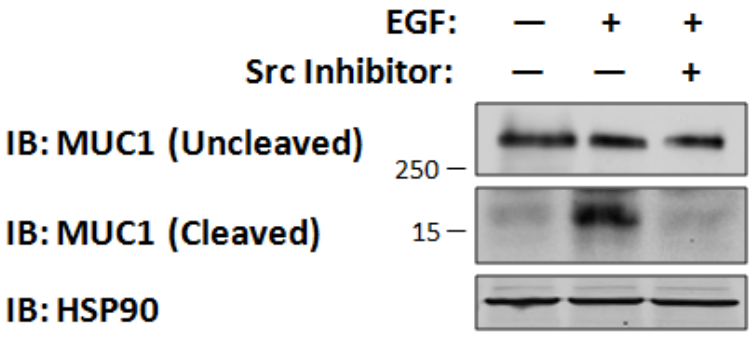

Figure 4.14: Src activity is required for EGF-induced MUC1 cleavage. Whole cell lysates from FG cells treated with EGF (50 ng/mL) in the presence or absence of Src inhibitor (bosutinib, 500 nM) were probed for MUC1 by immunoblot. Similar findings were observed in three independent experiments.

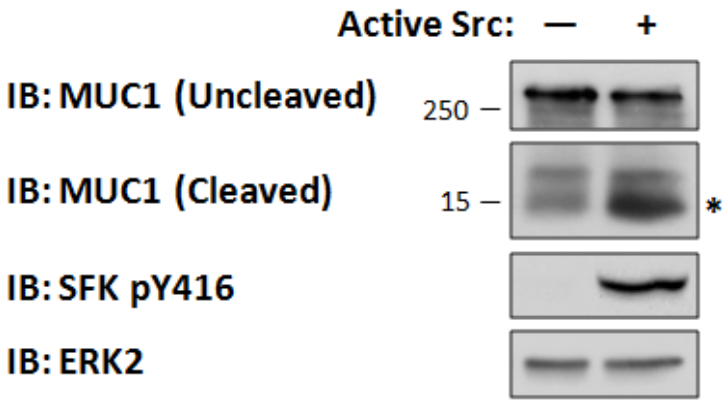

Figure 4.15: Src activity promotes MUC1 cleavage. Whole cell lysates from FG cells transfected with either vector control or constitutively active Src were probed for MUC1 by immunoblot. Similar findings were observed in three independent experiments.

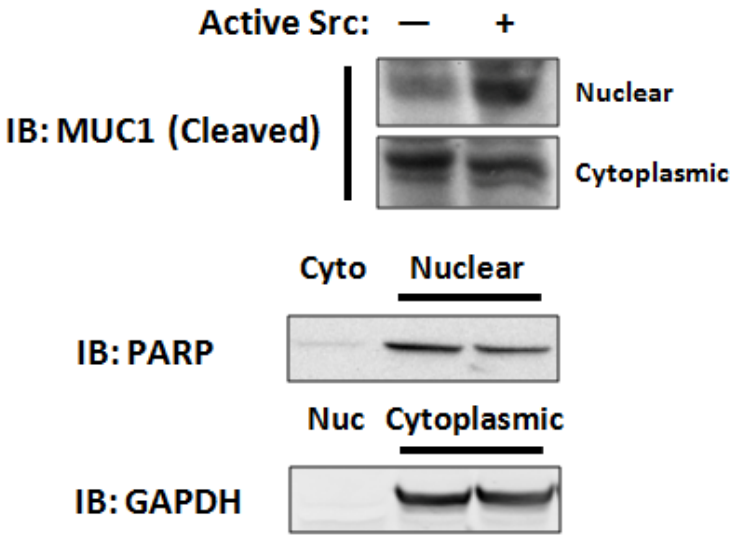

Figure 4.16: Src activity promotes MUC1 nuclear localization. Immunoblot detecting MUC1 showing enrichment of MUC1 cytoplasmic domain in the nuclear fraction of FG cells transfected with constitutively active Src compared to vector control. Fraction purity and loading were determined by immunoblot for PARP (Nuclear, Nuc) and GAPDH (Cytoplasmic, Cyto). Similar findings were observed in three independent experiments.

cleavage (**Figure 4.14**). Conversely, expression of constitutively active Src in FG cells enhanced both MUC1 cleavage (**Figure 4.15**) and its nuclear localization (**Figure 4.16**). Therefore, both pharmacological and genetic approaches suggest that Src kinase activity promotes MUC1 cleavage and nuclear translocation.

4.3.10 MUC1 is required for Src-dependent cell migration, but cell migration mediated by MUC1.CD is Src-independent

Since active Src promotes spontaneous cell migration mediated by integrin αvβ5 [41] and our data indicates that Src kinase activity regulates cleavage of MUC1 (**Figure 4.15**), we asked whether Src-dependent cell migration required MUC1. FG cells expressing constitutively active Src were transfected with either non-silencing control siRNA or MUC1 siRNA and were then allowed to migrate through a Boyden chamber coated with a vitronectin or a collagen substrate. As expected, active Src enhanced cell migration on a vitronectin substrate (**Figure 4.17**). Interestingly, knockdown of MUC1 expression selectively blocked Src-dependent migration on vitronectin but had no effect on Src-independent migration on a collagen substrate (**Figure 4.17**).

Since MUC1.CD also promotes spontaneous cell migration on a vitronectin substrate, we asked whether this required Src kinase activity. FG cells expressing MUC1.CD were pre-treated for 30 minutes with vehicle

control or the Src inhibitor bosutinib prior to migration. MUC1.CD enhanced cell migration on a vitronectin substrate as expected, and this was insensitive to inhibition of Src kinase activity (**Figure 4.18**). Therefore, while MUC1 is required for cell migration driven by Src, Src kinase activity is not required for cell migration mediated by MUC1.CD.

4.3.11 Pharmacological inhibition of Src inhibits MUC1 cleavage and tumor cell metastasis *in vivo*

Given that Src regulates MUC1 tyrosine phosphorylation, cleavage and nuclear localization in FG cells *in vitro*, we considered whether Src-mediated MUC1 cleavage in tumors *in vivo* might represent an unexpected effect of pharmacological inhibition of Src. To test whether Src was required for MUC1 cleavage *in vivo*, we took advantage of an orthotopic pancreatic tumor mouse model in which Src is required for metastasis to various secondary sites.[79] Briefly, FG cells are orthotopically implanted into the pancreata of nude mice and, over the course of six weeks, tumor cells spontaneously metastasize to the hepatic hilar lymph nodes, mesenteric lymph nodes and other tissues. Treatment of mice with a Src inhibitor (dasatinib, 30 mg/kg, twice daily for four weeks) inhibited both primary tumor growth and metastasis (**Figure 4.19**).[79] To test whether inhibition of Src activity in these tumors was associated with decreased levels of MUC1 cleavage, we assessed primary tumor lysates for the presence of cleaved MUC1. We observed that inhibition of Src activity

was associated with a decrease in levels of MUC1 cleavage product (**Figure 4.19**). These findings suggest that pharmacologic treatment of tumors with a Src inhibitor can block MUC1 cleavage and is associated with the suppression of tumor cell metastasis.

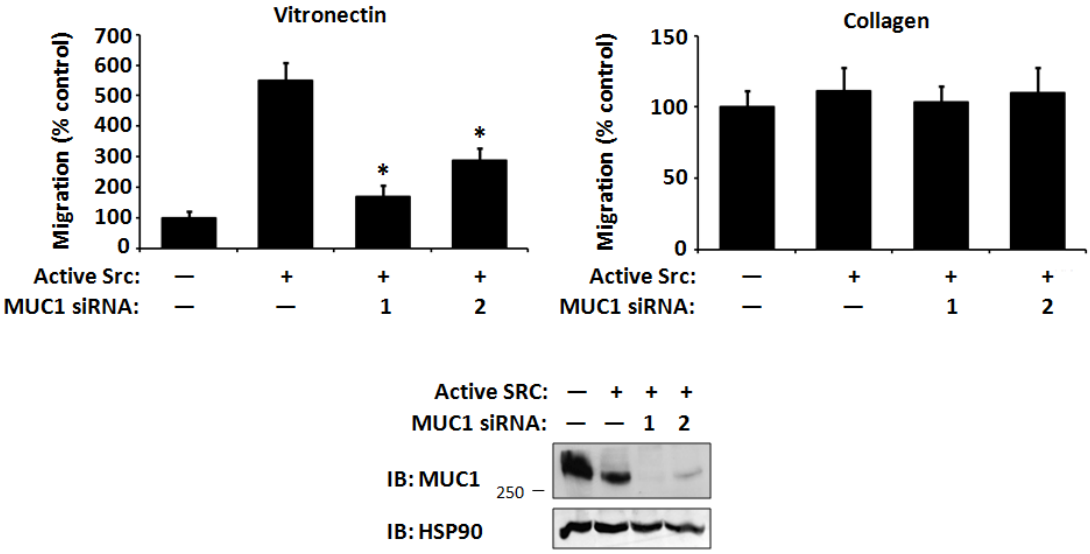

Figure 4.17: MUC1 is required for cell migration driven by Src. Migration assays on a vitronectin (left) or a collagen (right) substrate comparing FG cells co-expressing constitutively active Src and either a control siRNA or one of two unique MUC1 siRNAs. Results are expressed as mean ± s.e.m. of three replicates. Immunoblot detecting MUC1 expression (bottom). Similar findings were observed in three independent experiments. $P < 0.0001$ comparing cells expressing vector control or active Src, $P < 0.0001$ comparing cells expressing control siRNA or MUC1 siRNA, $P = 0.2$ (ANOVA) for migration on collagen.

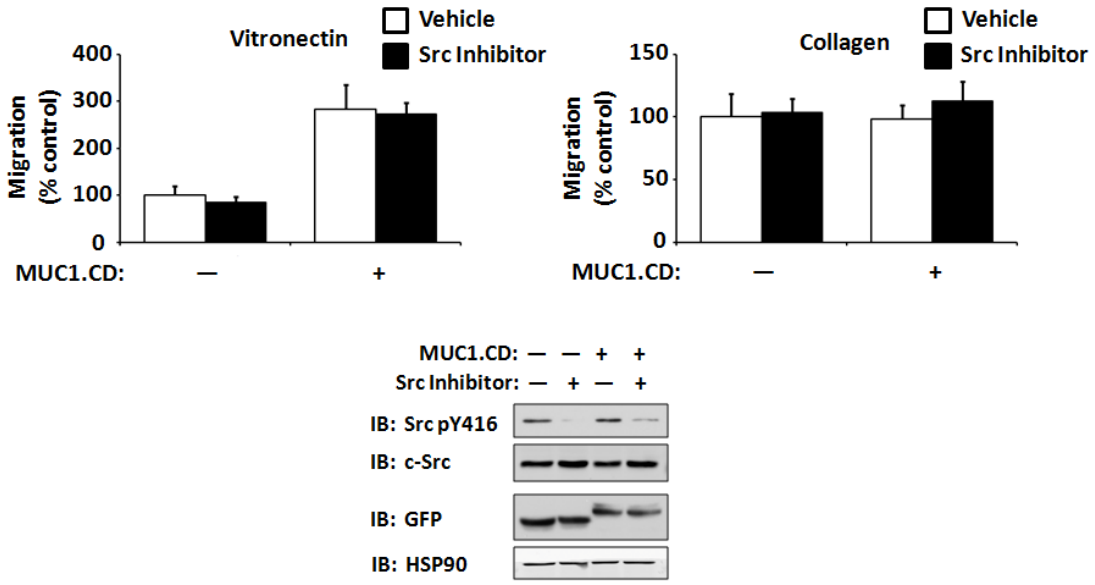

Figure 4.18: Cell migration driven by MUC1.CD is independent of Src activity. Migration assays on a vitronectin (top) or a collagen (middle) substrate comparing FG cells expressing MUC1.CD pre-treated with or without a 30 minute pre-treatment of Src inhibitor (bosutinib, 500 nM). Cells were washed with PBS prior to inoculation on Boyden chambers to remove Src inhibitor. Results expressed as mean ± s.e.m. of three replicates. Similar findings were observed in two independent experiments. $P = 0.7$ for cells expressing MUC1.CD treated with vehicle control or Src inhibitor.

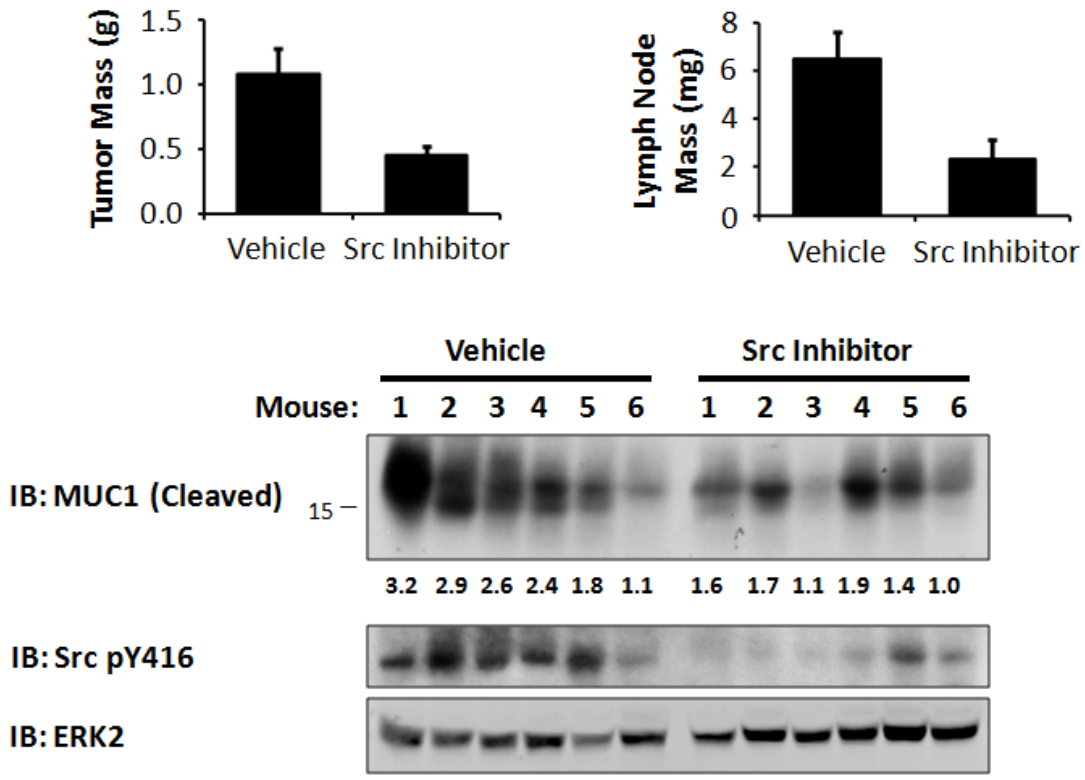

Figure 4.19: Src inhibitor blocks MUC1 cleavage *in vivo*. Primary tumor (top left) and hepatic hilar lymph node (top right) masses were assessed in a murine orthotopic pancreatic tumor model of FG cells treated with or without Src inhibitor (dasatinib, 30 mg/kg).[79] Immunoblot (bottom) detecting MUC1 cytoplasmic domain revealing decreased levels of MUC1 cleavage product with Src inhibitor treatment in primary tumor lysates. Densitometry quantification of MUC1 cytoplasmic domain (below immunoblot) is normalized to ERK2.

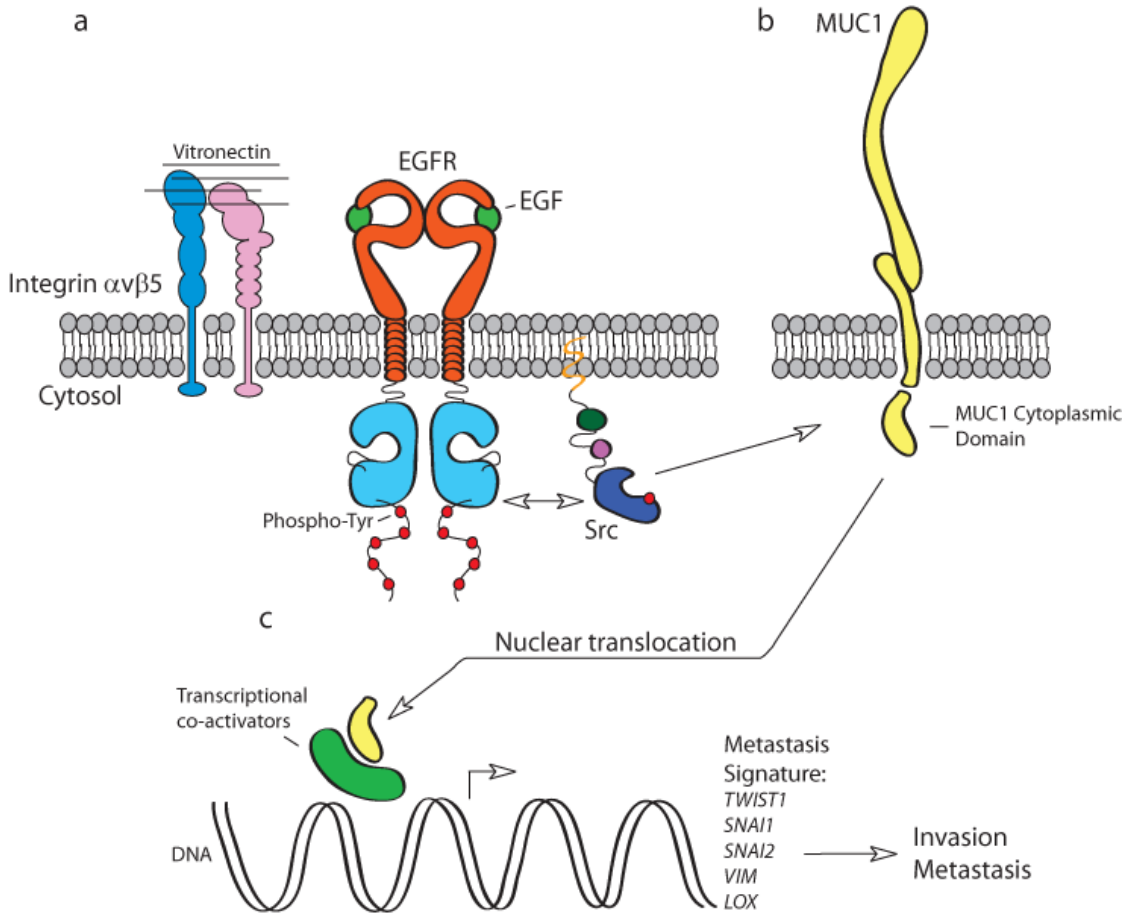

Figure 4.20: Model for MUC1 in metastasis dependent on EGFR and Src. (**a**) Migration and metastasis mediated by integrin αvβ5 requires EGFR and Src activation. (**b**) EGFR and Src activity promote proteolytic cleavage of MUC1 and release from the plasma membrane. (**c**) The MUC1 cytoplasmic domain translocates to the nucleus in association with transcription co-activators such as β-catenin, resulting in the transcription of a metastasis signature including *TWIST1* and enhancement of tumor cell invasion and metastasis.

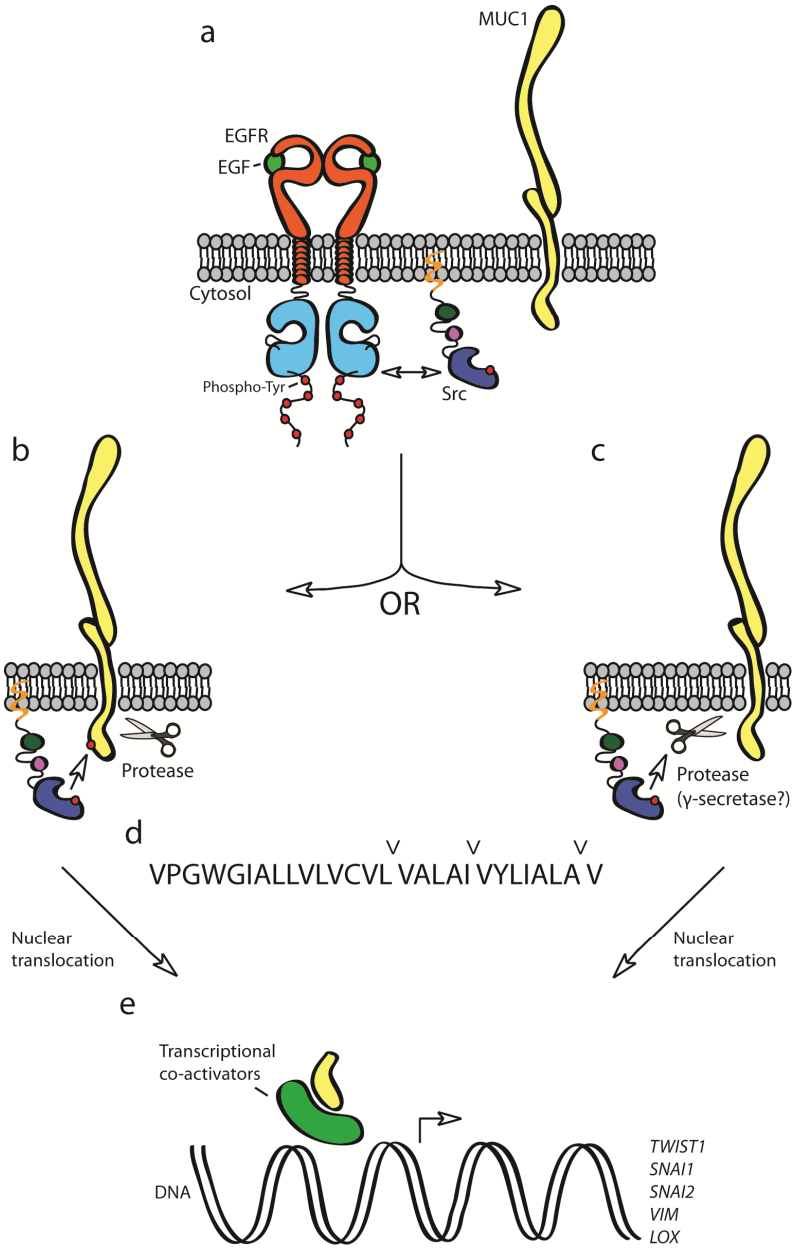

Figure 4.21: Hypotheses for mechanism of MUC1 cleavage dependent on EGFR and Src. (**a**) MUC1 interacts with EGFR and Src. (**b**) Src activity promotes tyrosine phosphorylation of the MUC1 cytoplasmic domain, enhancing recognition of MUC1 by a protease. (**c**) Alternatively, Src enhances the activity of a protease which recognizes MUC1 as a substrate. Only γ-secretase, which is activated by Src activity[165], has been proposed to cleave MUC1.[166] (**d**) Protein sequence of the MUC1 transmembrane domain showing the predicted sites of γ-secretase cleavage.[166]

4.3.12 Summary of Results

- MUC1 is required for EGF-induced cell migration on vitronectin and metastasis in the chick CAM model
- EGF promotes MUC1 cleavage and nuclear localization
- The MUC1 cytoplasmic domain is required for EGF-induced cell migration on vitronectin
- The MUC1 cytoplasmic domain is sufficient for spontaneous cell migration on vitronectin and metastasis in the chick CAM model
- EGF treatment and the MUC1 cytoplasmic domain promote transcription of *TWIST1, SNAI1, SNAI2, VIM* and *LOX*
- Src kinase activity is required for EGF-induced MUC1 cleavage
- Constitutively active Src promotes MUC1 cleavage and nuclear localization
- MUC1 is required for cell migration on vitronectin dependent on Src kinase activity, but cell migration mediated by the MUC1 cytoplasmic domain is insensitive to Src inhibition
- Treatment with a Src inhibitor in a murine orthotopic pancreatic tumor model suppresses spontaneous tumor metastasis and MUC1 cleavage

4.4 Conclusions

EGF treatment induced cleavage of MUC1 and nuclear localization of the MUC1 intracellular domain, and this enhanced the transcription of genes linked to metastasis such as *TWIST1*. Furthermore, expression of only the MUC1 cytoplasmic domain promoted spontaneous metastasis without affecting primary tumor formation. Importantly, EGF-induced MUC1 processing was dependent on Src kinase activity, and inhibition of Src prevented MUC1 cleavage and spontaneous metastasis *in vivo*.

These findings shed new light on how EGFR and Src contribute to tumor cell metastasis. Interestingly, elevated tumor-associated MUC1 is also associated with *de novo* resistance to EGFR inhibitors.[143, 144] Conversely, seroconversion against MUC1 is associated with prolonged survival for breast and pancreatic cancer patients.[92, 93] Our studies suggest MUC1 as a relevant target for the prevention and treatment of metastatic disease.

Chapter 4, in part, is currently being prepared for submission for publication of the material. Lau SKM, Shields DJ, Murphy EA, Desgrosellier JS, Anand S, Huang M, Lim ST, Stupack DG, Schlaepfer DD, and Cheresh DA. The dissertation author was the primary investigator and author of this material.

Chapter 5

Discussion

5.1 EGFR and Src signaling in tumor metastasis

Tumor metastasis is an important contributor of cancer morbidity and mortality. Metastasis is a complex process resulting from a sequence of events including local invasion, intravasation, transport and survival in the vasculature, extravasation, and colonization. The mechanisms employed by tumor cells to this end are rooted in normal processes such as embryologic development and the leukocyte immune response. However, it is remarkable that not all tumor cells comprising the primary tumor are capable of successfully metastasizing.[5, 6, 7, 11, 12]

What distinguishes highly metastatic tumor cells from those with lower propensity for metastasis? This question is presently the subject of much scrutiny. Can we determine what cellular and molecular events dictate the likelihood that an individual cell will successfully colonize a distant site? The work in this dissertation addresses these fundamental questions in tumor biology by describing a signaling pathway that enhances tumor cell metastasis.

EGFR and Src are key signaling nodes both in normal and in pathological cellular processes including tumorigenesis and metastatic spread. The clinical use of targeted therapies for EGFR [33, 34] and Src [58, 59, 60, 61, 62] has been approved for several years, but cancer continues to be a leading cause of death in the United States. Since metastasis is responsible

for most cancer deaths, elucidation of the molecular mechanisms driving tumor cell invasion and metastasis are essential for devising new strategies for the treatment of human cancers.

Integrins mediate tumor cell metastasis in several ways. Their primary function is to regulate cell adhesion, migration and invasion. While integrins promote cell death in the absence of ligation [78], integrin signaling in tumor cells deficient for this integrin-mediated death actually enhances metastasis.[79] Previous studies have revealed that cross-talk between receptor tyrosine kinases and integrins promote tumor cell metastasis.[41, 82] Importantly, we recently reported that EGFR and integrin αvβ5 cooperatively regulate metastasis.[41] In the absence of growth factor stimulation, integrin αvβ5 is unable to initiate cell migration/invasion.[81] However, EGF stimulation enhances cell migration *in vitro* and metastasis *in vivo* that is mediated integrin αvβ5.[73, 82] Moreover, EGF-dependent metastasis mediated by integrin αvβ5 requires Src activation.[41] Thus, an EGFR/Src/αvβ5 signaling axis provides instructions to cells to initiate metastasis.

EGFR and Src signaling have essential roles in tumor cell metastasis, and are among the most well studied targets of modern therapies for cancer. Nonetheless, the precise contributions of EGFR and Src to metastasis remain poorly understood. Our *in silico* approach synthesized a wealth of information

collected from multiple sources and revealed novel effectors downstream of EGFR and Src signaling that were shown to be required for integrin αvβ5-mediated tumor cell migration and metastasis. It is remarkable that other candidates identified by our approach, such as EGFR and CAS (also known as p130CAS, Crk-associated substrate and breast cancer anti-estrogen resistance protein 1), have been demonstrated to be required for cell migration mediated by integrin αvβ5.[41]

We methodically assessed the requirement of six lead candidates – all associated with tumor cell motility – in EGF-induced metastasis. One lead candidate, the pro-apoptotic protease Caspase-8, enhances the metastasis of apoptosis-resistant NB7 neuroblastoma cells by acting independently of its proteolytic activity as a scaffold for calpain-2.[167] Src-dependent phosphorylation of another lead candidate, Cortactin, is associated with pre-cancerous pancreatitis in a rat model [168] and invadopodia formation in MDA-MB-231 breast carcinoma cells.[169] While Caspase-8 and Cortactin were found to be required for EGF-induced cell migration *in vitro*, it is notable that other lead candidates (*c*-Abl, Stat3 and Villin-1) were not. We cannot exclude the possibility that residual expression of these candidate effectors was sufficient for EGF-induced cell migration.

Thus, EGFR and Src activity may contribute to tumor cell metastasis through multiple signaling pathways. This is supported by additional findings

that knockdown of either Src family kinases *c*-Src or Yes can suppress EGF-induced cell migration.[Miller Huang, unpublished observations] As such, our data indicate that EGFR and Src signaling could promote the metastasis of distinct subpopulations of tumor cells by means of activating different downstream partners. However, further studies will need to be completed to validate this hypothesis.

5.2 MUC1 in tumor progression and metastasis

The MUC1 transmembrane glycoprotein is normally found on the apical surface of ductal epithelial cells.[88] Malignant transformation results in loss of cell polarity, and MUC1 expression is frequently upregulated.[88] Since MUC1 expression is associated with distant metastasis and poor prognosis, MUC1 has become a target of interest for the treatment of human cancers.[88, 89, 90, 92] Here we identify MUC1 as a critical effector of EGF-induced FG cell migration *in vitro* (**Figure 3.3**) and metastasis in the chick CAM model (**Figure 3.4**).

MUC1 is synthesized as a single polypeptide chain, but is thought to be auto-proteolytically cleaved into a stable heterodimer.[117, 118] Interestingly, both MUC1 subunits have been linked to tumor progression and metastasis. The large extracellular subunit extends well beyond the glycocalyx [119] and inhibits cell-cell and cell-matrix adhesion mediated by E-cadherin [120] and β1

integrins [121, 158], respectively. Conversely, the MUC1 ectodomain promotes adhesion and invasion by binding to endothelial ICAM-1 [122, 123] and E-selectin [124] and neural Siglec-4a.[125] Binding of MUC1 to these substrates has been reported to activate migration machinery such as the small Rho family GTPases Rac1 and Cdc42 via Src kinase.[123] Studies focused on the small transmembrane subunit have been focused on its role in signaling downstream of RTKs including ErbB family members [101, 102, 105, 128, 129], *c*-Met [130], FGFR [131] and PDGFR [132]. These signaling pathways direct the MUC1 cytoplasmic domain to various intracellular locations. For example, stimulation with either EGF or its related family member HRG promotes nuclear translocation of MUC1 in association with β- or γ-catenin, respectively.[128, 129, 142] Once in the nucleus, MUC1 enhances the expression of genes associated with tumor metastasis such as *TWIST1*.[130, 131, 133, 134] HRG stimulation is also reported to enhance MUC1 mitochondrial localization, where MUC1 promotes tumor cell survival in the presence of genotoxic agents.[135, 136] The requirement for MUC1 in EGF-induced cell migration *in vitro* and metastasis in the chick CAM model might be explained by involvement of either or both MUC1 subunits.

We presented evidence supporting a critical role for the MUC1 cytoplasmic domain in EGF-induced tumor cell migration mediated by integrin αvβ5. EGF treatment induced MUC1 cleavage (**Figure 3.9**), nuclear localization (**Figure 3.15**) and the expression of MUC1 target genes (**Figure**

3.18). While expression of shRNA-resistant full-length MUC1 rescued EGF-induced cell migration in cells with MUC1 knockdown, expression of MUC1 lacking the cytoplasmic domain did not (**Figure 3.11**). Furthermore, expression of only the MUC1 cytoplasmic domain, which spontaneously localized to the nucleus, was sufficient to drive cell migration *in vitro* (**Figure 3.12**) and metastasis *in vivo* without enhancing primary tumor growth (**Figure 3.13**). Surprisingly, expression of MUC1 lacking the cytoplasmic domain (MUC1.CT3) also enhanced spontaneous metastasis to the lungs, albeit to a lesser degree than the MUC1 cytoplasmic domain (**Figure 3.13**). Since MUC1.CT3 also enhanced cell proliferation *in vitro* (**Figure 3.14**) and tumor growth *in vivo* (**Figure 3.13**), MUC1.CT3 may promote metastasis via proliferation and survival. Alternatively, MUC1.CT3 may mediate metastasis by enhancing transendothelial migration through its previously reported association with ICAM-1 [122, 123] and E-selectin.[124] These data suggest that both the extracellular and intracellular domains of MUC1 contribute to metastasis through different mechanisms. Notably, the MUC1 cytoplasmic domain functions downstream of EGFR signaling to enhance integrin αvβ5-mediated cell migration, whereas the MUC1 ectodomain participates through a mechanism independent of cross-talk between EGFR and integrin αvβ5.

Two regions of the MUC1 cytoplasmic domain have been reported to be required for its nuclear localization. First, a RRK sequence proximal to the plasma membrane has been proposed to function as a nuclear localization

signal (more specifically, to the nucleoli) for MUC1-γ-catenin complexes following HRG stimulation.[129] Second, the CQC sequence also proximal to the plasma membrane is thought to mediate oligomerization of the MUC1 cytoplasmic domain and association with nucleoporin p62.[170] Additional studies will be required to test whether these regions of the MUC1 cytoplasmic domain are required for EGF-induced tumor metastasis.

5.3 Interaction between MUC1 and Src promotes tumor formation and metastasis

Does the MUC1 contribute to spontaneous tumor formation and progression? Several groups have studied the role of MUC1 in spontaneous tumorigenesis in murine models dependent on EGFR and Src activity. For example, whereas wild-type mice expressing transgenic TGFα uniformly develop mammary tumors which spontaneously metastasize, tumor formation in a *Muc1*$^{-/-}$ background was delayed and metastasis was abolished.[104] Similarly, spontaneous tumor formation, growth and metastasis is suppressed in *Muc1*$^{-/-}$ mice compared to wild-type mice in a well-establsihed murine breast tumor model driven by polyoma middle T antigen.[138] Tumor development in this model is dependent on activation of Src [139], and tumors developing in *Muc1*$^{-/-}$ mice exhibit decreased Src activity.[140] Interestingly, expression of transgenic MUC1 under control of the MMTV promoter results in spontaneous mammary tumor formation, and the MUC1 cytoplasmic domain is

required.[137] These findings suggest the MUC1 cytoplasmic domain has oncogenic characteristics. While transgenic MUC1 accelerates spontaneous pancreatic tumor formation and progression in mice expressing oncogenic *Kras*^{G12D} [108, 171], the relationship between MUC1, EGFR and Src in spontaneous pancreatic tumorigenesis is unknown. We recently reported that activation of Src cooperates with oncogenic *Kras*^{G12D} to accelerate pancreatic tumor development.[172] Crossing these mice with mice expressing transgenic MUC1 or cytoplasmic domain-deleted MUC1 and *Muc1*$^{-/-}$ mice would expand our understanding of how MUC1 contributes to pancreatic tumor formation and progression.

Src is known to interact with and phosphorylate the MUC1 cytoplasmic domain [107, 136, 140], but how does this promote malignancy? As described above, tyrosine phosphorylation of MUC1 mediates its interaction with other proteins including β-catenin [142], HSP90 [136] and CrkL.[123] While the functional significance of these phosphorylation-dependent interactions is poorly understood, it is clear that MUC1 is a direct substrate of Src.[123, 136, 141, 142] We demonstrated that Src kinase activity is required for EGF-induced tyrosine phosphorylation of the MUC1 cytoplasmic domain (**Figure 3.19**). Furthermore, we observed an unexpected effect of interaction between MUC1 and Src, wherein Src activity promotes MUC1 cleavage (**Figure 3.22**) and nuclear localization (**Figure 3.23**). What is the functional consequence of Src-dependent MUC1 cleavage? Src activity drives cell migration mediated by

integrin αvβ5.[41] Here we reported that MUC1 is required for Src-dependent cell migration (**Figure 3.24**). In contrast, expression of the MUC1 cytoplasmic domain induces cell migration mediated by integrin αvβ5 that is insensitive to pharmacological inhibition of Src activity (**Figure 3.25**). We also demonstrated that pharmacological inhibition of Src activity in an orthotopic mouse model of pancreatic cancer suppressed both primary tumor growth and distant metastasis and was associated with decreased MUC1 cleavage product (**Figure 3.26**). Our findings are consistent with the hypothesis that Src-dependent MUC1 tyrosine phosphorylation promotes its cleavage, leading to cell migration mediated by a specific integrin. Alternatively, Src activity could enhance the ability of a yet undefined protease to cleave MUC1. Further studies will be required to establish whether either or both of these possibilities are true.

5.4 Therapeutic applications

Substantial progress has been made towards the development and use of pharmacological inhibitors of EGFR [33, 34] and Src [58, 59, 60, 61, 62] in the treatment of human cancer. Our findings may explain in part how inhibitors of EGFR and Src can suppress the metastatic potential of tumor cells expressing MUC1. Likewise, targeting integrin αvβ5 represents a therapeutic approach to preventing the metastatic spread of tumors expressing EGFR, Src or MUC1. In addition, MUC1 has recently become a

target of clinical interest, and several MUC1 vaccines designed to initiate and sustain an immune response to extracellular MUC1 on tumor cells are currently in phase 3 clinical trials. However, given that the MUC1 cytoplasmic domain promotes tumor metastasis independent of the MUC1 ectodomain, our studies suggest that such therapies targeting the extracellular domain of MUC1 may provide limited clinical benefit. Therefore, recent preclinical studies using peptide [145, 146, 147] and small molecule [148] antagonists of the MUC1 intracellular domain are of particular interest.

5.5 Summary

Metastasis is a fearful step in tumor progression associated with morbidity and mortality and comprised of a sequence of complex events including local invasion. Great attention is being paid to the cellular and molecular mechanisms leading to metastasis. However, we are just beginning to understand the cues that stimulate and sustain tumor dissemination to secondary sites. The work in this dissertation sheds new light on a signaling pathway instructing human carcinoma cells to invade and metastasize.

Signaling from EGFR and Src lends instruction to the seemingly stochastic process of carcinoma cell metastasis, specifically that which is mediated by integrin αvβ5. However, the downstream molecular mechanisms initiated by these key signaling nodes to enhance metastasis are not well-defined. MUC1 is a well-established cancer antigen, and contemporary

reports are revealing previously undiscovered signaling roles for MUC1 in tumor progression. Our work demonstrates that activation of Src kinase by EGFR signaling leads to cleavage and nuclear translocation of MUC1, which promotes transcription of genes associated with metastasis including *TWIST1*. Therefore, the work presented herein proposes that EGFR-dependent expression of MUC1 target genes, in cooperation with integrin αvβ5, regulate tumor cell metastasis.

Appendix

Sources consulted for substrates of tyrosine phosphorylation following EGF treatment and Src substrates and interactants are as follows:

- Biomolecular Interaction Network Database (BIND, http://www.bind.ca) accessed May 5, 2009
- Human Protein Reference Database (HPRD, http://www.hprd.org) accessed March 24, 2008
- Molecular Interactions Database (MINT, http://mint.bio.uniroma2.it/mint) accessed May 8, 2009
- IntAct (http://www.ebi.ac.uk/intact) accessed May 7, 2009
- EGF signaling phosphotyrosine: [173, 174, 175, 176, 177]
- Src signaling phosphotyrosine: [178, 179, 180]

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