THE ROLES OF MUC1 AND EGFR IN BREAST CANCER PROGRESSION AND MAMMARY LACTATION

By

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DEDICATION

I would like to dedicate my work to my mother, Carina Teresita Tumaliuan Tiam White Pinch, who is my biggest supporter and my greatest blessing. I hope I have made you proud.
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ABSTRACT

The relationship between MUC1 and EGFR has been characterized by our lab to be highly tumorigenic. A peptide therapeutic was developed in our lab to block the cytoplasmic interaction of MUC1 and EGFR by competing with the EGFR-binding domain of MUC1. The peptide, PMIP, reduced invasion and proliferation in vitro and reduced tumor growth and metastasis in vivo. These studies demonstrated the potency of MUC1/EGFR interactions in tumor progression, and we sought to explore this concept further. We wanted to clarify a mechanism by which MUC1 and EGFR together drive breast cancer metastasis, and we identified c-Met as a mediator of MUC1 and EGFR-driven cell motility. In two separate assays, we demonstrated that c-Met activity was necessary for MUC1 and EGFR to promote migration and invasion. In addition, we wanted to identify the role of EGFR membrane localization in membrane identity and tumor initiation. We established several EGFR localization mutants to compare to wild-type basolateral EGFR and we performed proof-of-concept experiments to show that these mutants will be useful in future studies. Finally, we studied the effect of MUC1 and EGF loss on tissue architecture and function in the lactating mammary gland. EGF is the primary ligand for EGFR during lactation, and MUC1 is highly expressed during this period of mammary development. In addition, it has been shown that EGFR and MUC1 interact at the apical cell surface of lactating mammary ducts, yet there is no link between lactation and tumor formation. We hypothesized that MUC1 and EGFR interaction may have a role in maintaining tissue architecture and lactation function in the mouse.
mammary gland. We found instead that the loss of MUC1 and EGF had no noticeable effect on lactation and did not result in tissue defects. These studies further clarified the relationship between MUC1 and EGFR in several different contexts, showing a role for their interaction in metastatic progression, and showing that their ablation has no effect in the lactating mammary gland. Future studies will elucidate the role of MUC1 and EGFR interaction in tumor initiation, and we have taken several steps in our studies toward that goal.
I. INTRODUCTION

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Cancer

Cancer is a disease characterized by uncontrolled cell growth and disregard for tissue organization. Mutations in the DNA of a single cell or group of cells confer advantageous growth and survival and enable the cancer cells to ignore cues from the environment [2]. Normal cells in a multi-cellular organism cooperate to contribute to the overall health of the organism, whereas cancer cells have adopted a program of self-preservation and regeneration. Cancer cells must gain several characteristics in order to become a threat to the livelihood of the host, including the ability to control their own growth and survival, and the ability to move and invade through the tissue to a secondary location, among others [3]. These attributes are good for the survival of the cancer, but when the cancer metastasizes to vital organs, it can compromise the function of those organs, making metastasis the cause of death of the cancer patient in most cases [3], killing both the host and the cancer. Cancer originates in many organs, and loss of epithelial polarity, which will be described next, allows for tumor initiation in all epithelial cancers. The primary focus of this work is breast cancer.

Breast cancer

Epithelial polarity is important for the function of many tissues, and loss of epithelial polarity is an early step in the establishment of carcinomas. Our studies will further focus on breast cancer and we will use the models mentioned above to study the role of polarity in breast cancer initiation.
Breast cancer is a deadly disease, claiming nearly half a million women per year worldwide, even with modern methods of detection and treatment[4]. Breast cancer is the second most common cancer diagnosed after lung cancer, and it is the leading cause of cancer death in women [5]. Because this disease is so widespread and so deadly, it affects many lives and breast cancer fatality must be reduced through the introduction of novel therapies and treatment regimens.

Breast cancer is a class of diseases and not one distinct affliction. The majority of breast cancers are invasive ductal carcinomas, characterized by cancer formation within the milk ducts of the breast and invading into nearby tissues, though cancer can also arise within breast lobules. A specific breast cancer may be distinguished by the mutations it has accumulated and which are crucial for its growth and survival. Subtypes of breast cancer include Estrogen Receptor (ER) and/or Progesterone Receptor (PR)-expressing, ER and/or PR and HER2-expressing, HER2-expressing only, triple-negative or basal-like, and normal breast-like [6]. Currently, these subtypes of breast cancer have distinct treatment regimes. Surgery is usually included in the treatment schedule for all of these subtypes to remove the primary tumor from the breast. Chemotherapy and radiation may be used depending on the spread of the cancer from the primary tumor. In addition, targeted therapies exist to disrupt activation of the Estrogen and Progesterone hormone receptors, either through inhibition of hormone production, or through perturbation of hormone binding to the receptor [7]. These hormone ablation therapies are very successful, as a recent meta-analysis of selective Estrogen Receptor modulators (SERMs) found that reducing Estrogen Receptor activity for five years in women with Estrogen
Receptor-driven breast cancers drastically reduced the incidence of recurrence and death up to 15 years after treatment [8].

HER2-positive breast cancer is also treated with targeted therapy. HER2 is a receptor tyrosine kinase on the cell surface of cancer cells, and it is a member of the ErbB family of receptor tyrosine kinases. When activated by dimerization with another member of the family, such as ErbB1 (the Epidermal Growth Factor Receptor (EGFR)), ErbB3 or ErbB4, the dimer pair signals to cause growth of the cancer cells. HER2 is an attractive target for therapeutics because HER2-positive cancers are it for growth [9]. Trastuzumab and its derivatives are antibodies which bind to the extracellular portion of HER2 and prevent it from becoming activated (Fig 1.1). These targeted therapeutics have drastically reduced the number of breast cancer deaths due to HER2 expression, and they are being improved upon to reduce side effects [10]. Normal breast-like tumors are often small and minimally invasive, and are generally treated by surgery.
Figure 1.1. ErbB2 dimerizes with ErbB1 (EGFR) or another member of the ErbB family of receptor tyrosine kinases to activate signaling. Trastuzumab (Herceptin) prevents dimerization, inhibiting ErbB2 signaling.
Triple negative breast cancers do not rely on the receptors previously mentioned and are thus commonly unresponsive to Estrogen or Progesterone ablation therapies or HER2-targeted antibodies. Triple-negative cancers are frequently aggressive and morphologically basal-like, resembling the myoepithelial cell layer of the breast [11]. Myoepithelial cells surround the inner luminal epithelial cell layer to aid in contraction of the ducts for milk ejection. These cells express more mesenchymal cell markers than the luminal epithelial cells, and are poised to migrate when the tissue requires it [12]. Basal-like cancers which resemble myoepithelial cells are generally more aggressive and invasive than the Estrogen or Progesterone Receptor-positive cancers, and many triple-negative cancers fit this type [13]. There are currently no viable targeted therapeutics for triple-negative breast cancer, and so the standard of care for these cancers is a combination of surgery, chemotherapy, and radiation [13]. Due to the aggressive nature of triple-negative breast cancers, they account for a disproportionately high percentage of breast cancer-related deaths [14]. While advancements in breast cancer therapy have reduced fatality due to certain subtypes of cancer, more targeted therapies for triple-negative cancers are necessary to further reduce the number of deaths due to breast cancer each year.

**Epidermal Growth Factor Receptor (EGFR)**

A subset of breast cancers, and especially triple-negative breast cancers, overexpress ErbB1, the Epidermal Growth Factor Receptor (EGFR) [15]. EGFR, like HER2, is a member of the ErbB family of receptor tyrosine kinases, and it is
overexpressed in 20-30% of breast cancers. EGFR has the ability to promote growth and survival of cancer cells, in addition to cell motility, making it a method by which cancers initiate and metastasize [16].

EGFR is an oncogene which is overexpressed, overactivated, or mutated in a variety of cancers, including breast [17], prostate [18], lung [19], colorectal [20], and brain cancers [21]. EGFR is a transmembrane receptor on the cell surface which has an N-terminal extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane domain adjacent to the inner leaflet of the plasma membrane, and a C-terminal kinase domain [22]. The ligand-binding domain binds to several ligands, including the Epidermal Growth Factor (EGF), Amphiregulin, and Transforming Growth Factor-α (TGFα) [16]. Upon ligand binding, EGFR homo-dimerizes or hetero-dimerizes with one of the other ErbB family members, ErbB2 (HER2), ErbB3, or ErbB4 [16] (Fig 1.2). The receptors transphosphorylate and recruit binding partners to resulting phosphorylated tyrosine residues. In this way, EGFR and the other ErbB family members are capable of establishing massive signal transduction cascades which result in biological outputs such as proliferation, survival, and migration [23]. The main pathways activated in response to EGF ligand binding include the phospho-lipase C-γ (PLC-γ) pathway [24], the Ras MAPK pathway, the phosphoinositide-3-phosphate kinase (PI3K) pathway, and signal transducer and activator of transcription 3 (STAT3) activation [23].
Figure 1.2. ErbB1 (EGFR) has several functional domains. Upon ligand binding, EGFR homodimerizes or heterodimerizes with another member of the ErbB family of receptor tyrosine kinases to promote downstream signaling.
Upon ligand-binding, EGFR recruits PLC-γ, which converts phosphoinositide-2-phosphate into inositoltriphosphate (IP3) and diacylglycerol (DAG) [24]. IP3 then allows for calcium release from the endoplasmic reticulum (ER) and DAG promotes activation of protein kinase C (PKC). Calcium release into the cytoplasm and PKC activation have many implications for such processes as proliferation, cell morphology, differentiation, etc. [25]. In addition, EGFR can recruit Grb2, an adaptor protein, and son of sevenless (SOS), a guanine nucleotide exchange factor which primes the GTPase function of Ras [26]. Ras then recruits Raf to the membrane, which results in MEK phosphorylation, Erk translocation to the nucleus, and activation of several transcription factors, such as Elk1 [27]. This pathway results in Cyclin D expression, enabling progression through the cell cycle and promoting cell proliferation, among many other effects involved in cancer initiation, progression, and metastasis [28].

EGFR also recruits Gab1 which recruits PI3K to the receptor, though PI3K can also bind EGFR directly with lower affinity. PI3K phosphorylates phosphoinositide-2-phosphate in the plasma membrane, promoting the recruitment of AKT through its pleckstrin-homology domain. AKT activates the mammalian TOR complex and drives protein translation. AKT also inhibits Bad, a negative regulator of apoptosis, enabling proliferation and survival among other effects [29]. Ras is also known to activate the PI3K pathway in addition to the MEK/Erk pathway, demonstrating a role for signal crosstalk in cancer progression [30]. Finally, EGFR phosphorylation recruits JAK2, which phosphorylates STAT3 and promotes its nuclear translocation. STAT3 regulates many genes; among them is Twist1, a master transcriptional regulator of the epithelial to
mesenchymal transition (EMT) [31]. Additionally, STAT3 positively regulates the expression of MUC1, a mucin protein which is important for EGFR biology and is implicated in many epithelial cancers [32]. These canonical signaling activities contribute to EGFR-driven tumor progression.

Because the signaling cascade activated upon EGFR activation is potent, the signal is quickly halted through receptor degradation. Upon ligand-binding, EGFR undergoes clathrin-mediated endocytosis [33, 34]. EGFR localized in endosomes near the plasma membrane may be recycled back to the cell surface or trafficked to the multi-vesicular body, the docking site for endosomes. From the multi-vesicular body, endosome-bound EGFR may be degraded in the lysosome, reducing EGFR signaling activity and reducing the number of EGFR molecules at the cell surface [34]. Through this mechanism, EGFR activity is quenched. Alternatively, EGFR may be trafficked from the multi-vesicular body to other cellular compartments, as will be discussed later.

*Epidermal Growth Factor Receptor localization*

The Epidermal Growth Factor Receptor (EGFR) is basolaterally localized on the plasma membrane [35]. On the basolateral side, EGFR is poised to receive ligands from the surrounding stroma of the tissue [36]. In addition, it is able to interact with other proteins which are also localized basolaterally, such as integrins and other receptor tyrosine kinases, and these proteins are spatially separated from proteins on the apical side of the cell [37] [38]. Apical localization of EGFR has been achieved through the overexpression of EGFR, similar to the scenario in EGFR-driven cancers, and apical
EGFR still induces proliferation through the MEK/ERK pathway. Apical EGFR, however, does not interact as strongly with integrins, presumably because integrin localization is still intact, though apical EGFR interacts more robustly with β-catenin, a component of adherens junctions, effector of Wnt signaling and a powerful driver of oncogenesis [39]. This demonstrates that mislocalized EGFR alters signaling and cellular phenotypes, but the full potential of mislocalized EGFR in tumorigenesis has not been explored. It is clear that loss of specified protein localization disrupts function, and membrane localization is regulated by epithelial cell polarity.

**EGFR in cancer**

EGFR is an indicator of poor prognosis in gastic cancer [40], head and neck squamous cell carcinoma [41], colorectal cancer [20], invasive breast cancer [17], renal cell carcinoma [42], medulloblastoma [43], non-small cell lung cancer [19], and prostate cancer [18], among others. Because EGFR is a powerful inducer of signals leading to phenotypes that are important in development, such as proliferation, survival, and motility, EGFR is often exploited in cancer. In many cancers, such as lung cancer and glioma, activating mutations in the kinase domain or ligand-binding domain of EGFR enable constitutive activation of the receptor, promoting cancerous growth [44]. In other cancers, such as breast, EGFR is amplified, overexpressed, or overactivated, but not mutated [45]. TGF-α, one of the ligands for EGFR, is found overexpressed in many breast cancers and confers poor prognosis [46]. We found that treatment of MCF10A mammary epithelial cells with excess amounts of EGF ligand to activate EGFR induces
luminal filling in 3D cell culture (Fig 1.3). Luminal filling in this system is analogous to Ductal Carcinoma in Situ (DCIS), indicating that excess activation of EGFR leads to loss of polarized growth, loss of contact inhibition, and evasion of apoptosis, all of which are essential for tumor growth. These data demonstrate that though EGFR is an important moderator of normal cell signals, its activities are also used by cancer to promote aberrant growth.
Figure 1.3. Polarized mammary epithelial cells lose polarity upon EGFR overstimulation. MCF10A cells were seeded in Matrigel and grown for 21 days in either 5 ng/mL EGF (top row) or overstimulated with 100 ng/mL EGF (bottom row). Shown from left to right is a sequence of slices from one side of the acinus to the middle. Experiment performed by Dr. Ina Menzl.
Non-canonical EGFR functions

In addition to the canonical signal transduction cascades, EGFR participates in several non-canonical activities, including its nuclear function as a transcription co-factor and trafficking to the mitochondria. To traffic to the nucleus, EGFR is first internalized upon ligand-binding through clathrin-mediated endocytosis. From the endosome, EGFR may be trafficked to the multi-vesicular body for sorting, or it may traffic directly to the Golgi apparatus. While in the Golgi, the amino-terminal end of the protein projects into the interior of the Golgi, whereas the carboxy-terminal end protrudes into the cytoplasm. From the Golgi, EGFR is trafficked to the Endoplasmic Reticulum through a COP1-dependent retrograde mechanism [47], and EGFR then transfers to the inner nuclear membrane through the action of the Sec61 translocon and interaction with Importin β [48] to enter the nuclear pore and become embedded in the nuclear membrane.

In the nucleus, EGFR has been shown to interact directly with the DNA, regulating gene expression. EGFR, together with RNA Helicase A, binds to A/T-rich response sequences in the promoters of specific genes and uses a transactivation domain in its C-terminal domain to activate transcription [49, 50]. EGFR has been shown to bind to the promoters of several genes which are important for tumor development and therapeutic resistance as well as genome instability, all of which aid in cancer progression. Several proteins have been found to interact specifically with nuclear EGFR affecting specificity of EGFR-DNA binding and/or allowing EGFR to identify DNA targets for them to bind. EGFR has been shown to interact with E2F1 transcription factor to cooperatively bind the promoter of b-Myb [51]. Likewise, EGFR interacts with
STAT3 to activate transcription of Cox-2 [52] and iNOS [53], and interacts with STAT5A to promote expression of Aurora-A [54]. In addition, EGFR complexes with Src and STAT3 to stimulate transcription of c-Myc [55], joins with HER2 to increase expression of thymidylate synthase [56], and binds directly to the promoters of CyclinD1 [49], and BCRP [57]. MUC1 is a transmembrane mucin which is known to act as a transcriptional co-factor, and only in the presence of MUC1 does EGFR bind to the CCND1 promoter, leading to expression of Cyclin D1 as mentioned above [58].

Nuclear EGFR is also implicated in DNA damage repair mechanisms [59]. EGFR interacts with proliferative cell nuclear antigen (PCNA) and DNA-dependent Protein Kinase (DNA-PK) in the nucleus [60]. These proteins are involved in DNA replication and DNA double strand break repair, enabling chemotherapeutic resistance during cancer treatment [61].

Mitochondrial EGFR has been shown to be driven by the induction of autophagy and apoptosis and to be protective against cell death [62]. In addition, mitochondrial translocation of EGFR is driven by drug therapies, and is known to be involved in drug resistance [63], potentially through its effect against cell death. Studies have also shown that mitochondrial EGFR interacts with and phosphorylates COX-II, a cytochrome c oxidase subunit, directly affecting energy production and/or cytochrome c release and thus controlling apoptosis [63]. These are some of the known non-canonical functions of EGFR, but more studies will likely reveal roles for EGFR in many other cellular processes.
EGFR transcriptional regulation

EGFR is kept at steady state levels in the cell by the Sp1 transcription factor, a transcription factor which is involved in cell cycle regulation [64]. EGFR can be upregulated by several pathways, including the Wnt, parathyroid hormone, and ErbB2 pathways. Activation of the Wnt pathway leads to β-catenin protein stabilization and nuclear translocation, promoting TCF/LEF gene transcription, allowing the transcription of genes that are responsive to TCF/LEF [65]. EGFR has a TCF binding site in its promoter, and in addition EGFR has been found to be a direct transcriptional target of β-catenin itself in prostate cancer cells, which leads to significant Erk 1/2 and Stat3 activity. These findings demonstrate that EGFR is strongly expressed in response to Wnt signaling resulting in increased EGFR activity [66].

In addition, parathyroid hormone has been shown to increase EGFR expression 2-3 fold in osteoblast cells. Parathyroid hormone has been shown to be involved in bone growth despite that fact that parathyroid hormone inhibits growth in other tissues. The finding that EGFR expression is induced by parathyroid hormone in this tissue at least partially explains this phenomenon [67]. Furthermore, basement membrane detachment of epithelial cells results in cell death through anoikis due to loss of integrin-matrix interactions, and decreases EGFR expression. These effects are reversed in ErbB2-overexpressing cells, wherein ErbB2 activity results in EGFR upregulation, allowing anchorage-independent growth [68].

Among transcriptional repressive mechanisms of EGFR are the activity of the Estrogen Receptor and p53. Removal of estradiol from Estrogen Receptor positive breast
cancer cells resulted in a 3-6 fold increase in EGFR mRNA. In addition, estrogen depletion sensitized breast cancer cells to the proliferative effects of EGF and TGFα stimulation. This indicates that EGFR is negatively regulated by Estrogen Receptor activation [69]. Lastly, p53 knockdown strongly induces EGFR transcription in keratinocytes. Loss of p53 through loss of heterozygosity or viral inactivation induces many cancer-promoting oncogenic proteins, and upregulation of EGFR is yet another avenue to drive tumor progression [70]. These proteins have been revealed to regulate EGFR transcriptional activity, though there will likely be new mechanisms uncovered in the near future. Next, mouse models used to study EGFR will be discussed.

**Mouse models of EGFR**

Systemic EGFR knockout mice die perinatally [71]; therefore, other methods have been employed to use mouse models to examine EGFR function. Mice lacking one or more ligands for EGFR have defects in the epithelial cells of the skin, lung, gastrointestinal tract, eyelids and teeth, but are viable. TGF-α knockout mice have defects in their hair follicles, leading to a wavy hair phenotype [72]. Amphiregulin knockout mice have slow mammary development, though following parturition, female mice are able to nurse their pups. Mice lacking Amphiregulin, TGF-α, and EGF experience hair loss, dermatitis, skin ulcerations, eyelid defects and weight loss in addition to markedly greater mammary impairment than single ligand knockouts, with only a small percentage of female mice able to nurse following parturition [73]. This
indicates that there is some redundant EGFR activation between these ligands during mammary development.

Dominant negative EGFR mutants have been used to confirm the importance of EGFR in the skin, eyelid, and mammary tissues. Mice expressing dominant negative EGFR have impaired mammary ductal outgrowth and branching [74], in addition to impaired development of skin and hair follicles [75]. These studies conducted with ligand knockouts and dominant negatives show that EGFR is involved in epithelial cell growth and differentiation in specific tissues.

Overexpression of EGFR and its ligands identify it as a powerful regulator of epithelial cell growth. TGF-α overexpression drives epithelial hyperplasia in several carcinomas, including pancreatic and breast [76]. In contrast, EGF is not found overexpressed in carcinomas, and in fact, exogenously overexpressing EGF in mice and rats resulted in stunted growth [77]. Further exploration of this phenomenon revealed that EGF regulates binding proteins for the Insulin-like Growth Factor Receptor (IGFR), affecting signaling through the receptor and inhibiting mitogenic signaling [78]. This shows that in certain contexts, EGFR inhibits growth, which does not confer an advantage to tumor cells.

EGFR ligands are differentially expressed temporally throughout mammary gland development. TGF-α and β-cellulin are highly expressed from early puberty until late pregnancy. Amphiregulin and Epigen are upregulated during puberty and are downregulated again in late pregnancy. Heparin-bound EGF (HB-EGF) expression also stays constant throughout puberty and pregnancy and is reduced shortly before lactation.
EGF alone is drastically upregulated in late pregnancy and stays at constant high levels throughout lactation, declining again in early mammary gland pruning after lactation (involution) [79]. EGFR ligands are expressed in mammary epithelial cells during specific stages of mammary development, making particular ligand knockouts well suited for studies of EGFR function in defined stages.

EGFR is expressed during all stages of mammary development, but its localization is not constant. EGFR is generally basolaterally localized in order to ensure ligand stimulation from the stroma, but during lactation, EGFR is apical [80]. High volume of milk fat globule secretion from epithelial cells in the lactating mammary gland enable cell membrane domain mixing, resulting in apically localized EGFR. This difference in membrane localization of EGFR is a topic that will be discussed further below.

**EGFR targeted therapies**

Because EGFR is overexpressed and mutated in a number of carcinomas, it has been extensively therapeutically targeted, with limited efficacy. Classes of EGFR therapies include extracellular monoclonal antibodies, such as Cetuximab, intracellular tyrosine kinase inhibitors, such as Gefitinib and Erlotinib, and small peptides (Fig 1.4). Monoclonal antibodies have shown to be moderately effective in metastatic colorectal cancer [81]. In addition, non-small cell lung cancer patients with high EGFR expression treated with Cetuximab in combination with chemotherapy had significantly increased survival compared to patients without high EGFR expression and treated with Cetuximab
with chemotherapy [82]. Gefitinib has shown promise in non-small cell lung cancer patients as well [83]. These data demonstrate that both monoclonal antibodies and tyrosine kinase inhibitors have some effect on specific cancer types. Neither of these classes of therapeutics has been effective in treating breast cancer patients overexpressing EGFR [84], with the exception of Lapatinib, a tyrosine kinase inhibitor which specifically inhibits both EGFR and HER2. Lapatinib has shown only a 25% response rate in metastatic breast cancer patients, and this is the best response observed with any current EGFR-targeted therapy, signifying a need for additional therapeutics [84].

Several peptides have been shown in pre-clinical trials to effectively inhibit EGFR, though none of these has made it through clinical trials yet. These peptide therapeutics operate by selectively targeting EGFR-overexpressing cells for chemotherapy, or by competitively inhibiting EGFR activity.

A hybrid peptide containing an EGFR-binding protein conjugated to a membrane lytic peptide, which disrupts the cell membrane through the use of cationic amino acids, specifically lysed cancer cells overexpressing EGFR in vitro and in vivo. This hybrid peptide has shown efficacy against many cancer cell types, including glioma cells and breast, pancreatic, lung, and prostate cancer cells [85]. In addition, an EGFR-binding peptide conjugated to a polyethylene glycol (PEG) lipid inserted into the cell membranes of EGFR-expressing cancer cells, triggering endocytosis of the peptide [86] and effectively killing lung cancer cells. These peptides reveal effective and specific drug delivery methods to target EGFR-expressing cancer cells.
The EV peptide was delivered via nanoparticle and mimicked the Y845 site of EGFR, which is essential for transphosphorylation and complete activation of the receptor. EV prevented EGFR activation and downstream signaling in lung cancer cells, inhibiting cell proliferation and inducing apoptosis [87]. Finally, PTD4 MUC1 Inhibitory Peptide (PMIP), a peptide designed to mimic the sequence of MUC1, a transmembrane mucin which interacts with EGFR, competitively inhibited MUC1-EGFR interactions. We and others have shown that MUC1 contributes to EGFR activity by promoting EGFR recycling to the plasma membrane, inhibiting EGFR degradation, and specifying gene promoters for transcriptional activation by EGFR [58, 88]. PMIP blocked the interaction of MUC1 and EGFR and has been shown to inhibit cancer progression in models of breast and lung cancer [89, 90]. These peptides show promise for a new wave of EGFR-targeted therapies.
Figure 1.4. EGFR targeted therapies. Cetuximab antibody binds the extracellular domain of EGFR. EGFR BP (binding protein) conjugated to a membrane lytic peptide selectively binds EGFR and lyses EGFR-expressing cells. EGFR BP (binding protein) conjugated to PEG (polyethylene glycol) lipid selectively disrupts membrane integrity of cells overexpressing EGFR. EV peptide prevents EGFR activating phosphorylation. Gefitinib, Erlotinib, and Lapatinib inhibit ATP binding. PMIP peptide blocks EGFR binding to MUC1.
MUC1

Next, we will further discuss MUC1, a protein which has a profound effect on EGFR biology. MUC1 is a transmembrane mucin which protrudes from the cell surface and its extracellular domain acts to sterically inhibit binding by molecules to the apical cell surface, similar to the function of traditional secreted mucins. MUC1 contains a variable number tandem repeat (VNTR) region which makes up the bulk of the extracellular domain of the protein, which is heavily glycosylated on serine and threonine residues adding to the bulk of the extracellular domain [91-93]. Adjacent to the VNTR region, the protein is cleaved and subsequently non-covalently bonded to the remainder of the protein to make a heterodimer [93]. The transmembrane region is adjoined by a small cytoplasmic domain, which binds to and is phosphorylated by a number of proteins, including c-Met, Src, EGFR, and β-catenin [94-96]. The activities of the extracellular and cytoplasmic domains have been implicated in primary tumor and metastatic cancer progression and will be discussed further.

MUC1 in cancer progression
(excerpts from the following sections were published in [1])

MUC1, a transmembrane member of the mucin family, has long been associated with cancer and metastatic progression, both clinically and experimentally. MUC1 is involved in tumor progression through both its extracellular, O-glycosylated serine/threonine repeat region (the ‘mucin’ domain, MUC1-ECD), as well as through activities of its intracellular cytoplasmic domain (MUC1-CD). The role of MUC1 in
metastatic cancer progression is highlighted by the frequent observation of MUC1 overexpression in metastatic tissues and circulating tumor cells from patients with advanced adenocarcinoma, and the ability to use anti-MUC1 antibodies as diagnostics for metastatic disease. Mechanistically, MUC1 (both ECD and CD) engages in intercellular and intracellular interactions with other transmembrane proteins, such as ICAM-1 and EGFR, which have protumorigenic capacity themselves. In addition, MUC1 can engage cytoplasmic signaling proteins, such as Src and β-catenin, thereby driving changes in the proliferation, cytoskeleton and adhesive capacity of the transformed cell. Finally, MUC1 can directly drive transcription of genes, through the proteolytic cleavage and nuclear translocation of MUC1-CD. These interactions will be discussed further below.

**MUC1 in cancer**

In many tumor types, MUC1 expression correlates with aggressive, metastatic disease, poor response to therapy and poor survival. While MUC1 expression is limited to the apical surface of most ductal epithelia, in advanced disease, MUC1 is overexpressed and becomes localized throughout the cell [97]. This has perhaps been most intensively studied in breast cancer, in which MUC1 expression has been evaluated clinically at the level of immunohistochemistry [98, 99], RNA [100], shed MUC1 in sera, and biochemically [101], and has correlated with poor disease-free and overall survival, as well as axillary node metastases [99]. MUC1 expression is seen in all subtypes of breast cancer, including hormone receptor-positive, HER2+ and triple-negative, although
in each of these cancer types, expression is highest in those tumors that have metastasized [99, 102].

In other hormonally responsive cancers, including ovarian and prostate, a similar overexpression of MUC1 is observed in advanced disease. In ovarian cancer, patients with metastatic, treatment-resistant disease display elevated levels of MUC1, with greater than 90% of these patients producing antibodies to MUC1 [103]. Additionally, MUC1 expression is high in both primary epithelial ovarian cancers and in metastatic ovarian cancer (>90%) [104], with MUC1 cytoplasmic expression correlating with poor overall survival and invasive capacity [105]. Likewise, in prostate cancer, less than 60% of primary lesions were found to express MUC1 in one study, whereas 90% of lymph node metastases expressed MUC1 [106], indicating that MUC1 is enriched in metastatic tumors.

In the gastrointestinal system, MUC1 is also strongly correlated with metastatic progression. In gastric cancer, MUC1 is not only expressed in metastatic disease, but also found to be highly expressed in virtually all isolated cancer cells invading throughout the stroma of the primary tumor, indicating it may be promoting initial spread [107, 108]. High MUC1 expression is also associated with invasive intraductal papillary neoplasms of the bile duct [109], metastatic liver cancer [110] and pancreas [111, 112], as well as lymph node metastasis and vascular invasion in oral squamous cell carcinoma [113]. As such, MUC1 was found as a useful biomarker to identify occult lymph node metastases in oral squamous cell carcinoma [114]. Similarly, MUC1 is associated with higher grade tumors and shorter metastasis-free survival in renal cell carcinoma,
malignant thyroid cancer, leukemias and lymphomas [115-117]. Overall, these studies reveal a strong link between MUC1 expression and cancer progression, inversely correlating MUC1 and disease free survival due to metastatic spread.

Mouse models of MUC1

MUC1 expression is strongly correlated with tumor progression in patient samples, both in tissue and circulated tumor cells. In order to further explore this relationship, mouse models of cancer have been employed which demonstrate a role for MUC1 in cancer, a number of which provide direct evidence for MUC1 in driving metastatic progression. The first model demonstrating a potential role for MUC1 in driving metastatic progression was the MMTV-PyMT mouse model of breast cancer, which was crossed onto a Muc1 knockout background in a study by Spicer et al [118]. The MMTV-PyMT transgenic mouse develops multiple tumors in the mammary gland by approximately 10-12 weeks of age, with greater than 90% of these animals displaying lung metastases [119]. When crossed onto a Muc1 knockout, incidence of lung metastases was found to be lower, although the reduction did not reach statistical significance [118]. In a separate study, MMTV-Muc1 transgenic mice were created, resulting in late-onset mammary gland tumors that were metastatic to the lung, but only in animals overexpressing full-length Muc1 (Muc1 lacking the cytoplasmic domain was not tumorigenic) [120]. In addition, our laboratory evaluated the role of Muc1 in Epidermal Growth Factor Receptor (EGFR)-driven breast cancer by crossing the WAP-TGFα transgenic model onto a Muc1 knockout background. The WAP-TGFα transgenic
mouse develops mammary gland carcinoma in 100% of multiparous females, with fewer than 25% of mice presenting lung metastases [121]. Upon crossing the WAP-TGFα onto a Muc1−/− background, mammary gland carcinoma was reduced to less than 50%, and presentation of lung metastases dropped to 0% [122]. Together, these studies implicate MUC1 as a promoter of metastatic progression in mouse models of breast cancer.

In addition to breast cancer, a role for MUC1 in driving pancreatic and lung cancer metastasis has also been examined. Evaluation of Muc1 during pancreatic cancer progression was performed in the KC or Cre-LSL-KRASG12D mouse model, a spontaneous mouse model of pancreatic ductal adenocarcinoma that relies on an activating KRAS mutation for tumor progression [123]. Crossing this mouse onto a Muc1 null background (KC-Muc1-null) resulted in reduced tumor burden and an increase in overall survival as well as a 50% reduction in distant metastasis compared to a KC mouse crossed with a Muc1 transgenic (KC-Muc1), which had Muc1 levels above wild-type. 61% of KC-Muc1 mice exhibited lung metastases, 33% had liver metastases, and 23% had peritoneal metastases, whereas only 30% of KC mice with wild-type Muc1 levels had lung metastases, 20% had liver metastases, and 10% had peritoneal metastases. The incidence of lung, liver, and peritoneal metastases was further reduced in the KC-Muc1-null mice, only 10% of which developed metastases in any of the three organs examined, defining a role for MUC1 in pancreatic tumor growth and metastasis in this model [123].

The KC, or Cre-LSL-KRASG12D, mouse crossed with a Muc1 transgenic has also been used to look at the effect of MUC1 in lung cancer progression. In this model, Cre-
LSL-KRAS$^{G12D}$ mice developed lung tumors nine weeks after the administration of a Cre-adenovirus. The addition of transgenic Muc1 (KC-Muc1) induced the formation of twice as many premalignant and malignant masses, and Muc1 positive mouse tumors exhibited many more infiltrating cells [124].

Orthotopic models of cancer have also been evaluated for a role of MUC1 in driving cancer progression. In a model for breast cancer metastasis to the brain, MUC1-expressing MA11 cells were injected into the left ventricle heart chamber of an athymic nude mouse. The MA11 cell line was derived from a breast cancer which had metastasized to the bone. To isolate the cells, bone marrow was enriched for mucin-expressing cells which were then purified and maintained in culture for a period of time before injection. 87% of mice injected displayed brain metastases, with accompanying neurological symptoms. MUC1 was detected in the serum of 82% of mice showing histological evidence of brain metastasis, demonstrating that MUC1 is prevalent in this cell line and correlating expression with metastatic progression [125]. Additionally, an orthotopic lung cancer model was used to assess the significance of Muc1 expression. In an orthotopic model of H358 lung cancer cells, cells which stably express MUC1 siRNA or control siRNA were evaluated for their ability to induce metastatic disease. While 71% of MUC1-expressing tumors progressed to lung metastases, only 14% of mice with non-MUC1-expressing tumors displayed any metastases [32]. Similar results were found in an orthotopic pancreatic cancer model wherein S2-013 pancreatic cancer cells were stably transfected with MUC1 siRNA or control siRNA and implanted subcutaneously into mice. Tumors from these mice were excised and a normalized amount of the tumor
was then implanted into ceca of recipient mice and metastasis was evaluated. 75% of S2-013-control-siRNA-injected mice displayed local invasion into lymph nodes versus 29% of S2-013-MUC1-siRNA-injected mice. Furthermore, 64% of S2-013-control-siRNA-injected mice had lung metastases whereas only 32% of S2-013-MUC1-siRNA-injected mice displayed lung metastases [126]. Together, these studies demonstrate a role for MUC1 in driving metastatic progression in transgenic, knock-out, knock-in and xenograft mouse models of cancer.

_Mechanism of MUC1 overexpression_

As we have described, MUC1 expression strongly increases during the clinical progression from normal tissue to metastatic disease. We will next summarize the observations regarding the mechanisms by which MUC1 expression is controlled and any correlations these have to cancer progression. MUC1 expression is controlled by multiple factors, including alterations in transcription, amplification and post-translational modifications, and many of these regulatory pathways are strongly activated in the invasive, migratory or metastatic state (Fig 1.5).
Figure 1.5. Schematic of MUC1 interactions, regulation, and downstream signaling.
MUC1 transcriptional regulation

MUC1 is regulated at the transcriptional level by multiple factors, including hypoxia, STATs, hormones and growth factors. In a recent study of clear renal cell carcinoma, MUC1 was found to be a transcriptional target of hypoxia inducible factor 1 alpha (HIF1-α), which is upregulated in metastatic cancer, and promotes migration and invasion [127]. Hypoxia-enhanced MUC1 transcription was also observed in a human lung adenocarcinoma cell line [128], demonstrating that control of MUC1 levels by hypoxia is not tissue-specific. Furthermore, MUC1 is transcriptionally upregulated by STATs in response to interferon gamma (IFNγ) and interleukin-6 (IL-6) signaling [129]. In addition, EGFR can also activate STAT1 and STAT3 in breast cancer tissues [130, 131] and EGFR activation promotes MUC1 expression [132]. Intriguingly, MUC1 and EGFR can interact in cancer cells, resulting in a MUC1-dependent prolongation of EGFR activity [95, 133]. This may represent an EGFR-STAT-MUC1 positive feedback loop that is a source of MUC1 upregulation in breast cancer, although further studies need to be done in this area.

In prostate cancer cell lines, androgen receptor was found to regulate MUC1 expression through interaction with a consensus AR-element in the MUC1 promoter, although this result was cell line specific [134]. In this study, AR was found to down-regulate MUC1 expression in androgen-dependent, but not androgen-independent cell lines. Interestingly, in another study examining the phenotypic behavior of AR and MUC1, androgen treatment was found to increase expression of MUC1, and increased MUC1 expression correlated with loss of cell-cell adhesion [135]. These varied results
indicate that there is more work necessary to fully sort out the role of androgen receptor in MUC1 transcriptional regulation.

Amplification and miRNA regulation of MUC1 expression

While transcriptional regulation of MUC1 is well-established as a mechanism for MUC1 regulation during cancer progression, amplification was recently also identified as a contributing factor for MUC1 overexpression. Examination of 83 patients for MUC1 gene amplification and protein expression found that 12% of benign lesions and 38% of primary invasive breast carcinoma samples displayed MUC1 genomic amplification [136]. Further-more, meta-analysis of 886 primary invasive breast carcinoma samples from 22 studies demonstrated that 44% had genomic gain of the MUC1 gene. MUC1 gene amplification also correlated significantly with protein expression, indicating that MUC1 gene amplification may be an untapped and important source for MUC1 protein in breast cancer samples, and that amplification of MUC1 may select cells for metastatic progression [136].

MUC1 expression and corresponding metastatic phenotypes are also regulated at the post-transcriptional level by microRNAs. Two miRs have been identified to regulate MUC1 translation, including miR-125b, which is upregulated by androgen receptor, and miR-145. The first study to identify miR regulation of MUC1 found that MUC1 translation is repressed by miR-125b, and mir-125b is downregulated in breast cancer cells [137, 138]. AR also promotes miR-125b expression, resulting in a suppression of MUC1 translation [134], and together with AR directly downregulating MUC1
transcription [134], this may indicate an androgen-dependent MUC1 downregulation program in certain circumstances. Finally, miR-145 was found to suppress MUC1 translation, and reduce MUC1-dependent cell invasion [139], and miR-145 is frequently found to be lost during colorectal cancer progression [140].

*Molecular mechanisms of MUC1 in tumor progression*

The many interaction domains of MUC1 allow for distinct mechanisms by which MUC1 promotes cancer progression and metastasis. These include interactions with proteins in the extracellular matrix, at the cell membrane, in the cytoplasm, and in the nucleus where MUC1 acts as a cofactor for gene transcription.

**MUC1 extracellular domain drives migration and invasion**

Tumor-specific MUC1 is underglycosylated, enabling interactions between the MUC1 core protein and many transmembrane receptors and components of the extracellular matrix, such as ICAM-1, an adhesion receptor on the surface of endothelial and peritumoral stromal cells [141, 142]. E-selectin, a receptor present on the endothelial cell surface, also interacts with epithelial cells either via interactions with underglycosylated MUC1 itself or through the binding of other E-selectin ligands present adjacent to MUC1 on the cell surface. In addition, interactions between MUC1 and E-selectin may promote MUC1 binding to ICAM-1 on the endothelial cell surface [143]. The MUC1-ICAM-1 interaction promotes the migratory capacity of tumor cells through the microenvironment, by facilitating interaction between epithelial and endothelial cells,
enabling adhesion of circulating cancer cells to the inner lining of the blood vessel, slowing cell velocity and allowing escape from the blood vessel (reviewed in [144]).

In addition to promoting the ability of transformed cells to interact with vascular endothelium, MUC1-ICAM-1 interactions alter the metastatic phenotype of the cancer cell itself. Upon interacting with ICAM-1, Src interacts with the MUC1-CD, an interaction that promotes Src-mediated cytoskeletal rearrangements [145, 146]. The Src family of nonreceptor tyrosine kinases, through their ability to regulate integrin activation and cytoskeletal function, has long been regarded as key mediators of metastatic progression (reviewed in Aleshin [147]). Interactions between MUC1 and Src induce pro-migratory Rac1- and Cdc42-dependent actin reorganization at sites of contact with endothelial cells, thereby promoting an invasive phenotype in the tumor cell [145]. Lamellipodia and filopodia formation as a result of these interactions are induced via Src-CrkL complexes with MUC1-CD, with Src-dependent kinase activity driving cytoskeletal rearrangements [145]. Overall, these studies demonstrate that MUC1 can drive intercellular interactions that promote metastatic spread, as well as intra-cellular interactions that promote migratory behavior.

In addition to studies focused on interactions of the MUC1 extracellular domain with ICAM-1 and E-selectin, a truncated version of MUC1 spanning only the external stem region, the transmembrane domain, and the juxtamembrane domain was demonstrated to be sufficient to induce cellular EMT, although a precise mechanism was not described [148]. In this study, mouse mammary carcinoma cells expressing this
truncated MUC1 showed mesenchymal morphology, decreased E-cadherin expression, increased vimentin expression, and increased invasion through a Matrigel matrix [148].

MUC1 expression promotes angiogenesis

One key aspect of cancer progression is angiogenesis, which provides nutrients to the primary tumor and an escape route for migratory tumor cells. MUC1 overexpression in non-small cell lung cancer and breast cancer was found to upregulate Vascular-Endothelial Growth Factor (VEGF), thereby promoting endothelial migration and tube formation [149]. Though MUC1 is a transcriptional target of HIF1-α as discussed above, MUC1 also interacts with HIF1-α in the cytoplasm and promotes transcriptional upregulation of HIF1-α targets such as leptin, TGFβ3, and VEGF [150-152]. Furthermore, MUC1 and VEGF expression correlate in human breast cancer cell lines, and MMTV-PyMT mice expressing human MUC1 display more angiogenesis than MMTV-PyMT mice on a Muc1 null background [153], further supporting a role for MUC1 in the onset of angiogenesis.

Promotion of pro-metastatic activities of transmembrane receptors

In polarized epithelium, the MUC1 heterodimer is apically localized. Alternatively, in primary and metastatic tumors, MUC1 is found throughout the plasma membrane, in the cytoplasm and in the nucleus. Membrane-bound MUC1 is constitutively recycled via endocytosis and trafficking through the Golgi, resulting in re-glycosylation of the MUC1-ECD [154]. During cancer progression, when apical and
basolateral proteins become co-localized due to a breakdown of tight junctions, MUC1 interacts with a number of previously sequestered transmembrane receptors, including EGFR [122]. Interactions of MUC1 with EGFR result in the manipulation of EGFR trafficking, with EGFR becoming preferentially recycled instead of trafficked to the lysosome for degradation [133], or to other compartments of the cell such as the nucleus [155] or mitochondria [156]. The result is prolonged EGFR signaling, which can drive tumorigenic interaction and/or regulation of integrins, cadherins, phospholipase Cγ (PLCγ), phopho-inositide 3 kinase (PI3K), and matrix metalloproteinases (MMPs) which contribute to disruption of cell adhesion, induction of cell motility, and degradation of the extracellular matrix (ECM) [157]. In addition, overexpression of MUC1 in primary canine malignant mammary tumors (CMMT) was accompanied by downregulation of galectin-3, which resulted in upregulation of endogenous MUC1, enabling an auto-feedback loop. Additionally, CMMT cells that have invaded into the vasculature expressed MUC1 and EGFR at focal adhesions as opposed to uniform cell membrane expression, further suggesting a role for MUC1 and EGFR in cell motility [158].

Recently, our laboratory published evidence that MUC1 and EGFR regulate the expression of c-Met, the Hepatocyte Growth Factor/Scatter Factor Receptor. c-Met is a receptor tyrosine kinase that is often upregulated in metastatic cancers and is involved in metastatic progression [159]. We found that treatment with a competitive MUC1 inhibitor downregulated c-Met in breast cancer cells, and that MUC1 promoted EGF- and c-Met-dependent cell motility, scattering and the formation of invasive protrusions [160]. In addition, MUC1 has recently been found to be upregulated in a subset of lung cancers
which also possess upregulated or constitutively active EGFR and c-Met [161]. In this study, lung cancer cell lines were analyzed for expression of genes associated with tumorigenesis, and MUC1, EGFR, and c-Met expression were positively correlated. Cell lines with high expression of all three proteins also displayed high expression of Rho-family GTPases, and SNAIL transcription factor, in addition to other genes involved in EMT and cell motility [161], suggesting that this subset of lung cancer is very motile and that MUC1, EGFR and c-Met expression may contribute to this motility.

Studies also show that MUC1 interacts with ErbB2, another member of the EGFR family of receptor tyrosine kinases, in breast cancer cells [162]. ErbB2 does not have a ligand-binding domain, but can heterodimerize and phosphorylate EGFR, ErbB3 and ErbB4, the remaining members of the family. ErbB3 and ErbB4 bind several ligands to become activated, among them Heregulin [22]. The interaction between MUC1 and ErbB2 was found to be driven by Heregulin-binding of ErbB3 or ErbB4 and heterodimerization of either of these proteins with ErbB2. Importantly, this interaction was then observed to promote the nuclear localization of a MUC1-γ-catenin complex [162]. γ-catenin, like β-catenin, is a transcription factor in the Wnt pathway, and can affect genes involved in motility and metastasis. γ-catenin suppresses cell motility and metastasis by downregulating fibronectin [163], a secreted component of the extracellular matrix, by organizing the actin cytoskeleton through modulation of Rho-family GTPases [163], and by upregulating Nm23-H1, a known metastasis suppressor [164]. The authors of this study speculated that MUC1 could be sequestering γ-catenin in order to promote cell motility [162].
Pro-metastatic cytoplasmic interactions

MUC1 has no kinase domain itself, but protein-protein interactions in the cytoplasm allow it to activate signal transduction cascades, many of which have direct roles in driving cancer progression (reviewed in Singh et al) [165]. Many of these interacting partners have been studied for their roles in affecting transformation directly, including inhibitory interactions with the pro-apoptotic protein Bax[166] and c-Abl kinase [167]. MUC1-CD can also directly activate the JAK-1/STAT3 signaling pathway, promoting tumor growth and metastasis in an orthotopic model of lung cancer [32]. Finally, interactions with the tyrosine kinase Src or PKCδ can modulate the interactions between MUC1-CD, GSK3-β and β-catenin [95, 168]. Many of these interactions play a role not only in primary transformation, but in metastatic progression as well.

β-catenin, serving as both an activator of oncogenic transcription and as a suppressor of invasion, has both a protumorigenic and anti-metastatic role to play. As such, its interactions with MUC1 have been shown to promote transformation, primarily through MUC1-β-catenin protein complexes driving transcription of such genes as Cyclin D1 [169]. In addition, a study by Yuan et al demonstrated that the downregulation of MUC1 promoted E-cadherin/β-catenin complex formation, reduced nuclear β-catenin, upregulated both E-cadherin and β-catenin expression, and decreased invasive potential in PANC1 pancreatic cancer cells and MCF-7 breast cancer cells [170]. MUC1 can also interact with the SH3 domain of Src via the RXPPXR domain in the MUC1-CD [171], and the SH2 domain of Grb2 through a phosphorylated tyrosine at the YTPNP motif.
of the MUC1 cytoplasmic domain [172]. These interactions result in an increase in MUC1-dependent metastatic cell behavior including cytoskeletal rearrangement leading to invasive protrusion [145].

In addition to the interactions described above, MUC1 was also found to induce the expression of Platelet-Derived Growth Factor (PDGF-A) a known effector of motility, thereby promoting cell migration [173]. In a mouse model expressing mutant KRAS$^{G12D}$ in the pancreas to drive pancreatic cancer, MUC1 influenced the expression and secretion of PDGF-A through interaction with HIF1-α, a known effector of PDGF-A expression [173]. In this system, MUC1-overexpressing cells were highly dependent on PDGF-A for growth and migration, and PDGF-A and MUC1 were necessary for nuclear translocation of β-catenin. MUC1 is phosphorylated by activated PDGFR-β at two tyrosine residues in the cytoplasmic tail, and PDGF-A expression increases nuclear colocalization of β-catenin and MUC1-CD. Although these interactions do not affect cell proliferation, PDGF-A expression does increase invasion in vitro and tumor growth and metastasis in vivo [174].

Pro-metastatic roles in the nucleus

In addition to the numerous roles for MUC1 in altering pro-metastatic signaling cascades described above, MUC1 can also directly affect gene transcription by acting as a transcriptional cofactor. Gamma-secretase-dependent MUC1 cleavage at the juxtamembrane domain can result in soluble MUC1-CD that is capable of interacting with a variety of proteins. MUC1 has a non-canonical nuclear import domain that
interacts with Importinβ1, a nuclear importer [175]. MUC1-CD can directly act as a transcription factor, altering the transcription of a number of genes that directly affect transformation, including p53 [176, 177]. Induction of MUC1 intracellular cleavage has been shown to be promoted by EGFR activation, as cells pulsed with EGF showed an increase in MUC1-CD relative to full-length MUC1 [178].

Once cleaved from the transmembrane domain, MUC1-CD can interact with proteins such as β-catenin, p120catenin, Src, estrogen receptorβ (ERβ) and EGFR. Furthermore, interaction between MUC1-CD and these proteins promotes their nuclear translocation (reviewed in Singh) [165]. Nuclear localization of MUC1 correlates with poor prognosis, tumor-node-metastasis staging and lower survival and MUC1 has been found co-localized with β-catenin at the invasion front in colorectal carcinoma [179]. Importantly, while all of the gene targets have yet to be mapped, these transcriptional cofactor interactions have been shown to drive an EMT phenotypic-switch. This includes the activation of Slug and Snail transcription factors and the resulting induction of EMT genes, such as vimentin, and N-cadherin [180]. In pancreatic cancer, ChIP-Chip promoter analysis and microarray demonstrated that MUC1-CD is a cofactor for transcription of genes related to invasion, angiogenesis, and metastasis [181]. Additionally, MUC1-CD promotes expression of connective tissue growth factor (CTGF), a mediator of ECM remodeling and angiogenesis [181].
**MUC1 targeted therapies**

The role of MUC1 in both transformation and metastatic progression has led to extensive focus on this protein for the development of targeted therapies to treat metastatic disease. A number of groups have developed vaccine-like therapies to target MUC1, largely focusing on the primary tumor [165]. Below, we summarize a number of therapeutic interventions with an emphasis on metastatic disease (Fig 1.6).
Figure 1.6. Targeted therapies directed against MUC1.
Peptide-based therapies

Upon the identification of protein-protein interactions that drive metastatic progression, we and others began the development of peptide-based therapeutics to block these interactions. In our laboratory, we developed a peptide therapeutic that mimicked the domain of MUC1 that interacts with β-catenin/Src/EGFR. The peptide, PTD-4 MUC1 Inhibitory Peptide (PMIP), was conjugated to the cell-penetrating peptide sequence PTD-4 to allow for cellular uptake [182, 183]. PMIP blocked interactions by serving as a decoy to endogenous MUC1 binding partners, resulting in an inhibition of both proliferation and invasion in vitro. Importantly, PMIP treatment resulted in a significant decrease in metastatic progression in orthotopic models of breast cancer, demonstrating that inhibition of MUC1 can directly inhibit metastasis [184]. Follow-up studies with PMIP further demonstrated the utility of PMIP as a therapeutic intervention for lung cancer [185, 186].

In the Kufe laboratory, the ability of MUC1-CD to form dimers was targeted with a peptide-based therapy termed GO-203[187]. GO-203 is a decoy peptide conjugated to the cell penetrating peptide and targeted to the juxtamembrane region of MUC1, which serves as the non-canonical nuclear localization signal and the dimerization domain [187]. Treatment of chronic myelogenous leukemia and non-small cell lung cancer cell lines demonstrated that blocking MUC1 dimerization resulted in cell cycle arrest and an increase in reactive oxygen species and apoptosis [188]. GO-203 treatment of non-small cell lung cancer (NSCLC) cell lines in xenograft tumors caused tumor regression [189], demonstrating efficacy of this dimerization-blocking drug against MUC1-driven cancer.
Anti-MUC1 antibodies have been utilized to directly target MUC1-positive tumors, with several showing potential in a variety of cancer types. GP1.4 antibody promoted the internalization of MUC1, resulting in decreased signaling of EGFR in pancreatic cancer cells, inhibiting ERK phosphorylation and decreasing both proliferation and migration of these cells [190]. Pancreatic cancer cells coated in MUC1-specific HMFG2 antibody conjugated to CpG effectively activated natural killer cells, and intratumoral injection of conjugated MUC1 antibody reduced tumor burden in mouse models of pancreatic cancer [191]. In addition, in a xenograft mouse model of ovarian cancer, a MUC1 antibody C595 reduced tumor growth, whereas the C595 in combination with docetaxel inhibited tumor growth and metastasis while increasing survival [192].

Additionally, in a study of 447 patients with epithelial ovarian cancer at a variety of stages and who had already undergone chemotherapy, patients were given either standard treatment or one injection of an yttrium$^{90}$-labeled anti-MUC1 antibody (murine HMFG1 antibody), referred to as $^{90}$Y-muHMFG1. This antibody was designed to bind MUC1 epitopes and kill cancer cells with the radioactive yttrium moiety. Patients who were given the $^{90}$Y-muHMFG1 treatment had higher circulating anti-MUC1 antibodies in response to the treatment, and their MUC1 serum assessments were lower, indicating that the levels of circulating MUC1 were being reduced in these patients. Patients with the highest levels of circulating anti-MUC1 antibodies did have higher overall survival (80th percentile of $^{90}$Y-muHMFG1 treated group), though there was no significant difference in
survival between the $^{90}$Y-muHMGF1 treated group as a whole and the group who received the standard treatment [193].

Vaccination against MUC1

In a study testing a vaccination against MUC1, 31 post-menopausal women with stage 2 breast cancer were treated with tamoxifen and either seven injections of oxidized mannan conjugated to a MUC1-GST fusion protein (M-FP), or seven injections of placebo after mastectomy [194]. While none of the patients treated with M-FP experienced recurrence, 27% of the patients treated with placebo recurred. Moreover, after the injection series, patients treated with M-FP elicited immunity when subjected to an additional injection of a different MUC1 fusion protein, indicating that the M-FP treatment acted as a vaccine against MUC1 [194].

Evaluation of a MUC1 cDNA as a vaccine has shown promise as a metastasis suppressor. In this study, wild-type C57/Bi6 mice were given weekly intradermal injections of a full-length MUC1 cDNA plasmid and assessed against mice injected with empty vector. B16-F10 melanoma cells stably overexpressing MUC1 were then intravenously introduced to the mice and animals were evaluated for the formation of lung metastases. Mice vaccinated with the MUC1 cDNA had significantly fewer lung metastases than mice injected with empty vector, providing evidence that vaccination with MUC1 cDNA suppresses MUC1-dependent lung metastasis development [195].

One MUC1 vaccine recently finished Phase 3 clinical trials to determine its efficacy against MUC1-driven cancers. The MUC1 vaccine, ImMucin, is composed of a
short 21-mer peptide sequence at the N-terminal signal peptide region of MUC1, and was shown in preliminary studies to bind to both human MHC Class 1 and 2 immune cell proteins derived from cancer patient tissues. Pre-clinically, mice injected with MUC1-overexpressing DA3 mouse mammary carcinoma cells experienced longer survival after vaccination with ImMucin than mice injected with the carcinoma cells and treated with vehicle [196]. Phase 1 and Phase 2 human clinical trials in which multiple myeloma patients were treated with the vaccine have shown promise but have not been formally reported [197].

Numerous factors affect the metastatic cascade, including cell motility and invasion, degradation of ECM, neo-vascularization, intra- and extravasation, and dormancy and survival at a secondary site [198]. Of these, MUC1 has been shown to affect tumor invasion and neo-vascularization, interactions with the vasculature and survival and growth at a secondary site. Clinically, MUC1 expression is highly correlated with advanced disease, poor survival and tumor dissemination. Mechanistically, MUC1 can drive metastatic progression by altering the interaction between tumor cells and their environment, altering the composition of the tumor microenvironment, and altering the genetic makeup of the tumor cell itself to produce a pro-metastatic phenotype. Due to the prevalence of MUC1 expression in metastatic disease, and the role of metastasis in patient mortality, directly targeting MUC1 would appear to be of paramount importance. While still in the pre-clinical and early clinical stages, MUC1 targeting has now begun in earnest, and appears to hold significant promise for a number of tumor types, including lung, pancreatic and breast.
c-Met signaling

One protein which, like EGFR and MUC1, is implicated in both cancer progression and metastatic progression, is c-Met, a receptor tyrosine kinase and receptor for the Hepatocyte Growth Factor (HGF). In recent years, c-Met has become an important cancer target due to increased expression of c-Met in many cancers, and especially in metastatic cancer. c-Met has a ligand-binding domain at the basolateral cell surface, a transmembrane domain and a cytoplasmic kinase domain. The extracellular ligand-binding domain is processed into an asymmetric heterodimer which associates in the extracellular space through non-covalent bonding [199]. HGF binds to c-Met, causing dimerization and subsequent activation of the receptor through transphosphorylation of the cytoplasmic domain, similar to the mechanism of action of EGFR (Figure 1.7). Phosphorylated tyrosines recruit SH2 domain-containing proteins such as Gab1, the adaptor protein through which c-Met propagates most of its signal [200]. Gab1 recruits PI3K, which leads to mTOR activity and repression of apoptosis through inhibition of pro-apoptotic Bcl2 family members [29], as well as Shp2, a phosphatase involved in many biological functions, including activation of the MEK/Erk pathway and consequent cell proliferation [201]. Both PI3K and Erk/MEK pathways are important for cancer progression, but in addition to survival and proliferation signals, these pathways are also both implicated in disassembly of tight junctions [202], and both affect cell motility [201, 203]. In addition, c-Met induces cell motility through downstream activation of Rac1 and Gab1 interaction with Crk leading to Rap1 activation [204, 205]. Delamination, the release of cells from epithelial cell layers, is an important
process in development, and c-Met has been shown to be one of two receptors which are known to activate this behavior. The role of c-Met in delamination may be why c-Met is particularly implicated in cell motility and metastatic progression [206].

*c-Met transcriptional regulation and therapy*

C-Met transcriptional regulation has not been thoroughly described. Sp1 and Sp3 general transcription factors having binding sites in the c-Met promoter, which may impart continuous low expression levels [207]. C-Met is also regulated by HIF1-α, a hypoxia-induced transcription factor which is often activated in cancer, and AP-1, a transcription factor that is downstream of HIF1-α and many receptor tyrosine kinases is implicated in c-Met transcription [208]. There are several Ets1 binding sites in the c-Met promoter [209], though Ets1 is thought to be strictly an endothelial and stromal cell transcription factor in normal tissues. Ets1 is upregulated in many cancers and promotes migration and invasion when expressed in cancer cells in vitro [210], leading to the suggestion that the effect of Ets1 on cell motility is due to c-Met upregulation. Mechanisms by which these transcription factors are activated to upregulate c-Met have not been described.

Targeted therapies against c-Met have been pre-clinically investigated and are currently in clinical trials. Therapies include antibodies raised to the extracellular ligand-binding domain of c-Met and specific and non-specific tyrosine kinase inhibitors. One specific kinase inhibitor, INCB28060, was recently approved for Investigational New
Drug status, enabling its use in select patients, though it has not been fully FDA-approved [211].

c-Met activation has been implicated in resistance to EGFR targeted therapies in numerous studies [212-214]. Resistant populations of EGFR-overexpressing cells often gain overexpression of c-Met to activate blocked downstream signals. New combinations of therapies which target both c-Met and EGFR have been shown to prevent this specific population of resistant cells from arising [215], defining a need for combination therapy against both receptors in cancer treatment and specifying a role for c-Met in EGFR biology.
Figure 1.7. HGF binds to the extracellular domain of c-Met, enabling Gab1 recruitment to the intracellular kinase domain, resulting in extensive downstream signal transduction.
Epithelial cell polarity

One important aspect of epithelial tumor initiation is the loss of cell polarity. Cell membrane apical and basolateral domains are known to be demarcated by tight junctions, enabling segregation of different membrane domains. Correct membrane orientation is important for proper cell signaling, because receptors must be poised at the basolateral membrane to receive ligands from the stroma. Cell polarity is also important for secretion at the apical side of the cell as well as ensuring the formation of membrane-associated protein-protein interactions, among other biological functions [216].

As tumor initiation progresses, cancer cells lose contact inhibition and gain the ability to grow out of contact with the basement membrane, the extracellular matrix secreted by the epithelial cells themselves which binds integrins at the basolateral cell surface. Normal cells undergo programmed cell death when basement membrane engagement is lost [217], but advantageous mutations in the cancer confer the ability to survive. Cells grown in an unorganized way may still have cell-cell junctions, but they have lost orientation due to absence of a basement membrane; therefore, apical and basolateral delineations are lost and membrane-associated proteins may localize to any area of the plasma membrane [218]. This allows cancer-specific oncogenic interactions to occur and promotes tumor progression as will be discussed in more detail shortly.

Loss of cell polarity is detrimental to the function of the tissue; therefore, its formation is highly regulated. Establishment of the cell membrane domains is due to the work of three protein complexes: the Par complex, the Scribble complex, and the Crumbs complex (Fig 1.8). The Par complex is comprised of atypical protein kinase C (aPKC),
PAR3 and PAR6. Together, this protein complex defines the apical domain of the plasma membrane. It localizes to the cell cortex above the tight junction and PAR3 and PAR6 use their postsynaptic density 95/Discs large/Zona occludens-1 (PDZ) domains as scaffolds to regulate aPKC kinase activity and substrate specification [219]. The Scribble complex is composed of Scribble (Scrib), Discs Large (DLG), and Lethal Giant Larvae (LGL), which localize to the lateral plasma membrane and define the basolateral domain through antagonism of the Crumbs complex. LGL is phosphorylated by aPKC to exclude it from the apical domain and cause its translocation to the basolateral domain, defining the basolateral domain [220]. The Crumbs complex contains PALS, PATJ, and Crumbs. It localizes to the subapical domain just above the tight junction and docks in the cell membrane due to the transmembrane Crumbs protein [221]. The concerted cooperation between these complexes defines the domains of the plasma membrane, and this governs membrane trafficking and membrane protein localization in the cell.
Figure 1.8. Epithelial cell polarity is established through the interactions of the Par, Scribble, and Crumbs complexes. Used with permission from [222]
Widely used assays to discern epithelial cell polarity include 3D cell culture in Matrigel, a basement membrane extract, and growth on permeable membrane filter supports. In the Matrigel cell culture system, cells are seeded on Matrigel and allowed to embed and grow to a solid ball of cells. Contact with the Matrigel induces secretion of basement membrane components from immortalized epithelial cells, allowing for the establishment of apical-basolateral polarity, with the basolateral side of cells in contact with the external basement membrane. Cells within the cell mass, or acinus, undergo anoikis due to lack of integrin engagement with the basement membrane. This allows for the establishment of an apical cell membrane domain and a single cell-layered sphere of cells (Fig 1.9). This acinus can be manipulated experimentally and polarity can be assessed by staining for proteins of known localization in normal epithelial cells [223].
Figure 1.9. Non-cancerous epithelial cells form polarized acinus structures when grown in Matrigel basement membrane extract. (Left to right) Single cells seeded on Matrigel proliferate into a solid ball of cells before lack of basement membrane contact induces anoikis in the internal cells. This process results in a monolayer sphere of epithelial cells that has a lumen and is analogous to a duct. Used with permission [224].
Growth of epithelial cells on permeable supports promotes cell polarization. Cells are seeded on a filter and submerged in growth media. Cells attach to the filter and secrete basement membrane components onto it, allowing for integrin engagement with extracellular matrix proteins (Fig 1.10). In addition, basolaterally localized receptors are primed to receive ligands through the filter, a scenario which is analogous to normal epithelial cell layer interactions with the stroma but which is absent in conventional cell culture. Growth of cells in this configuration allows for apical-basolateral cell polarity to be established [225]. These two polarity assays allow for experimental manipulation of cells and the assessment of resulting cell polarity changes. Specific uses of these assays in our breast cancer studies will be discussed below.
Figure 1.10. Growth of epithelial cells on membrane filters permits apical-basolateral cell polarity formation.
**Statement of the problem**

EGFR and MUC1 are both implicated in progression of many epithelial cancers, though there are many aspects of their tumor promotion that are not well characterized. EGFR is well-studied for its role in signal transduction leading to cell survival and cancer metastasis, and MUC1 is known for its pro-tumorigenic protein-protein interactions. EGFR and MUC1 have been shown to interact, and this interaction promotes EGFR activity and prevents its degradation, leading to tumorigensis. Inhibition of EGFR and MUC1 interaction slows tumor progression and inhibits cancer cell phenotypes and metastatic progression. We know that in normal cells, EGFR and MUC1 are stratified to opposite domains of the cell membrane, preventing their interaction, and that this localization is lost in cancer. We also know that MUC1 and EGFR are not segregated in the lactating mammary gland, yet lactation does not predispose to breast cancer. These facts led us to the following hypothesis: Mislocalization of MUC1 and EGFR promotes breast cancer metastasis by affecting epithelial cell polarity. To address this hypothesis, we have proposed the following questions:

I. How do MUC1 and EGFR promote cancer metastasis?
   a. Do MUC1 and EGFR affect cell motility?
   b. Do MUC1 and EGFR together transcriptionally regulate the cell motility program?

II. Do MUC1 and EGFR interactions cause a loss of epithelial cell polarity?
   a. Does mislocalized EGFR, and subsequent interaction with MUC1, cause a loss of membrane identity?
III. Do MUC1 and EGF expression affect function in the lactating mammary gland?
   a. Do loss of MUC1 and EGF cause an impairment of tissue architecture in the lactating mammary gland?
   b. Do loss of MUC1 and EGF reduce weaning litter size?
II. MATERIALS AND METHODS

Cell culture

MDA-MB-231, MDA-MB-453, and MDA-MB-468 cells were acquired from the American Type Culture Collection (ATCC) and grown in RPMI 1640 (Cellgro) with 10% FBS (MDA-MB-468 cells were grown in 5% FBS) (PAA Laboratories) and 1% Pen/Strep (Cellgro). BT20 cells were acquired from ATCC and grown in EMEM (ATCC) with 10% FBS and 1% Pen/Strep. MCF10A cells were acquired from ATCC and grown in DMEM/F12 (with L-glutamine) supplemented with 5% Horse Serum, 20 ng/mL Epidermal Growth Factor (EGF), 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin (Sigma Aldrich), 10 μg/mL insulin, and 1% Pen/Strep. Primary HMEC cells were acquired from Invitrogen and grown in serum-free HuMEC Ready Medium supplemented with Bovine Pituitary Extract (Invitrogen) and 1% Pen/Strep. All cells were grown in 5% CO₂. Cells were selected and grown with 1 mg/mL G418 for MDA-MB-231, or 0.5 mg/mL G418 for MDCK or MCF10A cells (Omega Scientific, GN-04) and with 5 μg/mL Puromycin for MDCK.

3D Matrigel cell culture

40 μL of Matrigel (BD) was plated on each well of 8-well chamber slides and allowed to dry at 37°C for 30 minutes. MCF10A cells were suspended in DMEM/F12 containing 2% Matrigel, 5% Horse Serum, 5 ng/mL EGF, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin (Sigma Aldrich), 10 μg/mL insulin, and 1% Pen/Strep and allowed to embed in the Matrigel at a density of 6,000 cells/well. Cells were grown for 21 days and
media was changed every four days. HMEC cells were suspended in HuMEC media containing 2% Matrigel, Bovine Pituitary Extract and 1% Pen/Strep and plated on Matrigel as above. HMEC cells were grown for 4 days and media was changed every two days.

**Immunofluorescence of cells in 3D cell culture**

The cells were fixed in 2% paraformaldehyde for 10 minutes, washed in PBS, and permeabilized (0.5% Triton X-100 in 10mM Pipes (pH 6.8), 50mM NaCl, 300 mM sucrose and 3mM MgCl2) for 10 minutes at room temperature. Following permeabilization, cells were blocked in 3% BSA/0.05% Tween in PBS for 30 minutes at room temperature. The cells were incubated in primary antibody overnight at 4°C, and following antibody incubation were washed every 10 minutes for two hours in PBS at room temperature. The cells were then incubated overnight at 4°C in Alexa fluor-conjugated secondary antibodies and then washed for two hours in PBS. Cells were then mounted with SlowFade Gold Antifade Reagent with DAPI (Invitrogen) and visualized using a Zeiss Confocal Microscope and LSM510 Software.

**Immunofluorescence/immunohistochemistry of tissues**

Tissues were dissected and fixed in 10% buffered formalin or Methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid), embedded in paraffin and sectioned using the Tissue Acquisition and Cellular/Molecular Analysis Shared Service (TACMASS) of The Arizona Cancer Center. Tissue deparaffinization and immunofluorescence was
performed as described in [226]. Antigen retrieval was performed in 1mM EDTA or 10 mM sodium citrate at 100°C.

Mice
MUC1-/- mice were a kind gift from Sandra Gendler (Mayo Clinic, Scottsdale, AZ). EGF-/- mice were a kind gift from David Lee (University of North Carolina, Chapel Hill, NC). MUC1-/- and EGF-/- mice were bred to yield double heterozygotes from which all mice were generated. Genotypes were maintained on a C57Bl/6 background and bred to yield MUC1-/- EGF-/-, MUC1+/- EGF-/-, MUC1-/- EGF+/-, and MUC1+/- EGF+/-.

Microarray
BT20 breast cancer cells were treated for one hour with 50µM PMIP, 50µM control peptide, or peptide vehicle (PBS) and RNA was collected after 24 hours. Six CodeLink Human Whole Genome Bioarrays were hybridized, and data analysis was performed by the University of Arizona Genomics Facility Core. The False Discovery Rate method of statistical significance was employed to interpret the data [227], in addition to the GeneSpring program by Agilent.
RT-PCR

RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). The Superscript III First-Strand Synthesis System for RT-PCR was used to generate cDNA (Invitrogen). Polymerase chain reaction was performed using Crimson Taq DNA Polymerase (New England Biolabs) and the following gene-specific primers:  

- **MET**: sense 5’-ACTCCCCCTGAAAAACCAAAGCC-3’, antisense 5’-GGCTTACACTTCGGGCACTTAC-3’; 
- **ACTB**: sense 5’-AAGAGAGGCATCCTCACCCT-3’, antisense 5’-TACATGGCTGGGGTGTGTTGAA-3’ [228].

Cycling conditions were:  

- **MET**: denaturing at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 68°C for 30 s; 
- **ACTB**: denaturing at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 68°C for 30 s. 

The PCR products were separated on a 1% agarose gel. Gels were imaged and densitometry performed using CareStream Molecular Imaging Software (v 5.0.4.46).

qRT-PCR

RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) and cDNA was generated through the use of Transcriptor First Strand cDNA Synthesis Kit (Roche). qPCR was performed using the following primers synthesized by Operon: 

- **MET**: 5’-AAATGTGCATGAAGGAA-3’ and 5’-TCTCTGAATTAGAGCGATGTTGA-3’; 
- **ALAS1**: 5’-GCCTCTGCAGTCCTCAGC-3’ and 5’-AACAAACACTCTCCATGTTCAGG-3’.

Cycling conditions were: initial denaturing at 95°C for 2min, 55 cycles of: denaturing at 95°C for 10 sec, annealing at 62-52°C for 10
sec (temperature was reduced 0.5°C per cycle), and extension at 72°C for 5 sec, final extension at 72°C for 2 min, and a final cooling to 37°C for 2 min. Products were detected using Roche Universal Probe Library gene-specific probes. PCR was performed on a Lightcycler 2.0 (Roche).

**Plasmids and transfection reagents**

pCDNA3 plasmid containing the full length MUC1 cDNA was a kind gift from MA Hollingsworth (University of Nebraska Medical Center). pEGFP-N1 plasmid containing EGFR cDNA was a kind gift from G Carpenter (Vanderbilt University School of Medicine). Lipofectamine 2000 (Invitrogen) was used to transfect constructs into cells according to the manufacturer’s specifications.

**Cloning**

fusion protein cDNAs were amplified from pEGFP-N1 and ligated into pLVX-Tre3G using the Clontech InFusion Cloning System according to manufacturer protocols.

**Reagents and antibodies**

Human recombinant EGF was from Fisher. SU11274 was from EMD Chemicals. Human recombinant HGF was from R&D Systems. PMIP was produced by GenScript (sequence: NH$_2$-YARAAARQARAPYEKVSAGNGGSSLSCOOH) and reconstituted in PBS at a concentration of 1 mM. MUC-1 antibody was obtained from Thermo Scientific (AB-5), and β-actin antibody was obtained from Sigma (AC-15). Phospho-tyrosine, EGFR 1005, c-Met, and aPKC antibodies were obtained from Santa Cruz Biotechnologies. Phospho-Met antibody was obtained from Cell Signaling Technologies. E-cadherin antibody was obtained from Invitrogen. GM130 was from BD. Laminin γ5 was from Millipore.

**Western blot**

Cells were lysed in cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 5 mM EDTA, pH 8.0, and 10 mM sodium fluoride, 2 mM sodium orthovanadate, 50 μM ammonium molybdate, and Complete (Roche) protease inhibitors). Cell lysates were vortexed briefly, centrifuged, and the supernatant stored at -80°C. Protein concentrations were determined by BCA assay (Pierce). Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Millipore). Membrane was blocked in 3% nonfat milk in PBS (0.1% Tween20 (Fisher Scientific)) or 3% BSA in TBS (0.1% Tween20
(Fisher Scientific) and immunoblotted. The membrane was then treated with Super Signal West Pico Chemiluminscent Substrate (Pierce), visualized on Full Speed Blue film (American Digital Imaging & Medical Products, Inc.), and developed with a Konica SRX-101A. Blot images were manipulated as follows: images were converted to black and white and brightness was adjusted using Adobe Photoshop Elements Version 9.0.

**Scattering assay**

MDA-MB-231 cells were seeded onto Matrigel at a density of 6.0 x 10³ cells per 8-well chamber and incubated in RPMI with 10%FBS, 1% Pen/Strep, and 1 µg/mL G418. Cells were treated with 10 ng/mL EGF, 20 ng/mL HGF, both ligands, or were left untreated. Media and treatment were changed every other day. To count single scattered cells, cells less than 50 pixels in diameter were considered a single cell. We performed a two-tailed student t-test in order to define statistical significance. Images were manipulated as follows: all images were converted to black and white, shadows were lightened, highlights were darkened, and brightness and contrast were adjusted using Adobe Photoshop Elements Version 9.0.

**Scratch assay**

MDA-MB-231 cells and BT20 cells were seeded and allowed to grow to complete confluence. A scratch was introduced and media was replaced with complete media containing either 10 ng/mL EGF, 50 µM PMIP, 5 µM SU11274 or no treatment. Cells were observed at 6 hours (MDA-MB-231) or 24 hours (BT20) after scratching. Image J
software was used to analyze the area of the scratch, and we performed a two-tailed student t-test to determine statistical significance. Images were manipulated as follows: all images were converted to black and white, shadows were lightened, highlights were darkened, and brightness and contrast were adjusted using Adobe Photoshop Elements Version 9.0.
III. MUC1 DRIVES C-MET-DEPENDENT MIGRATION AND SCATTERING

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Conflict of Interest: JAS is also the chief scientific officer of Arizona Cancer Therapeutics

Note: The contents of this chapter were previously published in:

Experiments in Fig 3.2A, B and Supplemental Figure 3.1 were performed by Benjamin G. Bitler, Supplemental Figure 3.2 were performed by Derrick M. Broka, and several replicates of experiments in Fig 3.5 were performed by Jeanne M. Louderbough.

**Introduction**

MUC1 is a hetero-dimeric O-glycosylated transmembrane mucin highly expressed in ductal carcinomas, including carcinomas of the breast, lung, pancreas, colon and ovary [230-234]. MUC1 expression has been shown to correlate with increased severity of disease and metastatic progression in gastric, prostate, breast and pancreatic cancer in addition to thymic epithelial tumors [235-240] and MUC1 cellular mislocalization has been shown to correlate with disease progression in non-small cell lung cancer [241]. In addition, MUC1 expression is increased specifically in metastatic colorectal cancer [242], highlighting the importance of MUC1 for cancer progression and metastasis.

MUC1 has been implicated as a driver of metastatic progression, via specific activities of both the extracellular domain and its intracellular domain (MUC1-C) [243-245]. The extracellular domain of MUC1 has been shown to increase endothelial cell adhesion and promote intravasation and extravasation through interactions with ICAM-1 and galectin-3 [246, 247]. In addition, localization of MUC1 can alter integrin-dependent binding and cell adhesion [248]. Furthermore, MUC1 can drive invasion and migration via the upregulation of matrix metalloproteinase 13 (MMP13), a protein that aids in cell invasion [249], and through the induction of epithelial to mesenchymal transition in
pancreatic cancer cells [180]. These analyses demonstrate a role for MUC1 in promoting cell migration, invasion, and metastasis. In addition, MMTV-MUC1 transgenic models of breast cancer displayed lung metastasis that was dependent upon expression of the cytoplasmic portion of MUC1 [250].

MUC1-C has been shown to interact with a number of oncogenic proteins, promoting either their membrane function or activity in the nucleus [80, 251-255]. One such interaction is via the Epidermal Growth Factor Receptor (EGFR), wherein MUC1 expression promotes the oncogenic properties of EGFR by prolonging the activity of EGFR and promoting its nuclear function [58, 88]. Studies in the WAP-TGFα model of EGFR-dependent breast cancer showed that loss of Muc1 expression suppresses tumor formation by greater than 60% and significantly blocks early hyperplasia (note: mouse Muc1 and human MUC1 are denoted as such). In addition, loss of Muc1 expression completely abrogates metastatic progression in this model [256]. Interactions between MUC1 and EGFR reduce the ligand-dependent degradation of EGFR, and alter EGFR trafficking from the lysosome, resulting in preferential recycling to the plasma membrane [88]. In addition, MUC1 co-expression promotes retro-translocation of EGFR to the nucleus, where interaction with MUC1 promotes the binding of EGFR to the CCND1 promoter [58]. MUC1-C has also been shown to interact with β-catenin and p120-catenin to promote their translocation to the nucleus and their activity as transcriptional cofactors [245, 257]. Overall, MUC1 promotes the intracellular localization and activity of a number of proto-oncogenes, including EGFR, FGFR, PDGFRβ, β-catenin, p120 catenin, src, estrogen receptor, p53, HSP70 and HSP90 [58, 88, 243-245, 257-260].
In previous studies, we have generated a MUC1 ‘decoy’ peptide to block protein-protein interactions between MUC1 and EGFR and MUC1 and β-catenin. This was accomplished by synthesizing a 15-amino acid region of MUC1-C that was previously shown to be required for interactions between these proteins in tandem with a Cell Penetrating Peptide (CPP) [89, 261-263]. The CPP allows adjacent peptide sequences to be taken up into cells across the plasma membrane, where interactions with endogenous intracellular proteins can occur. The MUC1 peptide was termed Protein Transduction Domain – MUC1 Inhibitory Peptide (PMIP), and in vitro studies demonstrated that treatment of breast cancer cells with PMIP resulted in an inhibition of interaction between MUC1 and EGFR as well as an inhibition of the colocalization between MUC1 and β-catenin [89]. PMIP significantly inhibited the growth and invasion of breast cancer cell lines in vivo and the initiation and progression of mammary gland tumors in the MMTV-pyMT transgenic model. In animals treated with PMIP, analysis of remaining mammary glands and tumors revealed a reduction in MUC1 expression after treatment. In addition, PMIP significantly suppressed the ability of primary breast tumors to form secondary metastasis in a MDA-MB-231 orthotopic model of breast cancer [89]. Subsequent to this work, Klinge et al., reported PMIP treatment of lung cancer cells resulted in a decrease in proliferation, decreased Estrogen Receptor α (ERα)-dependent gene transcription, and altered subcellular localization of MUC1, ERα and ERβ [90].

In the current study, we investigated the mechanism by which MUC1 promotes metastatic progression and whether PMIP could inhibit this phenotype. Analysis of
MUC1-induced migration in Matrigel revealed an induction of both migration and cell scattering. Using microarray technology, we identified c-Met mRNA as being significantly downregulated by PMIP. Further characterization of c-Met regulation demonstrated a role for c-Met in driving MUC1 and EGFR-dependent migration and scattering.

Results

PMIP inhibits cell motility

MUC1 promotes EGFR-dependent transformation in the WAP-TGFα model of breast cancer, inhibits ligand-dependent degradation of EGFR and promotes EGFR-dependent Cyclin D1 expression [58, 88, 256]. The cell penetrating peptide to the MUC1-cytoplasmic domain, PMIP, blocks MUC1 and EGFR interactions and inhibits tumor progression in both the MMTV-pyMT transgenic model of breast cancer and the scid-MDA-MB-231 orthotopic model of breast cancer [89]. In addition to blocking tumor progression, PMIP treatment results in a reduction of metastasis in the scid-MDA-MB-231 mouse model [89]. In order to determine whether inhibition of metastasis is due to decreased cell motility upon PMIP treatment, we performed an in vitro wound healing assay. For this assay, parental BT20 breast cancer cells or MDA-MB-231 breast cancer cells transfected with either a CMV-MUC1 overexpression construct (hereafter referred to as MDA-MB-231-MUC1) or the CMV empty vector (MDA-MB-231-control) were used (Figure 3.1N). Cells were seeded on Collagen IV-coated tissue culture plastic, allowed to grow to complete confluence and then a scratch was introduced into the
monolayer. Complete media alone (Figure 3.1 G and H), complete media plus 10 ng/mL EGF (Figure 3.1 A-D and I-J), or complete media plus 10 ng/mL EGF and 50 µM PMIP (Figure 3.1 E-F and K-L) was added to cells. Activation of phospho-tyrosine by EGF was observed in EGF-treated groups, although PMIP treatment reduced total pEGFR/EGFR levels (Figure 3.1P) as was previously shown [89]. Cells were observed at either 6 hours (MDA-MB-231) or 24 hours (BT20) after treatment to assess motility into the scratch. Six hours after treatment, it was found that MDA-MB-231-MUC1 cells filled the scratch 23% more than MDA-MB-231-control cells (p=0.002), and PMIP treatment reduced this behavior back to baseline (p=0.0004) (Figure 3.1M). 24 hours after treatment of BT20 cells, PMIP treatment reduced EGF-dependent migration by 28% (p=0.017) (Figure 3.1O). This indicates that MUC1 is a driver of cell motility, and that PMIP blocks this activity. These data indicate that PMIP functions to block EGF-dependent migration.
Figure 3.1. PMIP treatment inhibits in vitro wound healing. MDA-MB-231-control or MDA-MB-231-MUC1 cells (A-F), or BT20 cells (G-L) were grown to complete confluence on Collagen IV-coated tissue culture plates and a scratch was introduced. Cells were untreated (G and H), treated with 10 ng/mL EGF alone (A-D and I-J) or EGF
and 50 µM PMIP (E, F, K, and L). Area filled was determined by demarcating cell front as demonstrated by dotted line and measuring the area of the center space. Area filled at end of experiment is quantified in M (for MDA-MB-231) and O (for BT20). MUC1 expression in MDA-MB-231-MUC1 and MDA-MB-231-control cells is shown in N. Phospho-tyrosine expression in response to EGF (Santa Cruz PY99), EGFR (Santa Cruz 1005-G), and MUC1 (NeoMarkers AB-5) expression are shown in P. Scale bar indicates 100 µm. *p<0.05, ** p< 0.01, *** p< 0.001.

PMIP treatment results in downregulation of c-Met transcript.

To analyze a potential mechanism by which PMIP reduces metastasis, we performed a Codelink Whole Human Genome Bioarray in BT20 breast cancer cells. Cells were treated with PMIP, cell penetrating peptide (PTD-4) or vehicle (PBS) for 24 hours, RNA was collected and hybridized to the array (Figure 3.2A). We observed differential regulation greater than 3-fold in ninety-seven genes affecting a variety of processes with a majority of the downregulated genes involved in cell cycle, cell proliferation, and signal transduction (Figure 3.2A and B, and Supplemental Figure 3.1). Among the downregulated genes was MET, which was downregulated 3-fold with PMIP treatment. To verify that MET expression was regulated by PMIP treatment, we performed semi-quantitative RT-PCR on BT20 cells treated with 10 µM or 20 µM PMIP, compared MET expression to untreated cells, and found a dose-dependent reduction of MET expression in response to PMIP treatment (Figure 3.2C). Additionally, we performed qRT-PCR on BT20 cells treated with 50 µM PMIP, the same concentration that was used in the microarray study. We compared MET expression in these cells to cells treated with vehicle (PBS) and found a significant reduction in MET expression upon PMIP treatment (Figure 3.2D). Next, the effect of PMIP treatment on MET expression was evaluated by
RT-PCR in MCF10A cells, a non-transformed breast epithelial cell line (Figure 3.2E). While MET is expressed in MCF10A, no significant reduction was found upon PMIP treatment, indicating that the effect of PMIP may be tumor-specific. These data indicate that one potential mechanism by which PMIP blocks metastasis is through the down-regulation of MET.
Figure 3.2. PMIP treatment changes gene transcription. BT20 breast cancer cells (A-D) or MCF10A immortalized breast epithelial cells (E) were treated with 50 µM PMIP, control peptide (TAT), or vehicle (PBS) and analyzed for differences in gene expression. (A) Induction of gene expression (red) and repression of gene expression (green) are shown for the ninety-seven expression changes detected. The accession number for c-Met is highlighted in yellow. (B) Changes in gene expression grouped by biological process. (C) RNA was extracted from untreated BT20 cells or cells treated with PMIP and analyzed for relative MET expression by RT-PCR. MET expression is normalized to ACTB expression. (D) RNA was extracted from BT20 cells treated with PMIP or vehicle (PBS) and analyzed for relative MET expression by qRT-PCR. MET expression is normalized to ALAS1 expression. (E) RNA was extracted from MCF10A cells treated with PMIP or vehicle (PBS) and analyzed for relative MET expression by RT-PCR. * p<0.05, ** p<0.01, *** p<0.001, NS=no significance.
Inhibition of c-Met activity decreases cell motility.

PMIP is hypothesized to act by blocking MUC1-dependent functions in the cell. To determine whether MUC1-dependent migration relied on c-Met activity, we performed an in vitro wound healing assay on Collagen Type IV and Fibronectin extracellular matrix substrates with the c-Met kinase inhibitor, SU11274. MDA-MB-231-control and MDA-MB-231-MUC1 cells were seeded onto Collagen Type IV or Fibronectin and scratched as described in Figure 3.1. To determine the effect of c-Met activity on MUC1-dependent migration, cells were either treated with DMSO vehicle and 10 ng/mL EGF (Figure 3.3A, B, C and D) or supplemented with 5 µM SU11274 (Figure 3.3E and F). As seen before, MDA-MB-231-MUC1 cells exhibited greater wound healing capacity than MDA-MB-231-control cells (Figure 3.3D vs B). Addition of SU11274 significantly decreased scratch filling in MUC1-overexpressing cells (Figure 3.3F vs D), demonstrating that MUC1-driven cell motility requires activation of c-Met. On Collagen Type IV, MUC1 increased migration by 19% (p=0.002), which was repressed by c-Met inhibitor back to baseline levels (p=0.0002). On Fibronectin, MUC1 increased migration by 36% (p=0.032), which again, was repressed by c-Met inhibitor to control levels (p=0.033) (Figure 3.3G and H). In addition to evaluating migration in MDA-MB-231 cells, migration was also assessed in BT20 cells which were treated with either complete media (Figure 3.3I and J), complete media plus 10 ng/mL EGF (Figure 3.3K and L), or complete media plus 10 ng/mL EGF and 5 µM c-Met inhibitor (Figure 3.3M and N). Analysis of c-Met activity revealed an induction of phospho-Met in response to EGF compared to control (Figure 3.3P middle lane vs left lane), while this induction is blocked.
by the c-Met kinase inhibitor (Figure 3.3P right lane vs middle lane). Note that treatment with either EGF or the c-Met kinase inhibitor does not alter total MUC1 levels. When evaluated in BT20 cells (Figure 3.3I-N), EGF-stimulated migration (on both Collagen IV and Fibronectin) was again blocked by Met inhibitor. As seen in MDA-MB-231 cells, the c-Met kinase inhibitor blocks wound healing in BT20 cells. On Collagen IV, the c-Met kinase inhibitor reduced migration of BT20 cells by 28% (p=0.013) (data not shown), and on Fibronectin, migration was reduced by 27% (p=0.0001) (Figure 3.3O).
Figure 3.3. c-Met kinase inhibitor (SU11274) inhibits MUC1-dependent in vitro wound healing. MDA-MB-231-control and MDA-MB-231-MUC1 cells (A-F) or BT20 breast cancer cells (I-N) were plated and scratched as described in Figure 1 on both Collagen IV and Fibronectin substrates, after which cells were treated with either DMSO vehicle (A and B), complete media (I and J), 10 ng/mL EGF (A-D and K-L), or 10 ng/mL EGF plus
5 μM SU11274 (E-F and M-N), a selective c-Met kinase inhibitor. Images shown are on Collagen IV substrate. Area filled was determined as in Figure 1. Quantification of area filled is shown in G and H (MDA-MB-231) and O (BT20). (P) Protein lysates were created from BT20 cells grown on Fibronectin overnight in either complete media (left lane), complete media plus 10 ng/mL EGF (middle lane), or complete media plus 10 ng/mL EGF and 5 μM SU11274 (right lane). 40 μg of lysate per treatment were separated by SDS-PAGE and probed by immunoblot to evaluate c-Met phosphorylation (Cell Signaling 3D7), total c-Met (Santa Cruz C-28), and MUC1 expression (NeoMarkers AB-5). Scale bar represents 100 μm. * p<0.05, ** p<0.01, *** p<0.001.

*MUC1 and EGF promote cell scattering and invasive branching.*

One of the hallmarks of c-Met activity is its ability to induce cell scattering. To evaluate the effects of MUC1 and EGF on cell scattering, cells were plated in Matrigel and evaluated for single-cell scattering away from primary colonies. For this assay, MDA-MB-231 cells were seeded in Matrigel and grown for thirteen days in the presence of 10% serum. During this time, cells formed colonies and dispersed from the periphery of the colonies through the surrounding matrix (Figure 3.4, arrowheads). To determine the effect of MUC1 expression on scattering in this assay, MDA-MB-231-MUC1 cells or MDA-MB-231-control cells were seeded in Matrigel and single cells were counted in the matrix surrounding the cell colonies. We observed that MDA-MB-231-MUC1 exhibited greater cell scattering than MDA-MB-231-control cells (Figure 3.4B and D vs A and C, arrowheads, and quantified in Figure 3.4E). We also noticed that colonies of MDA-MB-231-MUC1 cells occasionally had branches invading into the surrounding Matrigel matrix, whereas we did not see these branches in MDA-MB-231-control cells. (Figure 3.4B and D vs A and C, arrows). These results show that MUC1 promotes cell scattering in breast cancer cells grown on Matrigel.
To evaluate the role of EGFR activity on MUC1-dependent cell scattering, we treated MDA-MB-231 cells with 10ng/ml human recombinant EGF to stimulate EGFR and evaluated scattering as described above. We found that EGF-treated MDA-MB-231-MUC1 cells exhibited more cell scattering than MDA-MB-231-MUC1 cells grown in the absence of EGF (Figure 3.4C and D vs A and B, arrowheads, and quantified in Figure 3.4E). We also observed that EGF-treated MDA-MB-231-control cell colonies possessed invasive branching similar to those seen in MDA-MB-231-MUC1 cells without EGF treatment, and the invasive phenotype of the cell colonies was most apparent in cells both overexpressing MUC1 and treated with EGF (Figure 3.4C and D vs A and B, arrows). These results indicate that EGFR activation promotes scattering similar to that seen with MUC1 expression in three-dimensional culture.
Figure 3.4. MUC1 expression and EGF treatment promote cell scattering and matrix invasion. MDA-MB-231-control (A, C) or MDA-MB-231-MUC1 (B, D) were seeded on Matrigel in complete media (A, B) or complete media with 10ng/mL EGF (C, D). Arrowheads indicate single cells, and arrows indicate invasive protrusions. Images were
taken after 8 days, and (E) single cells around the cell colonies were counted after 13
days for eight images per treatment in three independent experiments. Shown is
quantification from one experiment, though similar results were found for all three. Scale
bar represents 50 μm. * p<0.05, ** p<0.01, ***p<0.001

c-Met activation drives invasive branching in MUC1-expressing cells.

Our observation that MUC1 and EGF induced cell branching in addition to cell scattering
prompted us to evaluate the branching phenotype. We treated MDA-MB-231-MUC1 and
MDA-MB-231-control cells in Matrigel with no exogenous ligand (Figure 3.5A and E),
10 ng/mL EGF (Figure 3.5B and F), 20 ng/mL HGF, the Hepatocye Growth Factor and
the ligand for c-Met (Figure 3.5C and G), or both 20 ng/mL HGF and 10 ng/mL EGF
(Figure 3.5D and H). MDA-MB-231-MUC1 cells were found to form branches in the
presence of EGF or HGF (Figure 3.5F and G, arrows). While MUC1 expression was
required for the branching phenotype induced by either ligand alone, both ligands
together could synergize and form branches in the absence of MUC1 (Figure 3.5D,
arrows). These data indicate that MUC1 and EGFR together promote cell scattering and
branching, a phenotype that is enhanced by the c-Met ligand, HGF.
Figure 3.5. HGF stimulation drives invasive branching. MDA-MB-231-control (A-D) and MDA-MB-231-MUC1 (E-H) cells were seeded in Matrigel with 10% serum alone (A and E), 10% serum and 10 ng/mL EGF (B and F), 10% serum and 20 ng/mL HGF (C and G) or 10% serum and both 20 ng/mL HGF and 10 ng/mL EGF (D and H). Arrows indicate cell branching. Cells were imaged after five days of growth. Scale bar represents 50 µm.

**MUC1 and EGF-dependent cell scattering is due to activated c-Met.**

To explore whether the MUC1 and EGF-driven scattering effects observed in three-dimensional culture were due to c-Met activity, we treated MDA-MB-231-MUC1 cells with 10 ng/ml EGF and a selective c-Met kinase inhibitor, SU11274, or DMSO vehicle and cells were imaged after eight days of treatment. In MDA-MB-231-MUC1 cells treated with EGF, scattering (Figure 3.6B, arrowheads), and branching (Figure 3.6B, arrows) were observed as shown in Figures 4 and 5. Alternatively, the c-Met kinase inhibitor completely blocked the MUC1 and EGF-induced phenotype (Figure 3.6C). Importantly, SU11274 does not affect proliferation in MDA-MB-231-MUC1 or MDA-
MB-231-control cells (Supplemental Figure 3.2). These results indicated that MUC1 and EGFR-dependent cell scattering is dependent upon c-Met kinase activity.
Figure 3.6. c-Met kinase inhibitor (SU11274) inhibits MUC1 and EGF-driven cell scattering and invasive phenotype. MDA-MB-231-control cells (A) or a MDA-MB-231-MUC1 cells (B) were seeded in Matrigel. MDA-MB-231-MUC1 cells were treated with 10ng/mL EGF and DMSO (B) or 5 µM SU11274, a selective c-Met kinase inhibitor (C). Arrowheads indicate single cells, and arrows indicate branching. Images were taken after 8 days. Scale bar represents 50 µm.
Supplemental Figure 3.1. Top ten downregulated (A) and upregulated (B) genes upon PMIP treatment, along with their function and fold-change.
Supplemental Figure 3.2. MUC1 overexpression in MDA-MB-231 cells does not affect cell proliferation. MDA-MB-231-control (A) and MDA-MB-231-MUC1 (B) were grown for 7 days and proliferation was assessed. There was no significant difference in growth over this period between the two cell lines.
Discussion

In the current study, we have identified c-Met as an important mediator of MUC1-dependent migration, scattering and branching of breast cancer cells. Microarray analysis of genes with altered expression in response to PMIP treatment identified a number of potential metastatic mediators, including c-Met. In BT20 breast cancer cells RT-PCR verified that PMIP downregulates c-Met expression in a dose-dependent fashion. Further evaluation of c-Met determined that MUC1 expression resulted in an increase in cell migration on Type IV collagen, and this increase in migration can be inhibited by the selective c-Met inhibitor, SU11274. Furthermore, when placed in Matrigel, cell scattering and branching is increased dramatically upon MUC1 overexpression and EGF treatment, and this phenotype is also blocked by treatment with the c-Met kinase inhibitor.

Previously, MUC1 was shown to promote cell motility and invasion through upregulation of MMP13 and through the induction of epithelial to mesenchymal transition (EMT) in pancreatic cell lines [180, 249]. The breast cancer cells we utilized in this study, MDA-MB-231 have a mesenchymal phenotype which is not dependent on MUC1. The second cell line utilized, BT20, have an epithelial phenotype, and this was not altered by either MUC1 downregulation or PMIP treatment (data not shown). It is possible that the EMT phenotype previously identified is specific to pancreatic cell lines. In the microarray analysis we did not find that MMP13 transcription was altered following PMIP treatment (data not shown). Alternatively, we did find LAMA5 was
downregulated by PMIP treatment (Supplementary Fig. 1), and \textit{LAMA5} has been shown to be involved in cell migration [264]. Whether \textit{LAMA5} is a direct or indirect target of MUC1 will be the subject of future studies. Overall, we initially identified ninety-seven genes significantly upregulated/downregulated at the level of the microarray, and further analyzed six targets by qRT-PCR. Of these six, only \textit{MET} and \textit{LAMA5} were confirmed by qRT-PCR in 3 or more replicates (data not shown).

Our data indicate that MUC1 expression can drive c-Met-dependent scattering and branching. These data are in opposition to a previous study in which MUC1 was shown to interact with and be phosphorylated by Met [34]. In that study, the authors observed that MUC1 expression reduced MMP1 and decreased cell invasion in pancreatic cells [94]. Whether this is a tissue-type specific response, or whether c-Met negatively regulates MUC1 is unclear. Of note, all of our c-Met-dependent migration required EGF, which may be an important difference between our experiments and those in the previous study. In the current study, we have shown that MUC1 promotes the expression of c-Met while EGF promotes the activation of c-Met. Together, MUC1 expression and EGFR activation would result in the activated expression of c-Met, driving metastatic progression. We previously demonstrated that PMIP treatment increases EGF-dependent degradation of EGFR [89]. This data, in combination with the current study, may indicate that PMIP blocks metastatic progression by blocking both c-Met expression and its activation via EGFR.
Overall, the body of literature indicates that both MUC1 and c-Met promote metastatic phenotypes [170, 265-267]. A recent study by Matsubara et al. that demonstrate that MUC1, EGFR and c-Met are coordinately upregulated in aggressive lung cancer patient samples [268]. This human data correlates with both our current study demonstrating that MUC1 expression can promote c-Met activity and with our previous study demonstrating that MUC1 promotes EGFR protein stabilization[88]. We are currently investigating the mechanism by which MUC1 and/or EGFR promote increased MET expression.

Using metastatic breast cancer cells, we demonstrated that MUC1 promotes cell motility using an in vitro wound healing assay. This behavior is reversed upon treatment with PMIP, illustrating that MUC1 promotion of metastatic behavior can be targeted therapeutically. This is the first demonstration of the therapeutic targeting of MUC1 activity as it relates specifically to metastasis. Recently, Raina et al demonstrated another decoy peptide, GO-201, that is designed to block MUC1 multimerization and nuclear translocation. Results from that study indicated a loss of tumor growth, but no effects on metastatic potential were reported [269]. Interestingly, [185] recently found that PMIP blocks smoke-induced EMT in airway epithelial cells, another mechanism by which PMIP may be capable of blocking metastasis.

Previous work regarding the role of MUC1 in promoting metastatic progression indicated that the extracellular domain of MUC1 was required for its function in this process [270]. None of our studies separated the effects of the extracellular domain of
MUC1 from MUC1-C per se, in that we either overexpressed the intact protein or knocked down the entire protein via siRNA. While PMIP is designed to block protein-protein interactions between MUC1 and EGFR and/or MUC1 and beta-catenin, we cannot rule out the possibility that PMIP also affects the localization of the MUC1 or EGFR as a whole and therefore alters the interactions of MUC1 with proteins at its extracellular domain.

Downregulation of c-Met by PMIP demonstrated a reduction in an important oncogene and metastasis promoter [271]. In addition, c-Met is known to play a role in resistance of EGFR-positive cancers to EGFR-directed therapies, such as specific tyrosine kinase inhibitors and monoclonal antibodies. Bean et al assessed c-Met expression in EGFR-expressing tumors that are resistant to EGFR-targeted therapies and compared them to EGFR-expressing tumors that had never been exposed to therapies. In their study, they found that 21% of the tumors with acquired resistance overexpressed c-Met while only 3% of the unexposed tumors overexpressed c-Met [272]. This study implicated a c-Met dependent mechanism for acquiring resistance to EGFR-targeted therapies. Conversely, it has also been seen that EGFR activation can contribute to resistance to c-Met-directed therapies. Bachleitner-Hofmann et al examined the effect of a c-Met kinase inhibitor on c-Met-overexpressing gastric cancer cell lines. They found that inhibition of c-Met prevented receptor tyrosine kinase crosstalk; specifically, EGFR and HER3 downstream signaling was reduced upon c-Met kinase inhibition. However, they found that stimulating cells with EGF effectively circumvented c-Met kinase
inhibition by activating downstream signaling pathways, such as MAPK and PI3K, describing one mechanism by which c-Met kinase inhibition can be thwarted [273]. Therefore, it may prove that targeting the activity of both of these receptors may prevent this resistance from occurring.

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IV. THE ROLE OF EGFR IN LOSS OF EPITHELIAL CELL POLARITY AND IN TUMOR FORMATION

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Introduction

Epithelial cell polarity is essential for proper tissue architecture and function. Tight junctions delineate apical from basolateral cell membrane domains, preventing mixing of the two membrane compartments [216]. Tight junctions also provide a barrier to intramembrane diffusion, preventing permeation of luminal components into the tissue stroma. This barrier function is very important for tissue integrity, especially with regard to epithelial tissues, which act as barriers to separate body compartments. Furthermore, apical and basolateral membrane identity is essential for proper paracellular transport, as transporters must be properly localized in order to ensure transport from the lumen to the stroma or vice versa. Breakdown of tight junctions and/or membrane identity sacrifices tissue function by allowing for inappropriate ion and nutrient diffusion [274].

In addition to tissue function, membrane identity is often necessary for protein function. Proteins which are functional at the apical or basolateral membranes must be properly localized, otherwise they may not receive the signals necessary for function, such as ligands or activation by nearby proteins. Due to its importance for cell and tissue function, epithelial cell polarity is tightly regulated. The Par, Scribble, and Crumbs complexes form a network of mutually exclusive interactions to define and enforce epithelial cell polarity [219-221].

Loss of polarity also contributes to tumor formation. Membrane identity orients cell growth so that only a single layer of cells acts as a barrier between the lumen and stroma. Loss of membrane identity allows for intracellular protein-protein interactions which contribute to cell proliferation, and membrane receptor mislocalization can
promote survival signals through interactions with ligands in the lumen of the tissue, enabling cell growth in the absence of basement membrane contact. Aberrant adherens junction formation allows cell adhesion between cells in the epithelial cell layer and cells growing into the lumen. These chain reactions of aberrant growth, survival, and adhesion allow for the formation of in situ tumors.

Many proteins have the potential to disrupt cell polarity through aberrant protein localization. The Epidermal Growth Factor (EGFR) is a receptor tyrosine kinase which is normally found on the basolateral cell surface of polarized epithelia [16]. MUC1 is a transmembrane mucin which protrudes from the apical cell surface [91]. These proteins do not interact in non-cancerous epithelial tissue, but they are often found to interact in certain types of carcinomas. EGFR binds ligand in the extracellular matrix, allowing it to commence extensive signal transduction through the use of its kinase domain leading to cell survival and proliferation [23]. The C-terminal domain of MUC1 is known to partake in many protumorigenic interactions in the cytoplasm, with proteins such as β-catenin and Src, and it is known to be phosphorylated by and affect the activity of EGFR in cancer [88, 95, 168], though these interactions do not occur in normal polarized epithelial tissue. Our lab has shown that the interaction of MUC1 and EGFR leads to prolonged EGFR signaling capacity [88] and specifies promoters for EGFR to bind to activate transcription [58]. In addition, mouse models of EGFR-driven breast cancer are more potently tumorigenic in the presence of MUC1 [256], demonstrating a role for MUC1 in EGFR-driven cancers. We hypothesized that the forced interaction of MUC1 and EGFR at the cell membrane would lead to a loss of epithelial cell polarity.
We sought to explore whether mislocalization of EGFR to the apical cell membrane, and subsequent interaction with MUC1, would be disruptive enough to disturb cell polarity. To address this question, Tet-inducible EGFR expression constructs with different localization signals were produced and tested to determine their efficacy. These EGFR localization mutants which were designed to localize EGFR to the apical membrane and to non-specifically localize EGFR to the membrane. We tested the constructs to determine membrane localization and assess the use of these constructs as tools to study the effect of EGFR on loss of cell polarity and tumor formation.

**Results**

*MCF10A grown in 3D cell culture localize apical and basolateral proteins*

We established a 3D cell culture system utilizing MCF10A immortalized mammary epithelial cells modeled after a protocol described in [223] and tested the localization of membrane markers. MCF10A cells were allowed to embed into Matrigel basement membrane extract and grown for 21 days. Cells proliferated to form spheres, became hollow due to anoikis of internal cells, and polarized proteins to become analogous to functional mammary ducts lined with epithelial cells. At Day 21 of growth, cells were fixed in 2% PFA, permeabilized in PIPES buffer, and stained for GM130, an apically localized Golgi marker, and Laminin V, a basolaterally localized basement membrane component. We found that in our hands, GM130 and Laminin V were properly localized on the cell membrane (Figure 4.1), demonstrating that we could accurately recapitulate epithelial cell architecture using this system.
Figure 4.1. MCF10A cells polarize when grown in Matrigel. MCF10A cells were grown for 21 days in Matrigel before fixation with 2% PFA and staining with DAPI to visualize the nucleus (blue), and GM130, a Golgi marker (left, green), and Laminin V, a basement membrane component (right, green).
Fixative affects EGFR staining in MCF10A cells grown in Matrigel

We were interested in producing EGFR mutant constructs, so we wanted to discern whether we could dependably depict wild-type EGFR at the basolateral cell membrane in MCF10A acinar structures. We grew MCF10A immortalized mammary epithelial cells in Matrigel for 21 days to form hollow spheres, and used several fixatives with the objective of optimizing EGFR staining. They were fixed in either 10% formalin (Figure 4.2 A-C), Methacarn (Figure 4.2 D-F) or 2% paraformaldehyde (PFA) (Figure 4.2 G-I). Cells were stained with an EGFR antibody (Santa Cruz) (Figure 3.2 C, E, H) and DAPI to visualize nuclei (Figure 4.2 A, D, G). DAPI and EGFR overlays are shown in Figure 4.2 C, F, I. Formalin-fixed MCF10A cells showed EGFR around the nucleus, whereas Methacarn-fixed cells displayed EGFR on the basolateral cell membrane and paraformaldehyde-fixed cells showed diffuse EGFR expression. The localization of EGFR was drastically different in these three conditions, making it difficult to interpret which expression pattern was real and which was non-specific stain. This demonstrated to us that when comparing localization of EGFR mutants to the wild-type protein, consistency of fixation is essential to our interpretation of the results.
Figure 4.2. Fixative affects staining pattern of MCF10A cells with EGFR antibody in Matrigel cell culture. MCF10A cells were grown for 21 days in Matrigel with complete media. Cells were fixed in 10% Formalin (A-C), Methacarn (D-F), or 4% PFA (G-I) and mounted in ProLong Antifade with DAPI (Invitrogen) (A, D, G). Cells were stained with EGFR 1005 antibody (Santa Cruz) (B, E, H). Overlay is shown in C, F, and I.
MCF10A cells do not possess all characteristics of an epithelial monolayer

MCF10A cells are commonly used for 3D polarity studies, but we wanted to decipher whether these cells would be useful for 2D studies on membrane filters. MCF10A cells were grown on membrane filters, fixed with 1:1 Acetone:Methanol, and stained with DAPI to view nuclei (Figure 4.3), ZO-1 tight junction protein (Figure 4.3, A, green) and GM130, an apical Golgi marker (Figure 4.3, B, green). We found that there were areas of ZO-1 localized to the cell-cell junctions, and that in these areas, ZO-1 was laterally localized (Figure 4.3, C, green) as seen by a Z-line image. ZO-1 was not ubiquitously found in the MCF10A cell layer. Areas with ZO-1 expression were rare (data not shown). In addition, GM130 Golgi marker was not apically localized in these cells as would be expected of an epithelial monolayer. Instead, Golgi were localized laterally to the nucleus. These data showed that although MCF10A cells have the ability to localize ZO-1 tight junction protein, they do not do so absolutely, and furthermore, they do not polarize their Golgi as would a normal epithelial cell layer. We concluded from this that MCF10A cells were useful for polarity studies in 3D Matrigel cell culture, but in 2D membrane filter studies, they may not have all the signals necessary to fully polarize.
Figure 4.3. MCF10A cells grown on membrane filters form epithelial cell layers. MCF10A cells grown to 100% confluence on membrane filters were fixed in Acetone:Methanol and stained for ZO-1 (BD), a tight junction protein (A, C), and GM130, an apical Golgi marker (B).
EGFR mislocalization constructs show aberrant EGFR membrane localization

We hypothesized that mislocalization of EGFR to the apical membrane and subsequent interaction with MUC1 would disrupt cell polarity and lead to aberrant cell growth. In order to address this, the basolateral localization signal of EGFR was mutated to ablate basolateral targeting, resulting in non-specific membrane targeting of an EGFR-GFP fusion protein (Figure 4.5A). This EGFR expression construct contained a proline to alanine mutation at the 667 amino acid position, thus it was called EGFR-P667A, as it was previously characterized [35]. The EGFR-P667A mutant was further mutated to create a preferentially apically targeted EGFR. The apical membrane targeting element of MUC1 [275] was inserted into the ablated basolateral targeting sequence of EGFR, resulting in an EGFR construct which would be apically localized, and it was termed EGFR-Map (MUC1 apical). A wild-type basolaterally targeted EGFR construct was used as a control. All three inducible constructs contained EGFR genes fused to the GFP gene, resulting fusion proteins. In order to test the localization of these constructs, they were stably transfected into Madin-Darby Canine Kidney (MDCK) cells, which are widely used to look at membrane identity dynamics due to the fact that they are easily polarized on membrane filters. We opted not to use MCF10A cells for these experiments due to a lack of polarization on membrane filters. Low levels of expression were induced with 5 ng/mL Doxycycline in MDCK cells grown on membrane filters. Planar images (top-down view) were taken as well as Z-line images (side-long view) of the cells with the apical side of the cell “up” and the basal side of the cell “down” in the images (Figure 4.5 B-D). We found that wild-type EGFR localized to the basolateral side of the cell,
whereas EGFR-Map localized to the apical side and EGFR-P667A localized non-specifically, though these findings are preliminary. These results demonstrated that these constructs could be used as tools to assess the effect of EGFR on tumor initiation.

In order to ensure that exogenous wild-type EGFR expression or transfection of mutant constructs was not affecting polarized monolayer formation of MDCK cells, cells stably transfected with wild-type EGFR or EGFR-P667A were stained with tight junction proteins Claudin-1 and Occludin, respectively, though only wild-type EGFR-transfected cells were treated with Doxycycline to induce EGFR expression (Figure 4.5 E-F). We found that EGFR-expressing cells localized Claudin-1 to the subapical membrane, and cells stably transfected with EGFR-P667A but untreated with Doxycycline expressed Occludin at the lateral cell junctions, indicating that tight junctions were properly formed in both cases. These data demonstrated that transfection of constructs or wild-type EGFR expression did not disrupt tight junction formation, validating our perturbation of this system in future studies.
Figure 4.4. EGFR mislocalization constructs aberrantly localize EGFR. (A) EGFR expression constructs were made to alter membrane targeting of EGFR. (B) MDCK cells stably transfected with EGFR mutant constructs were grown on membrane filters and localization of EGFR mutants was assessed. Planar (top-down view) and Z-line (side-long view) images of cells show nuclei stained with DAPI and EGFR-mutant fusion proteins are shown in green. (E) MDCK cells stably expressing wild-type EGFR were grown on filters and tight junction formation was assessed by Claudin-1 stain. Upper left panel shows a planar (XY) image, and the XZ dimension is shown on the bottom while the YZ dimension is shown on the right. (F) MDCK cells stably transfected with EGFR-P667A but not treated with Doxycycline were stained with Occludin to assess tight junction formation.
Breast cancers are derived from epithelial cell layers, but undergo epithelial to mesenchymal transition when they gain the ability to migrate to other tissues. The breast cancer cell lines that are commonly used in studies display a spectrum of epithelial to mesenchymal traits. We hypothesized that breast cancer cell lines possessed varying degrees of intrinsic apical-basolateral cell polarity, with the most epithelial cell lines being more polarized when compared to mesenchymal cell lines. If this hypothesis was correct, we hoped to determine whether expression of mislocalized EGFR would have differential effects in breast cancer cells which are epithelial-like versus mesenchymal like. To test this idea, breast cancer cell lines were grown on membrane filter supports to determine whether they could be induced to polarize in this system which promotes monolayer growth. Cell lines used were: 1. MDA-MB-453, a luminal HER2+ breast cancer cell line which has epithelial characteristics, 2. MDA-MB-468, a cell line which highly expresses EGFR and which possesses basal A characteristics, defined as mixed basal and luminal with an intermediate epithelial-mesenchymal phenotype, and 3. MDA-MB-231, a cell line which is referred to as basal B, or very mesenchymal. Cells were grown on permeable filters in complete media, fixed in 1:1 Acetone:Methanol and stained with DAPI to visualize nuclei (Figure 4.4, blue) and GM130, an apical Golgi protein (Figure 4.4, green). We found that MDA-MB-453 cells had apical Golgi, as evidenced by the appearance of GM130 “on top” of the nuclei when imaged (Figure 4.4, A). MDA-MB-468 cells localized Golgi laterally (Figure 4.4, B), whereas MDA-MB-231 cells had haphazard Golgi localization with some Golgi appearing apically and some
laterally (Figure 4.4, C). These findings showed that breast cancer cell lines display a spectrum of apical-basolateral polarity in this system, with epithelial-like cancer cells being the most intrinsically polarized, and mesenchymal cancer cells being the least polarized. In future studies, we will express basolateral and apical EGFR at moderate levels and determine whether they affect protein localization and cancer phenotypes, such as migration and proliferation, in breast cancer cells.
Figure 4.5. Breast cancer cell lines polarize their Golgi differently. MDA-MB-453 (A), MDA-MB-468 (B), and MDA-MB-231 (C) cells were grown on polarizing PET membrane filters, fixed with Acetone:Methanol and stained with GM130 antibody (BD) (green), and mounted with SlowFade AntiFade (Invitrogen) with DAPI (blue).
Discussion

EGFR overexpression and excess activation has been linked to tumorigenesis in breast cancer through canonical signaling pathways [28]. We were interested in studying the effect of EGFR mislocalization, and subsequent interaction with apical proteins, such as MUC1, on tumor initiation through the loss of apical-basolateral cell polarity. In order to address this question, we made Tet-inducible EGFR constructs with altered EGFR membrane targeting and tested the effects of the following in our study: wild-type EGFR (basolateral), EGFR-Map (apical), and EGFR-P667A (non-specific membrane). We transfected the constructs into MDCK cells and grew them on membrane filters, and we found that these three constructs localized EGFR as designed, indicating that we may use them as tools in future studies to assess the role of EGFR in tumor initiation. To assess the effect of the EGFR localization constructs, we may look at differences in cell proliferation, cell cycle progression, anchorage-independent growth, migration assays, and other cancerous phenotypes using the MDCK immortalized cell line stably expressing these constructs. There were no obvious differences in proliferation observed among cells expressing the different EGFR localization constructs, though we did not conduct a controlled proliferation assay. In addition, stable cell lines used in this study were not clonal populations, so some cells within each population expressed more EGFR protein when treated with the same Doxycycline amount. For optimal results, clonal cell populations which express the same amount of each EGFR mutant or wild-type protein must be compared, and this is the way future experiments will be conducted.
ErbB family receptor tyrosine kinases, of which EGFR is a member, have already been implicated in the loss of epithelial cell polarity. It has been shown that ErbB2 interacts with PAR6 and dissociates the proteins in the Par complex, disrupting apical membrane identity [276]. In addition, ErbB2 homodimerization and synthetic activation has been shown to disrupt polarized epithelial cell growth in three-dimensional cell culture in Matrigel, causing luminal filling. Synthetic activation of EGFR homodimers did not disrupt polarity in this system [277], though EGFR in heterodimers with other ErbB family members could perhaps compromise polarity in a similar fashion. We found that overactivation of EGFR with excess amounts of EGF ligand did disrupt the polarity of MCF10A mammary epithelial cells grown in three-dimensional culture, showing that it is possible to perturb this system and cause a pro-tumorigenic phenotype (Figure 1.3).

Cell polarity is fundamental to tissue function, and loss of proteins that establish polarity contributes to tumorigenesis [278]. This demonstrates that cell polarity is not only essential to organ and tissue function, but that it is a tumor suppressor. In order to study the role of EGFR in loss of apical-basolateral polarity and in tumor formation, we sought to establish two models and use those models to test for apical and basolateral markers in both non-cancerous mammary cells and in breast cancer cell lines.

In MCF10A 3D Matrigel cell culture, we were able to show via polarity marker localization that the hollow spheres of mammary epithelial cells were polarized apical-basolaterally. We had a difficult time staining for EGFR, as different fixatives produced varied EGFR localization. We tried one other EGFR antibody (NeoMarkers, AB-1), but we did not see any specific stain with that antibody (data not shown). We will be careful
to consistently use the same fixative for future studies to allow for meaningful comparisons between treatments.

When we examined MCF10A cells on membrane filters, we found that instead of having uniformly apically localized Golgi, MCF10A cells had asynchronous Golgi which were laterally localized. Normal epithelial cells grown on membrane filters have apical GM130 Golgi localization, as was shown in a study utilizing MDCK cells [279]. The fact that MCF10A cells do not apically localize Golgi indicates that as an epithelial monolayer, MCF10A cells alone without extracellular matrix cues do not have proper tissue structure. We also found that there were patches of tight junction stain, as assessed by ZO-1 lateral localization, however, these areas were sparse. Based on a study published by Fogg in 2005, we were fortunate to have any areas of continuous ZO-1 lateral stain. That study found that MCF10A cells have low endogenous Crumbs3 expression, a member of the Crumbs-PATJ-PALS polarity complex which contributes to apical membrane identity [280]. As a result of low Crumbs3 expression, MCF10A cells grown in 2D cell culture have difficulty polarizing, as we saw on our membrane filters. We concluded from these experiments that MCF10A immortalized mammary epithelial cells are useful for polarity assessment when grown in 3D Matrigel cell culture, but lack of tight junctions in the 2D membrane filter support system made them impractical in that system.

We also looked at Golgi staining in several breast cancer cell lines to determine whether epithelial-like cancer cells are inherently more apical-basolaterally polarized than mesenchymal-like cancer cells. We found that in MDA-MB-453 cells, an epithelial-
like cell line, Golgi were uniformly apically polarized. In MDA-MB-468 cells, which are characterized as a mixed basal/luminal cell line, and are intermediate on the spectrum between epithelial and mesenchymal, Golgi were uniformly lateral. Finally, MDA-MB-231 cells, which are the most mesenchymal of the three cell lines studied, polarized Golgi haphazardly. In some cases, MDA-MB-231 cells deposited Golgi apically, and in some cases laterally. In comparison, MDA-MB-231 cells, which are very mesenchymal, localize their Golgi as individual cells as opposed to as a whole tissue. These findings may demonstrate that cancer cells that are more epithelial in phenotype are still subject to signals as a whole tissue, suggesting that aspects of a polarized epithelium may be restored in these cells to make them more normal. In a study conducted in our lab, human Lethal Giant Larvae (HUGL), a polarity protein and member of the Scribble basolateral identity complex, was expressed in MDA-MB-453 cells, which have lost HUGL expression. Re-expression of HUGL in these cells reduced proliferation, indicating at least a partial halt of the cancerous phenotype [281]. Apical Golgi localization may be an indicator of cells which are sensitive to polarization reprogramming, whereas random Golgi localization may indicate cells which are completely independent of tissue signaling and cannot deviate from the cancer program. In future studies, we will express basolateral and apical EGFR in epithelial-like and mesenchymal-like breast cancer cells and determine whether they enhance or reduce cancer phenotypes in these cell lines. Our hypotheses would predict that apical EGFR would disrupt any inherent apical-basolateral polarity present in epithelial-like cancer cells, contributing to their tumorigenic potential, and either basolateral or apical EGFR
expression in mesenchymal cells would allow them to become more migratory. These hypotheses will be tested in later studies.
V. MUC1 AND EGFR IN THE LACTATING MAMMARY GLAND

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**Introduction**

The mammary gland is a complex and dynamic tissue. At birth, only a rudimentary ductal tree grows from the nipple, and during puberty, the mammary tissue elongates and expands to create an extensive network of lobules and ducts which are specialized for milk production and secretion. During pregnancy, the breast becomes completely filled with alveoli to increase surface area of milk-producing cells for maximal production. After weaning, the alveoli undergo apoptosis to return the gland to the pre-pregnancy state through a process called involution [282]. This cycle of growth and reduction of mammary cells is delicately regulated in order to ensure proper mammary gland function. Improper regulation of these biological processes could predispose cells for breast cancer, though pregnancy and lactation are thought to be protective against breast cancer, at least in women with a family history of breast cancer [283]. This may be because involution of the mammary gland destroys pre-cancerous lesions and restores growth control.

MUC1 is a transmembrane mucin which is highly expressed in mammary epithelial cells. MUC1 protrudes from the apical cell surface into the lumen of the mammary ducts, secreting bulky sugar chains into the milk. MUC1 is overexpressed in greater than 90% of metastatic breast cancers, signifying that it imparts an advantage to cancer cells. It is known to participate in many cancer-specific interactions through mislocalization to the basolateral membrane in cancer. One of the proteins with which MUC1 interacts in cancer is the Epidermal Growth Factor Receptor (EGFR).
EGFR is a basolaterally localized receptor tyrosine kinase. It binds ligands in the extracellular matrix, causing dimerization, transphosphorylation, and subsequent canonical signaling events, including activation of the Ras-MAPK pathway [26], the PI3K-Akt pathway [30], and the PLC-calcium pathway [24]. The ultimate effects of these pathways include proliferation, survival, and cell motility, all of which promote normal tissue growth when properly regulated and cancerous growth when uncontrolled [23]. EGFR is overexpressed in 20-30% of breast cancers, and these cancers tend to be very aggressive [16]. In mammary epithelial cells, MUC1 and EGFR are polarized to opposite sides of the cell during early stages of mammary gland development, but not during lactation. In the lactating mammary gland, EGFR is observed on the apical domain of the plasma membrane, where it is free to interact with MUC1 [80]. Because we have shown in our lab that interaction of these two proteins leads to tumorigenesis [58, 88, 256], it is interesting to note that their interaction in this system is not deleterious. We decided to explore whether the expression of MUC1 or EGF, the primary ligand for EGFR during lactation [79], were necessary for lactation function or tissue architecture. We utilized a MUC1 knockout mouse and because EGF is the only ligand expressed at appreciable levels during lactation, we used an EGF knockout mouse as a model for inactive EGFR, since EGFR knockout mice are perinatally lethal.

We examined the lactating mammary glands of mice that lacked either Muc1 or Egf (the predominant ligand for EGFR during lactation [79]), or that lacked both proteins and assessed them for changes in tissue structure and litter size as well as assessing several proteins that have known cellular localization. We looked at the fourth day of
lactation because EGFR has been shown to be highly expressed early in lactation, which is a three-week period [79, 284]. Prior to our study, the Muc1-/− and Egf-/− genotypes were seen to be viable [73, 118], but the efficiency of lactation in these mice had not been investigated.

We found that there was no significant difference between tissue structure in the lactating mammary glands of wild-type compared to Muc1-/−, Egf-/−, or Muc1-/−Egf-/− mice. Milk fat globules were found in the lumens of all mice. There was no difference in the litter sizes of these genotypes at weaning, indicating that there was not a lactation phenotype. In addition, we stained for Egfr and found it strongly enriched on the apical side of the ductal epithelium in the wild-type mice, as we expected due to the excessive amount of exocytosis that occurs in the lactating mammary gland, enabling mixing of cell membrane compartments. We found Egfr also localized to the apical cell membrane in the Muc1-/−Egf-/− mice, indicating that there was no reliance on Egf expression for Egfr localization during lactation. Lastly, we found that aPKC, an apical protein, and E-cadherin, a lateral protein, were so localized in the wild-type and Muc1-/−Egf-/− mice, signifying that Muc1 and Egf expression is not necessary for localization of these proteins. This study indicated that Muc1 expression and Egfr activation do not affect lactational function or tissue architecture in the lactating mammary gland. Further work must be done to discover whether these proteins are important for mammary gland involution or another stage of mammary development.
Results

*Muc1 and Egf expression do not affect litter size*

We were interested in determining whether Muc1 and/or Egf affected the number of weanling pups per dam. Wild-type C57Bl/6 mice were compared to mice with mutant non-functional Muc1, Egf, or both. We counted the number of mice present at birth for all genotypes across several litters (Table 5.2), and the number of mice per litter had not changed at time of weaning. We found no statistically significant difference between mice possessing and mice lacking Muc1 or Egf. This demonstrates that Muc1 and Egf expression do not affect litter size in this background. Furthermore, the number of mice in each litter was not reduced between birth and weaning for any of the genotypes, indicating that there was no significant lactation defect in any of the female mice in these genotypes.
Table 5.1. Lack of MUC1 and/or EGF does not affect litter size at weaning. 

<table>
<thead>
<tr>
<th>Genotype (C57BL/6)</th>
<th>Average weanling litter size (# of mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>5.80</td>
</tr>
<tr>
<td>MUC1-/EGF+/+</td>
<td>6.81</td>
</tr>
<tr>
<td>MUC1+/EGF-/-</td>
<td>6.62</td>
</tr>
<tr>
<td>MUC1-/EGF-/</td>
<td>6.50</td>
</tr>
</tbody>
</table>

n=5 litters for MUC1+/+EGF+/+, 13 litters for MUC1-/EGF+/+, 8 litters for MUC1+/+EGF-/-, and 10 litters for MUC1-/EGF-/-.
*Muc1 and Egf expression do not affect milk fat globules or alveolar structure*

We next wanted to determine whether Muc1 and Egf affected milk fat globule formation or alveolar structure. Mammary glands were extracted from female mice on the fourth day of lactation for their first parturition. Mammary glands were fixed in Methacarn, paraffin-embedded, cut to 6 µm sections and stained with Hematoxylin and Eosin to visualize cellular architecture in the ducts. All genotypes possessed mammary glands full of alveolar structures for efficient milk secretion. Luminal epithelial cells were heavily stained with Hematoxylin nuclear stain in all the genotypes, and milk fat globules were visible in the lumens of all mice (Figure 5.1). There was no difference in lactating mammary gland architecture between mice expressing Muc1, Egf, or both proteins and mice lacking these proteins.
Figure 5.1. MUC1 and EGF expression does not affect milk fat globule formation and tissue architecture in the lactating mammary gland. Wild-type (A), MUC1+/EGF- (B), MUC1-EGF+ (C) and MUC1-EGF- (D) mammary glands were fixed in Methacarn, paraffin-embedded, cut, and stained with Hematoxylin and Eosin. Hematoxylin (purple) stains nuclei, and Eosin (pink) stains cytoplasm and collagen. n=2 mice for MUC1+/+EGF++, 4 mice for MUC1+/+EGF-/-, 6 mice for MUC1-/EGF+++, and 6 mice for MUC1--EGF--. Scale bar = 50 µm.
Muc1 and Egf do not affect apical aPKC expression

We wanted to determine whether apical protein localization was preserved in Muc1 and/or Egf knockout lactational mammary glands. Day 4 lactating mammary glands were fixed in Methacarn, paraffin-embedded, cut to 6 µm sections, rehydrated and stained for atypical Protein Kinase C (aPKC), an apically localized protein kinase involved in the maintenance of epithelial cell polarity. We sought to determine whether Muc1 and/or Egf expression was necessary for apical localization of this protein in the lactating mammary gland. We stained mammary glands with DAPI to view nuclei (blue) (Figure 5.2, A, D), aPKC antibody (green) (Figure 5.2, B, E) and overlaid the channels (Figure 5.2, C, F). Both the wild-type and MUC1/EGF double knockout mice displayed apical aPKC localization, indicating that Muc1 and Egf expression was not involved in aPKC localization, demonstrating that these proteins do not affect normal cellular components in this tissue.
Figure 5.2. MUC1 and EGF expression does not affect aPKC apical protein localization. Mammary glands were fixed in Methacarn and paraffin-embedded before being cut and stained with DAPI to visualize nuclei and aPKC antibody (Santa Cruz). n=2 mice for MUC1+/+EGF+/+, 3 mice for MUC1-/EGF-. Scale bar = 50µm.
**Muc1 and Egf do not affect E-cadherin lateral localization**

We next sought to establish whether Muc1 and/or Egf were important for lateral protein localization. Mice lacking Muc1 and/or Egf were fixed in Formalin, paraffin-embedded, cut to 6 µm sections, and stained with E-cadherin, a laterally localized adherens junction adhesion protein. E-cadherin is laterally localized in epithelial cells, and proper localization of this protein is necessary for the structure and function of the mammary gland. E-cadherin was laterally localized in lactating mammary glands from mice both expressing and lacking Muc1 and/or Egf (Figure 5.3 B, F, magnified in D, H) with nuclei stained with DAPI (Figure 5.3 A, E) and E-cadherin/DAPI overlay shown (Figure 5.3 C, G). In addition, we found that E-cadherin expression in lactating mammary glands was not significantly different between the Muc1, Egf, or double knockout mice (Figure 5.3 I). This indicates that expression of Muc1 and/or Egf is not necessary for adherens junction formation and E-cadherin localization.
Figure 5.3. MUC1 and EGF expression does not affect E-cadherin lateral protein localization. Mammary glands were fixed in Methacarn and paraffin-embedded before being cut and stained with DAPI (A, E) to visualize nuclei and E-cadherin antibody (B, F, magnified in D, H) and overlaid in C, G. Lactating mammary glands were lysed and 100 μg were run by SDS-PAGE and blotted for E-cadherin or β-actin (I). n=2 mice for MUC1+/+EGF+/+, 2 mice for MUC1-/-EGF-/- Scale bar = 50μm
*Muc1 and Egf do not affect Egfr localization*

Egfr is basolaterally localized in normal mammary epithelial cells, but during lactation, because of the high volume of milk being secreted from the gland and corresponding high rate of exocytosis from the epithelium, Egfr is enriched at the apical cell membrane. This is the only non-cancerous stage of mammary gland development in which Muc1 and Egfr are colocalized and able to interact. We have shown that the interaction of Muc1 and Egfr in cancer promotes tumorigenesis, and yet colocalization in lactation is not detrimental. We sought to determine whether Muc1 expression or Egfr activation via ligand binding were important for Egfr localization in the lactating mammary gland. Day 4 lactating mammary glands were fixed in Methacarn, paraffin-embedded, cut to 6 µm sections, and stained with DAPI (blue) (Figure 5.4, A, D), and Egfr (green) (Santa Cruz) (Figure 5.4, B, E), and overlaid (Figure 5.4, C, F). We found that Egfr was apically enriched in mice expressing Muc1 and/or Egf and mice lacking expression of these genes. This indicated that Muc1 expression and Egfr activation were not necessary for apical localization of Egfr in the lactating mammary gland.
Figure 5.4. Muc1 and Egf expression do not affect Egfr localization in the lactating mammary gland. Day 4 lactation mammary glands were fixed in Methacarn, paraffin-embedded, and stained with DAPI to visualize nuclei (blue, A, D) and EGFR antibody (Santa Cruz) (green, B, E) and overlaid in C, F. n=2 mice for MUC1+/+EGF+/+, 2 mice for MUC1-/EGF-. Scale bar = 50µm.
Discussion

The mammary gland is a highly active tissue, undergoing extensive proliferation during puberty and pregnancy, secreting large amounts of milk during lactation, and after weaning, reducing back to pre-pregnancy gland structure via apoptosis. EGFR is expressed during all phases of mammary gland development, though its ligands are differentially expressed throughout development, with TGFα and β-cellulin being highly expressed in the virgin gland, Amphiregulin and Epigen being highly expressed during mid to late pregnancy, EGF being highly expressed during lactation, and TGFα being highly expressed again during involution [79]. All ligands are expressed at some level during all phases of development, except that EGF is the predominant ligand during lactation and expression of all other ligands is minimal or undetectable. For this reason, we chose to use an EGF knockout mouse model to determine whether EGFR activation in conjunction with MUC1 is necessary during lactation.

MUC1 is highly expressed during lactation [285], as large amounts of the protein are secreted into the milk. It has been shown that MUC1 knockout mice are viable [118], though the effect on lactation was not described. EGF knockout mice are also viable, as are double and triple EGFR ligand knockouts, though double and triple EGFR ligand knockouts do have impaired mammary gland development, highlighting a role for the other ligands in mammary development. The effect of EGF knockout on lactation was also not fully described. We sought to use these models and a MUC1/EGF double knockout mouse to determine whether MUC1 and EGFR regulate lactation phenotypes.
In addition, it has been shown that while MUC1 and EGFR are spatially segregated to opposite cell membranes in normal epithelial cells, in the lactating mammary gland, because of extensive exocytosis of milk fat globules, EGFR can be found co-localized with MUC1 on the apical cell membrane. The observations that we have made regarding EGFR and MUC1 interactions suggest that lactation predisposes to cancer, though this is not the case. We sought to determine whether in lactation, the interaction of MUC1 and EGFR preserves the tissue architecture, instead of damaging it, as it does in cancer.

We examined litter size at weaning in the MUC1 and EGF single knockout mice and in the MUC1/EGF double knockout mice to determine whether the MUC1/EGF double knockout nurses fewer mice to weaning age. We found that there was no difference in litter size at weaning among any of the genotypes. This did not account for any minor lactation defects that might be observed, so we decided to examine Hematoxylin and Eosin-stained mammary glands and look for deficiencies in tissue architecture. We found milk fat globules in the lumens of all genotypes, and we didn’t find obvious differences in the density of alveoli present in the mammary glands, though this was not quantified. To further explore tissue structure, we stained for apical and basolateral epithelial cell markers. We found that in the wild-type, the MUC1 and EGF single knockouts, and the MUC1/EGF double knockout, aPKC was localized to the apical cell membrane, E-cadherin was localized to the lateral membrane, and EGFR was apical, as we expected from previous lactation studies. These findings all suggest that a lack of MUC1 and/or EGF does not affect lactation function in the mammary gland.
Previous studies have shown that many gene knockouts which impair lactation are genes that code for components of milk itself, such as α-lactalbumin and whey acidic protein [286, 287]. EGFR has been shown to be important in earlier stages of mammary gland development [288], but it appears that EGFR activation is not necessary for lactation. We cannot discount that EGF knockout mice may have upregulation of other ligands which are not normally present during lactation, though in an Amphiregulin knockout mouse, mammary gland development was severely impaired, indicating that the other ligands were not upregulated to compensate for loss of the Amphiregulin gene in that study [73]. It may be that EGFR is activated by EGF in lactation to set up conditions for post-lactation, and we did not investigate post-lactational involution phenotypes in these mice. Additionally, although MUC1 is a component of milk fat globules, it is not essential for milk fat globule formation. These findings indicate that although MUC1 and EGFR are important for mammary gland biology, they are not necessary for lactation.
VI. DISCUSSION

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Breast cancer killed 458,000 people in 2008, making it the fifth deadliest cancer worldwide. It is essential that we not only focus on developing new therapies to treat breast cancer, especially the more deadly, aggressive cancer types, but that we also concentrate on understanding how these cancers exploit normal biological processes to achieve transformation. MUC1 is an apical transmembrane mucin which is overexpressed in greater than 90% of metastatic breast cancers [285], and EGFR is a receptor tyrosine kinase which is overexpressed in 20-30% of breast cancers and which confers poor prognosis and aggressiveness of the cancer [16]. These proteins are often found colocalized in EGFR-positive breast cancers, and we have found that MUC1 promotes the pro-tumorigenic activities of EGFR [58, 88, 256]. In this study, we sought to further describe mechanisms by which the interaction and colocalization of MUC1 and EGFR promote cancer and affect mammary gland biology.

**Inhibition of MUC1 and EGFR**

The peptide therapeutic developed in our lab, PMIP, was designed to competitively inhibit the interaction of EGFR with MUC1. PMIP was previously found to inhibit proliferation and invasion, and prevent recurrence and contralateral metastases [89]. Further studies found that EGFR itself binds to the Cyclin D1 promoter in the presence of MUC1, but not in the absence of MUC1, implicating the interaction of EGFR and MUC1 in cell cycle progression [58]. We sought to further describe the effect of EGFR and MUC1 in migration and metastasis, functions which are necessary for cancer cells to spread throughout the body and form secondary tumors. We examined migratory
capacity of cells treated with PMIP, and found that compared to control cells, PMIP inhibits migration, indicating a mechanism by which PMIP treatment reduces metastatic progression. To further identify how PMIP exhibits this outcome, we performed microarray analysis of cells treated with PMIP and found that c-Met, the Hepatocyte Growth Factor Receptor and potent inducer of metastatic phenotypes, was downregulated 3-fold compared to control cells. c-Met activity has been found upregulated in metastatic cancer, and its remodeling of the actin cytoskeleton and induction of epithelial to mesenchymal transition are central to metastatic progression. We found that inhibiting c-Met kinase activity with a selective inhibitor reduced migration to the same extent as PMIP treatment, suggesting that migratory reduction due to PMIP is via c-Met downregulation. We did not look at actin-organizing small GTPases, such as cdc42 or Rac1, though we would expect that these would be inactive in PMIP-treated cells due to reduced EGFR activity and reduced c-Met expression.

**MUC1 and EGFR in metastatic phenotype**

Transcriptional regulation of c-Met has not been well characterized. It is not known how it is regulated in non-cancerous epithelial cells. In cancer, it is regulated by Ets1 [209], which is an endothelial and stromal cell transcription factor, though Ets1 function is often highjacked in epithelial cancer cells. Thus, Ets1 only regulates c-Met expression in neoplastic cells, and c-Met upregulation may be the main reason why Ets1 upregulation promotes migration and invasion in vitro [210]. c-Met is also regulated by HIF1-α, a hypoxia-induced transcription factor which is often activated in cancer, and
general transcription factors AP-1, a transcription factor that is downstream of both HIF1-α and many receptor tyrosine kinases [208]. Lastly, it is known that ubiquitous Sp1 and Sp3 transcription factors regulate c-Met expression [207]. All the transcription factors mentioned rely on signaling to activate transcription, and the signals for c-Met gene regulation are unclear. In our study, we found one novel method by which c-Met is upregulated in response to ligand-binding. It is indistinct from our study whether EGFR promotes c-Met transcription through canonical signal transduction and activation of AP-1, or whether EGFR binds to the c-Met promoter to affect transcription directly in a MUC1-dependent manner, and we cannot discount a combination of both methods. A preliminary analysis of the c-Met promoter indicated that there are 41 putative EGFR/RNA helicase A binding sites, which are characterized by an A/T-rich sequence (data not shown) [49]. Future studies will focus on discerning whether MUC1 and EGFR affect c-Met transcription through direct or indirect means.

We sought to determine whether c-Met was necessary for MUC1 and EGFR-driven cell migration and invasion by using a 3D Matrigel scattering assay. Breast cancer cells grown in Matrigel cell culture formed solid cell masses which dispersed cells into the matrix when expressing MUC1 or when grown with EGF to activate EGFR, analogous to metastatic cancer cells invading from a solid tumor into the surrounding extracellular matrix. In addition, cell masses formed branches of cells which protruded into the surrounding matrix, indicative of matrix invasion and metastatic phenotype. This showed that MUC1 and EGFR indeed drive cell migration and invasion. Additionally, cells treated with HGF to activate c-Met in combination with MUC1 expression or EGF
treatment displayed more invasive branching than EGF-treated cells alone, or HGF-treated cells alone, demonstrating a synergism between EGFR and c-Met which requires both ligands to fully potentiate invasive branching. Treatment of MUC1-expressing cells with EGF and a specific c-Met tyrosine kinase inhibitor resulted in a complete ablation of all cell scattering and branching phenotypes, indicating that c-Met is necessary for these phenotypes. We also saw a reduction in cell growth upon treatment with the c-Met kinase inhibitor. We were not surprised by this, since c-Met can promote proliferation through the Ras-MEK pathway [201], but we were unable to find a dose of the c-Met kinase inhibitor which affected only scattering and not growth. We seeded the same number of cells for each treatment, and when we compared the number of cell masses and the number of scattered cells for each treatment, we found that there were both more cell masses and more scattered cells in the MUC1-expressing and EGF-treated groups, and even more in the dual treatment. This indicated to us that there may have been an early scattering event which led to an increase in the number of cell colonies, and when we were counting scattered cells on day 13 of treatment, we might have been counting cells from a second scattering event. In any case, these migratory phenotypes were abolished in the group treated with the c-Met kinase inhibitor. These data demonstrated that MUC1 and EGFR drive c-Met transcription and that activated c-Met was necessary for migration and invasion phenotypes found in cells with MUC1 and activated EGFR. Expression of MUC1 has been associated with metastatic progression, as has EGFR activity, but in our study, we identified a new mechanism whereby EGFR and MUC1 promote transcription of c-Met, a protein known to be involved in cancer metastasis.
c-Met and EGFR have been implicated in instances of targeted drug resistance. Tumors that are resistant to EGFR-targeted therapeutics often overexpress c-Met [289], and tumors that are resistant to c-Met-targeted therapeutics often overexpress EGFR [273]. These two receptors drive the same signal transduction pathways, displaying one explanation for the upregulation of one receptor in response to inhibition of the other. Our study demonstrated a cooperative regulation of motility from these two receptors. Activation of both receptors showed more invasive branching than activation of either receptor alone. c-Met appeared to be absolutely required for the MUC1 and EGFR-driven motility phenotype, and although we did not treat cells with HGF and an EGFR-specific tyrosine kinase inhibitor, treatment of cells with HGF in the absence of EGFR only showed mild migratory phenotypes, indicative of the fact that both receptors are required for optimal cell motility. MUC1 enhanced the migration induced by either ligand alone, perhaps through its own enhancement of cytoskeletal rearrangements, or because it promotes the activities of these receptors, or through a combination of mechanisms. The interplay of c-Met, EGFR, and MUC1 is a potent inducer of migration and invasion. We showed in this study that PMIP inhibits the transcription of c-Met, leading to reduced cell motility and invasion, and previous studies showed that PMIP reduces the activity of EGFR and expression of MUC1 [89]. In order to treat c-Met and/or EGFR-driven cancers, we will have to rely on combination therapies which target both receptors in order to prevent drug resistance. PMIP itself acts as a combination therapy by targeting EGFR, c-Met and MUC1 at the same time, and I think we will find that strategies like this are effective with the least amount of toxicity.
Epithelial cell polarity

In addition to the effect of EGFR and MUC1 on metastatic progression, we were interested in the interaction of EGFR and MUC1 in tumor initiation, and specifically, we wanted to explore the role of EGFR and MUC1 in loss of membrane identity.

Cancer is characterized by disorganized cell growth. Normal epithelial tissues exist as a monolayer of cells lining ducts and organs of the body. These cells have directionality, with the apical cell membrane facing the lumen and the basolateral cell membrane facing the stroma. When epithelial cells lose membrane identity, they are no longer constrained to grow in a monolayer, and mutations which impart growth and survival allow growth of a tumor into the lumen. Loss of epithelial cell polarity has grown to be accepted as an early event in tumor growth. We know that cell polarity is regulated by three protein complexes [222], but we sought to determine whether EGFR and MUC1 could be upstream regulators of those protein complexes and contribute to loss of membrane identity.

For future studies along this vein, we established two systems to assess polarity. The first was a 3D Matrigel cell culture method previously described and widely used [223]. With this method, we could grow cells in a fashion analogous to in vivo ductal structure and determine whether polarity was established through the localization of apical and basolateral markers. We were able to grow MCF10A mammary epithelial cells using this method, and the resulting acinar structures did indeed polarize, positioning apical proteins and basolateral secreted proteins properly. We attempted to
discern EGFR localization in this system in order to determine whether EGFR was basolateral as expected. We found that localization of EGFR was completely dependent upon the fixation protocol used. Formalin-fixed cells showed strong perinuclear staining of EGFR, while Methacarn-fixed cells showed EGFR faintly basolateral and paraformaldehyde-fixed cells showed diffuse EGFR stain. These data demonstrated that the fixative used can easily skew results and must be considered when using this method.

The second system we used to assess polarity was culture of cells on membrane filters. In this growth condition, cells form true epithelial monolayers on the filters by virtue of integrin attachment and delivery of signaling molecules through the filter. Using this method, we discerned that while MCF10A cells do localize ZO-1 to cell junctions on occasion, they do not polarize their Golgi to the apical side of the nucleus as would be characteristic of polarized, non-transformed epithelial cells. This was not altogether surprising to us, considering that it had been shown previously that MCF10A cells grown on plastic have low endogenous expression of Crumbs3, a protein involved in apical membrane identity [280]. This results in a lack of epithelial tight junctions, as was shown in that study. The fact that we cannot induce tight junction formation even on membrane filters indicates that the cells need extracellular matrix (ECM), as is present in the 3D Matrigel cell culture system, in order to polarize fully. The factor that is missing in our membrane filter system may be a component of the ECM itself, such as Collagen, or it may be the semi-stiff support provided by the ECM. Studies have demonstrated that matrix stiffness contributes to cell function [290], and it may be that membrane filters and tissue culture plastic are too stiff to fully induce epithelial cell polarity in certain cell
lines. Other cell lines, such as Madin-Darby Canine Kidney cells (MDCK) polarize on membrane filters [291]. In future studies, we will focus on this or another cell line as an alternative to MCF10A cells on membrane filters.

Using the membrane filter protocol, we also found that breast cancer cell lines with various epithelial and mesenchymal phenotypes polarize their Golgi differently. We would expect for cancer cell lines which possess epithelial characteristics to localize Golgi apically, indicating that they still have the ability to respond to tissue organizational cues, and we saw that of the cell lines we examined, the epithelial-like cell line presented apical Golgi, the cell line with mixed phenotype presented lateral Golgi, and the mesenchymal cell line presented haphazard Golgi, displaying it sometimes apically and sometimes laterally. Relative Golgi localization may be used as a marker to reveal epithelial/mesenchymal cell characteristics, as well as an indicator of the ability to respond to a reestablishment of cell organization.

The establishment of these systems to study epithelial cell polarity will be important for future studies examining whether MUC1 and EGFR perturb polarity protein complexes and drive tumorigenesis through a loss of cell polarity.

**MUC1 and EGFR in epithelial cell polarity**

MUC1 and EGFR are spatially segregated on the cell membrane in normal epithelial cells. MUC1 localizes to the apical plasma membrane where it protrudes into the lumen, and EGFR localizes to the basolateral plasma membrane where it binds ligands in the extracellular matrix, enabling activation and subsequent signal
transduction. The separation of these two proteins on the plasma membrane prevents their interaction, which has been shown to be pro-tumorigenic via MUC1 prolonging and specifying EGFR activity [58, 88, 256]. We were interested in determining whether mislocalization of either MUC1 or EGFR and subsequent interaction of the two proteins could result in a loss of epithelial polarity and an induction of a tumor phenotype. MUC1 has been shown to be involved in loss of polarity in lung epithelial cells in response to cigarette smoke. When exposed to smoke, MUC1 becomes basolaterally localized, enabling an interaction with p120-catenin, disrupting adherens junction structure and leading to the degradation of E-cadherin and an epithelial to mesenchymal transition [185]. These results indicated that mislocalization of MUC1 was enough to drive a loss of epithelial polarity, though other confounding effects of the cigarette smoke and the direct cause of MUC1 mislocalization was not explored. In a study from the same group, cigarette smoke was shown to induce EGFR phosphorylation of MUC1, and mislocalized MUC1 was found to interact with β-catenin to modulate Wnt-responsive gene expression. The mislocalization of MUC1 upon smoke exposure was found to be EGFR-dependent, in that blocking EGFR kinase activity with a selective kinase inhibitor was found to prevent smoke-driven loss of epithelial polarity [292]. These findings suggested that MUC1/EGFR interaction was the driver for MUC1 basolateral localization and loss of epithelial polarity, though they focused on basolateral localization of MUC1 as a driver. These studies suggested a role for MUC1 and EGFR in loss of epithelial polarity, though they focused on smoke as a signal and did not demonstrate the specific effect of smoke on EGFR stimulation or localization.
Prior to this study, we knew that MUC1 and EGFR interaction promoted cancer progression. We wanted to determine whether mislocalization of EGFR to the apical side of the cell, enabling interaction with MUC1, would be disruptive enough to induce oncogenic signaling and initiate cancer phenotypes. In order to address this question, we generated Tet-inducible EGFR localization mutants which would target EGFR either to the apical side or non-specifically. We tested whether these constructs localized EGFR to the expected plasma membrane domains and found that they did, at least in our preliminary studies. Furthermore, we demonstrated that stable transfection of one of the uninduced mutant constructs and wild-type EGFR expression did not disrupt tight junction formation, which was an important control to perform before using the constructs to study polarity. Future studies will utilize these constructs to look at adherens junction and tight junction integrity in the context of aberrant EGFR localization. We will also look at the effect of EGFR mislocalization on MUC1 interacting partners and localization. These studies will help further elucidate the mechanism by which EGFR and MUC1 affect cell polarity and tissue architecture.

**MUC1 and EGF in the lactating mammary gland**

In addition to our studies of EGFR and MUC1 in cell polarity, we explored the role of these proteins in mammary lactation using a mouse model. The mammary gland undergoes widespread reorganization during puberty, pregnancy, lactation, and involution, and EGFR is expressed during all phases of mammary gland development. MUC1 is expressed on the apical surface of mammary epithelial cells, and EGFR is
basolaterally localized in the mature adult gland. In lactation, however, EGFR is found apically colocalized with MUC1, presumably due to the high volume of secreted vesicles resulting in shuttling of many proteins to the apical side. This is one instance wherein MUC1 and EGFR interaction does not result in tumorigenesis. We sought to understand whether in this biological circumstance, MUC1 and EGFR interaction was promoting maintenance of tissue architecture and lactation. We decided to investigate this question using knockout mouse models.

MUC1 knockout mice have been shown to be viable [118], whereas EGFR knockout mice have been shown to be perinatally lethal. EGF is the predominant ligand for EGFR during lactation, with all other ligands at very low levels, if detectable, and EGF knockout mice have been shown to be viable. For these reasons, we chose to use MUC1 and EGF single knockout mice in addition to MUC1/EGF double knockout mice to assess the role of MUC1 expression and EGFR activation in lactation.

In order to first assess whether MUC1-/-, EGF-/-, or MUC1/-/-EGF/- mice had lactation defects compared to wild-type mice, we examined litter size at weaning. Comparing the number of mice which were brought to weaning allowed us to directly compare lactational function in these genotypes. We found that there was no difference in litter size at weaning among any of the genotypes, indicating that there was not a significant difference in lactation among the dams. We decided to examine Hematoxylin and Eosin-stained mammary glands to look for deficiencies in milk fat globule secretion or tissue architecture or other subtle defects. We found milk fat globules in the lumens of all genotypes, and we didn’t find significant differences in the amount of alveoli present
in the mammary glands. To further explore tissue structure, we stained for apical and basolateral epithelial cell markers. We found that in all genotypes examined, aPKC was localized to the apical cell membrane, E-cadherin was localized to the lateral membrane, and EGFR was apical, as we expected from previous lactation studies. These data indicated that MUC1 and EGF were not necessary for proper protein trafficking or milk fat globule formation. Taken together, these findings all suggest that a lack of MUC1 and/or EGF deletion does not affect lactation function or tissue architecture in the mammary gland.

It appeared from our study that EGFR activation was not necessary for lactation, though EGFR may have a non-canonical role in the tissue which does not require activation, or it may be that EGFR is activated by EGF in lactation to set up conditions for post-lactation, and we did not investigate post-lactational involution phenotypes in these mice. Additionally, although MUC1 is a component of milk fat globules, it did not appear to be essential for milk fat globule formation. These findings indicate that although MUC1 and EGFR are important for mammary gland biology, they are not necessary for lactation.

Model of MUC1 and EGFR interaction in mammary gland biology and breast cancer progression

Our studies have demonstrated that the interaction of MUC1 and EGFR have several functions in addition to those previously characterized. Firstly, we showed that the interaction of MUC1 and EGFR leads to metastatic progression through the
expression of c-Met, a protein which is known to be involved in cell motility, a feature that is necessary for metastasis. Secondly, we generated tools to study the role of MUC1 and EGFR interaction in tumor initiation through the loss of epithelial cell polarity. Thirdly, we found that though MUC1 and EGFR interact in the lactating mammary gland and though that interaction does not promote cancer, the loss of either MUC1 or EGFR activity does not affect lactation or tissue structure of the lactating mammary gland, demonstrating that the interaction of MUC1 and EGFR does not have a functional consequence at this stage in mammary development. From these data, we present the following model: MUC1 and EGFR are spatially segregated in the adult mammary gland, resulting in low MUC1/EGFR interaction (Figure 6.1 A). During lactation, exocytosis of milk fat globules can increase membrane mixing, resulting in EGFR being apically localized and a surge in MUC1/EGFR interaction, though neither MUC1 nor EGFR are required for lactation function in the mammary gland (Figure 6.1 C). Loss of polarity, through various means, leads to loss of membrane identity and an increase in membrane mixing. This leads to increased MUC1/EGFR interaction (B), though increased MUC1/EGFR interactions may also be responsible for loss of membrane identity. Loss of polarity and increased MUC1/EGFR interaction in addition to further mutations which impart a growth advantage on unpolarized cells leads to cancer progression. Elevated MUC1/EGFR interactions leads to upregulation of c-Met, imparting migratory characteristics to the cancer cells and resulting in a metastatic phenotype (Figure 6.1 D). This study clarified the role of MUC1 and EGFR interaction in mammary gland biology and breast cancer progression further than was previously known. With this added
understanding, new questions can be asked regarding the role of aberrant protein interactions in cancer.

Figure 6.1. Model of MUC1 and EGFR interaction in mammary gland biology and breast cancer progression. (A) Normal adult mammary ductal epithelial cells polarize MUC1 (red) and EGFR (green) to opposite sides of the plasma membrane. (B) Loss of cell polarity locally enables mixing of membrane domains, enabling MUC1 and EGFR interaction (yellow). (C) In lactation, milk fat globule secretion results in MUC1 and EGFR colocalized at the apical membrane (yellow). (D) Interaction of MUC1 and EGFR promotes tumor formation and c-Met expression, resulting in a migratory phenotype that is conducive to metastasis.
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