

CHARACTERIZATION OF EFFECTS OF MUC1 EXPRESSION ON
EPIDERMAL GROWTH FACTOR RECEPTOR SIGNALING IN
BREAST CANCER

by

Mamata Rani Pochampalli

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2006

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation

prepared by Mamata R. Pochampalli
entitled Characterization of effects of MUC1 expression on Epidermal growth factor receptor
signaling in breast cancer

and recommend that it be accepted as fulfilling the dissertation requirement for the
Degree of Doctor of Philosophy

Dr. Joyce A. Schroeder _____ Date: 11/17/2006

Dr. Jesse D. Martinez _____ Date: 11/17/2006

Dr. Mark A. Nelson _____ Date: 11/17/2006

Dr. Roy R. Parker _____ Date: 11/17/2006

Dr. Tim G. Bowden _____ Date: 11/17/2006

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I hereby certify that I have read this dissertation prepared under my direction and recommend that
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Dissertation Director: Dr. Joyce A. Schroeder Date: 11/17/2006

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ACKNOWLEDGEMENTS

I am forever grateful to my mentor Joyce A Schroeder whose constant guidance, encouragement and support have helped me get to where I am today in my scientific career. No matter how big/small my problem was, no matter whether professional or personal, she always offered support and that meant a lot to me. I am definitely short of words to describe my gratitude to the most wonderful person I have ever met in my life.

I would like to acknowledge all my committee members for their valuable input throughout my research. I would like to thank all the faculty and staff of Molecular and Cellular Biology and Cancer Biology for helping me directly and indirectly at various times throughout my research. I thank all my past and current lab members for the fabulous time I got to spend with everyone, not just in the lab, but outside as well. For many, lab is like a second home. But for me, it has been almost like my first as I live far away from my family. I would not have been able to get to the finishing stage of my graduate career without their help, support and all the fun.

I am very fortunate to have an immense support from so many friends outside the lab as well. I thank all my family, especially, my little sister Karuna Pochampalli, who offered tremendous emotional support for me throughout. Their words of encouragement and a feeling of pride for my accomplishments have helped come a long way. Last, but not the least, my work would not have been possible if my husband, Raghu Muthyalampalli, did not become the most important part of my life. He never let me give up even though things were so hard not just for me, but for him too, for being so far away.

DEDICATION

I would like to dedicate this work to my mother Vijaya Lakshmi Pochampalli whose dream was always to see me as a very successful person, but unfortunately died very young, long before she got to see where I am today. But her dreams for me have always been and will continue to be the most driving force behind all my endeavors.

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ABSTRACT

EGF receptors are key regulators of cell survival and growth in normal and transformed tissues. Ligand binding results in formation of homo/hetero dimers of these receptors, followed by activation of the kinase activity and subsequent tyrosine phosphorylation of many downstream molecules. The activation of these receptors is not only mediated by the binding of their cognate ligands, but by transactivation by other molecules as well. Recent studies have identified an oncogenic glycoprotein MUC1 as a binding partner for EGFR and that MUC1 expression can potentiate EGFR-dependent signal transduction. After receptor activation, EGFR is typically downregulated via an endocytic pathway that results in receptor degradation or recycling. We report here that MUC1 expression inhibits the degradation of ligand-activated erbB1. In addition, MUC1 expression results in prolonged activation of Akt, but not ERK1,2 MAPKinase. The MUC1-mediated protection against degradation occurs with a decrease in EGF-stimulated ubiquitination of erbB1, and an increase in erbB1 recycling. We then utilized the WAP-TGF α transgenic mouse model of breast cancer and determined that a loss of Muc1 expression dramatically alters mammary tumor progression. While 100% of WAP-TGF α /Muc1^{+/+} mice form mammary gland tumors, only 37% of WAP-TGF α /Muc1^{-/-} form tumors. Furthermore, expression of cyclin D1 expression is significantly suppressed in tumors derived from WAP-TGF α /Muc1^{-/-} animals, and loss of Muc1 expression resulted in a significant inhibition in the formation of hyperplastic lesions in the mammary gland. We also observed metastatic pulmonary adenocarcinoma (1/29) and perivascular lymphoma of unknown origin (28/29) in the WAP-TGF α transgenic mice but not in the WAP-

TGF α /Muc1^{-/-} animals. To determine the effects of Muc1 expression on metastasis in a model lacking perivascular lymphoma, we crossed MMTV-Wnt-1 and MMTV-MUC1 transgenic mice and evaluated interactions between Muc1 and EGFR. Although the MMTV-Wnt-1 mice are non-metastatic, a majority (6/10) of the bitransgenic MMTV-Wnt-1/MMTV-MUC1 formed pulmonary metastases. Furthermore, overexpression of MUC1 increases the breast cancer cell invasion *in vitro*. The MUC1 induced increase in invasion is found to be EGF and EGFR-kinase dependent. Collectively, these data indicate that MUC1 expression contributes to many of the hallmarks of cancer and in addition, is an important modulator of EGFR-associated mammary tumor progression.

I. INTRODUCTION

Cancer

The term “cancer” is derived from the greek word for crab, namely “carcinoma” used by Hippocrates (460-370 B.C.) to describe an ulcer-forming tumor. Cancer encompasses a group of greater than 200 distinct and often fatal diseases caused due to uncontrolled proliferation and spread of abnormal cells into various sites in the body. Cancer is one of the leading causes of death in most of the developed countries and in fact, the American Cancer society estimates that more than 1500 people die of cancer every day. Hence, intense research focus is laid on understanding the mechanisms that convert a normal cell into a cancer cell.

Identification of oncogenes and tumor suppressors and the delineation of some of the pathways that are disrupted in cancers have provided valuable insights into defining the characteristics that distinguish a cancer cell from a normal cell. It is now clear that the process of carcinogenesis involves multiple stages and is extremely complex. Hanahan and Weinberg ,in a landmark review [7], defined six characteristic features that are common to most of the cancers (Figure 1.1). Therefore, research on any of the molecules that contribute to or inhibit any of these hallmarks, is pivotal to the development of diagnostic and therapeutic targets. The current study involves characterization of the role of two oncogenic proteins, namely, EGFR and MUC1, both of which contribute to breast cancer.

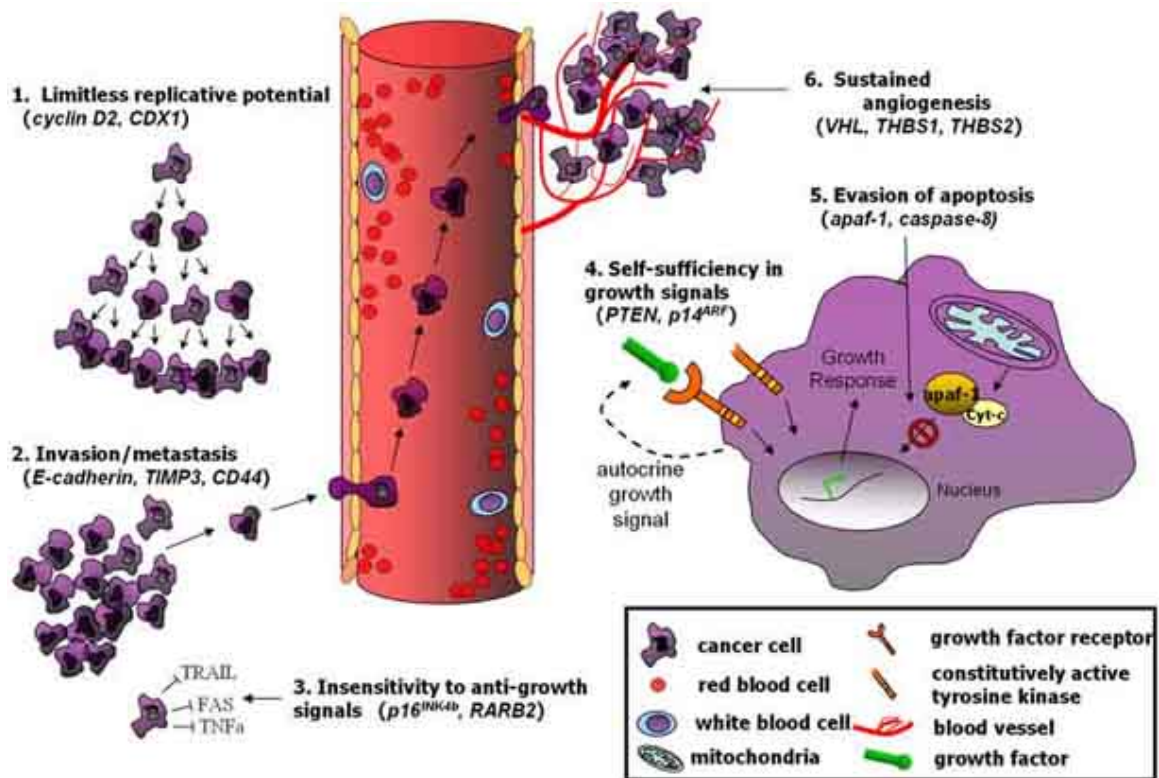


Figure 1.1: Hallmarks of cancer.

This figure depicts the six characteristic features that define the cancer cells and is adapted from [6]

Breast Cancer

Breast cancer is the most commonly diagnosed cancers in women worldwide. The American Cancer Society estimates that about 212,920 new cases of invasive breast cancer and 61,980 new cases of *in situ* breast cancer occur among women in US in 2006. Since the breast is composed of identical components in both male and females, the breast cancer can occur in males as well, although at a much lower frequency.

The structure of the breast together with the sites where the cancer can develop is depicted in the Figure 1.2. It is important to note that similar to many other solid tumors, the cause of deaths in breast cancer patients is not because of where it arises, but where it actually spreads to in the body. For example, in the case of breast carcinoma *in situ*, the abnormal cells are confined to the lobules or the milk ducts and do not spread to the surrounding and hence can be treated easily. In contrast, in the case of invasive breast cancers, tumors first develop in the lobules or ducts, which then break into the surrounding breast tissue and ultimately spread via the lymph nodes to several organs in the body such as liver, lungs and bones. This process of spread into distant sites is called metastases which ultimately results in the fatality.

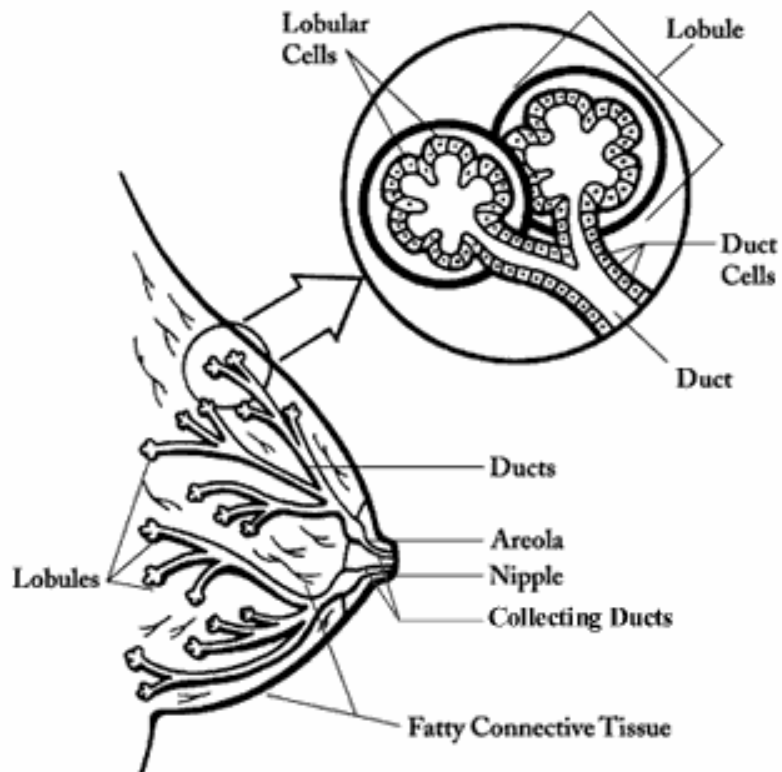


Figure 1.2: Structure of human breast

Although there has been a considerable progress made in the diagnosis and treatments of breast cancers, it still continues to be a life threatening disease for many women all over the world. Hence, the quest for other molecules that are important in the development of breast cancers is ongoing. One family of proteins that have been shown to play a vital role in the progression of benign mammary tumor to an aggressive malignant cancer and hence are therapeutic targets, are the EGFR family of receptor tyrosine kinases. Research in the past decade has led to the discovery of the role of a new class of proteins called mucins, in various malignancies, including breast cancer, and serves as important prognostic indicators. Recent studies suggest that EGFR, a receptor tyrosine kinase and MUC1, a mucin interact with each other. The present study is aimed at delineation of the functional and physiological consequence of their interactions.

The details about mucins, with special emphasis on MUC1, followed by description of the EGFR family of receptors are presented in this chapter.

Mucins

The term “mucin” was first used by DeSaussure (1835) to refer to the major component of mucous lining the surfaces of glandular epithelia as a viscoelastic gel (reviewed in [8]). The characteristic feature that defines a real mucin is the presence of variable number of tandem repeats with a high proportion of serines and threonines that are modified by O-glycosylation. The 20 Mucin-type glycoproteins identified to date are classified as a family based on the presence of an extensively O-glycosylated tandem repeat structure. (Human Genome

Organization Gene Nomenclature Committee; <http://www.hugo-international.org/hugo/>) [1].

However, it should be noted that this nomenclature has some exceptions due to the fact that splice isoforms of mucins, do not contain a repeat domain.

Types of Mucins

The discovery of MUC1 in 1990 led to the identification of many mucins that differ in their structure, chromosome location, tissue specificity and functions. The structural feature that is common to all mucins is the tandem-repeat domain, which comprises tandem repeats of identical or highly similar sequences that are rich in serine, threonine and proline residues. Accordingly, the human mucins have been classified into two main types: 1) Secreted 2) Membrane-bound /tethered mucins.

Secreted Mucins: These are the mucins that lack a transmembrane domain and are secreted into the extracellular space, remain at the apical surface and form a mucus gel. The secreted mucins form extremely large oligomers through linkage of the monomers by disulphide bonds. This class includes MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8 and MUC19 and constitutes the viscous mucus of the tracheobronchial, gastrointestinal and reproductive tracts (reviewed in [9] [2]).

Membrane-bound Mucins: These mucins contain a single transmembrane domain (hence type I) and a cytoplasmic tail at the C-terminus. The membrane bound mucins can

however be proteolytically cleaved and released into the extracellular space. These mucins do not form oligomers. This class includes MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20. The tandem repeat units of different mucins are not similar to each other either in number or the sequence of the amino acids.

The domain organization of the different mucins is depicted in the Figure 1.3. It is evident that the architecture of the multiple domains differs from one mucin to the other and this feature confers different properties to mucins.

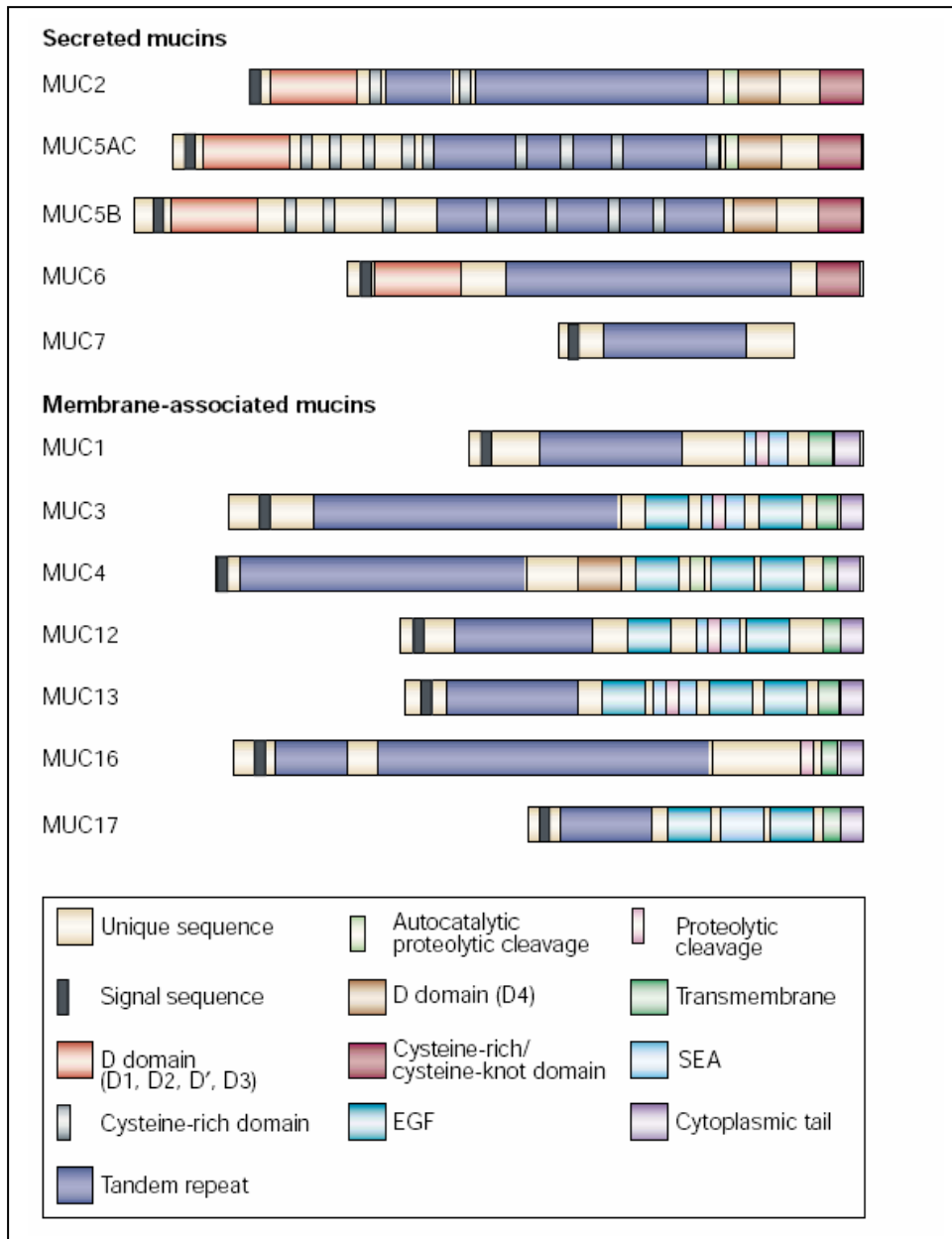


Figure 1.3: Schematic of domain structure of various mucins.

(Adapted from [2])

The functions of different mucin domains are summarized below [2]:

Cysteine-rich domain: This domain consists of many cysteine residues and is not heavily glycosylated.

Cysteine-rich/cysteine-knot domain: A domain that is conserved with von Willebrand factor and the cysteine knot of TGF- that mediates dimerization of mucin molecules.

Cytoplasmic tail: This domain is found on the cytoplasmic side of the cell-surface membrane. It often contains sites of phosphorylation that interact with mediators of signal transduction and might mediate association with cytoskeletal elements.

D domain (D1, D2, D', D3): These show sequence homology to the von Willebrand factor 'dimerization' domain and mediate trimerization of secreted mucin core proteins such as MUC2.

D domain (D4): A region of sequence homology to the D4 dimerization domain of von Willebrand factor; includes the GDPH autocatalytic site near the amino terminus of MUC2 and MUC5AC.

Epidermal-growth-factor (EGF)-like domains: Show homology to EGF and related growth factors and cytokines (for example, Cripto). They are believed to mediate interactions between mucin subunits and Erbb receptors.

GDPH autocatalytic proteolytic cleavage site: An autocatalytic proteolysis site in some mucins (demonstrated for MUC2, conserved in MUC5AC and MUC4) that cleaves

between GD and PH residues. A second step might involve formation of an autocatalytic ester linkage between the α carbon of the carboxy-terminal D residue and an internal *N*-acetylgalactosamine moiety on a chondroitin-sulphate glycosaminoglycan on the other subunit, forming a unique covalent bond by which mucin subunits are linked to other secreted molecules.

SEA domain: Named for a common domain found in sperm protein, enterokinase and agrin that was identified by sequence homology. It is widely distributed among cell-surface-associated proteins that are heavily *O*-glycosylated and is postulated to function in protein binding to carbohydrate moieties. Among mucins, this domain was originally identified in MUC1 and has subsequently been found in MUC3, MUC12, MUC13 and MUC17.

Signal sequence: Directs insertion into the endoplasmic reticulum for secretion or cell-surface delivery.

Tandem repeat: Rich in serine, threonine and proline residues. They are heavily *O*-glycosylated and are characteristic of mucin core proteins. There is a high degree of sequence similarity within any one repeat, but very little sequence conservation between different repeats or between orthologues. They can be highly polymorphic for length and sequence variability.

Transmembrane domain: Membrane-spanning domain that creates an integral membrane protein, which is found in cell-surface-associated mucins.

Proteolytic cleavage site: Conserved proteolytic cleavage site that is found within the SEA domains of some mucins and outside of the SEA domains in others. It facilitates the creation of mucin subunits that remain associated.

The mucin most relevant to breast cancer, and hence to our current study is MUC1, the founding member of the mucin gene family and is discussed below.

MUC1

The first human mucin gene was cloned and sequenced by four different groups of researchers in 1990 [10] [11] [12] [13] and was designated MUC1. The MUC1 molecule was first discovered in human milk by utilizing the mouse monoclonal antibodies raised against the human milk fat globule membrane [14]. Since then, MUC1 has been identified in various tissues and is referred to by multiple names: EMA (Epithelial Membrane Antigen), PAS-0 (PAS denotes positive staining for carbohydrate with periodic acid-Schiff's reagents), DUPAN-2, peanut-lectin reactive urinary mucin (PUM), CA1, NPGP (non-penetrating glycoprotein), NCRC-11 antigen, epitectin, DF3 antigen, HMFG antigen, Sebaceous Gland Antigen (SGA), MAM-6, polymorphic epithelial mucin (PEM), H23 antigen and episialin (epithelial glycoprotein of high sialic acid content) (reviewed in [15],[9]and[8]).

Structure of MUC1 gene

The MUC1 gene is located on chromosome 1q21-24 and spans about 4-7 kb of genomic DNA and is comprised of seven exons. The exon 2 encompasses the entire tandem repeat sequence, which is a stretch of 60 nucleotides repeated several times (ranging from 20-125) and encodes the 20-aminoacid repeat unit. Based on the number of tandem repeats, this region could represent as much as 50-80% of the whole gene [16]. The presence of variable number of tandem repeats (VNTRs) result in polymorphism at the DNA, RNA and protein levels and contributes to a high degree of heterogeneity among individuals. It has been shown that the VNTRs in Northern European population vary from 40-80 [17]. In addition, the genomic sequence of MUC1 has been shown to be unusual with respect to the G/C composition. In contrast to the 40% G+C composition observed in mammalian genomic DNA, the overall genomic sequence of MUC1 is 65% G+C rich, while the tandem repeat region has 82% G+C content [18]. The significance of the G/C composition will be discussed in the background section on regulation of gene expression.

Structure of MUC1 protein

MUC1 is a type I transmembrane glycoprotein with a high molecular weight of 300-600 kDa depending on the size of the VNTR domain. The mature glycoprotein is a heterodimer formed from a common precursor protein. The smaller subunit consists of a

hydrophobic tethered domain of 31 amino acids and a short cytoplasmic domain of 69 amino acids. The sequence and functions associated with cytoplasmic unit are discussed in detail in the later section.

The larger subunit comprises the majority of the VNTR domain flanked by non-repeat domains on the either side with a signal peptide sequence at the N terminus (Figure 1.4). The tandem repeat domain consists of up to 120-fold repeated icosapeptide sequences (PAPGSTAPPAHGVTSAPDTR) with the sequence variation (PAPGSTA PP/Q/A/TAH GVTAPD/ET/SR). One unique feature of mucins is the high content of five amino acids, namely, threonine, serine, proline, alanine and glycine. These five amino acids have been shown to account for 80% of the tandem repeat region [15]. Each tandem repeat has 3 Threonine and 2 Serine residues and hence has 5 potential O-glycosylation sites. MUC1 also contains five sites for N-glycosylation, four in the C-terminal region of the mucin subunit and one near the transmembrane of the membrane-associated subunit. The proline residues provide an extended form raising MUC1 well above the glycocalyx. Indeed, it has been suggested that the mucin domain of MUC1 extends at least 200-500 nm above the cell membrane, very far above any other membrane associated protein [19]. The smaller amino acids alanine and glycine are supposed to facilitate the close approach of the enzymes involved in glycosylation. Overall, MUC1 is a rigid extended molecule due to the presence of proline residues which confer the random coil feature and glycosylation that further adds to the rigidity (reviewed in [15]and [8]).

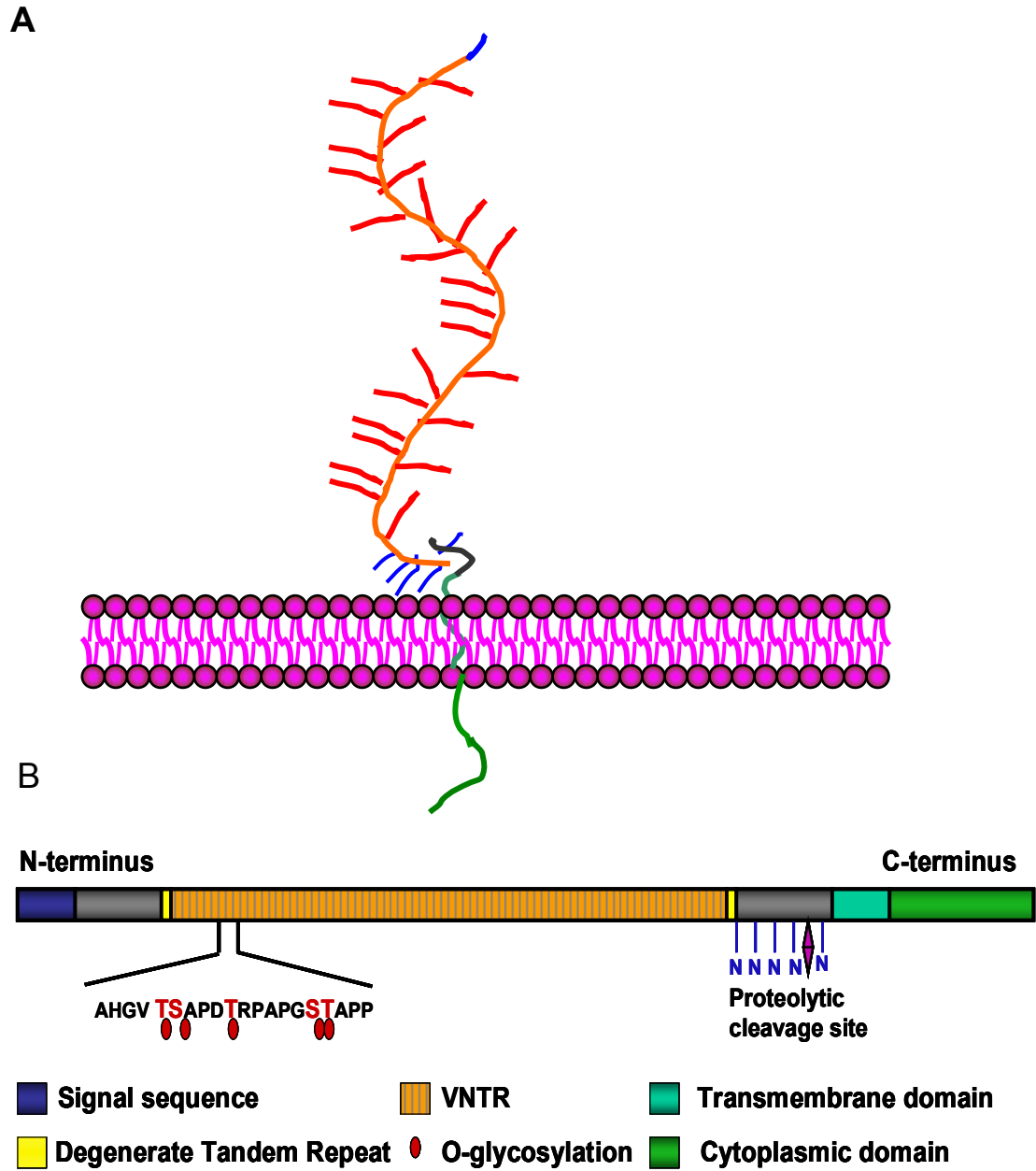


Figure 1.4: Schematic of protein domains and cartoon of MUC1

Isoforms of MUC1

MUC1 gene undergoes alternative splicing and can give rise to various isoforms, some having the mucin-like domain and some lacking this region [13]. The different isoforms are described below.

MUC/SEC isoform: This has an extracellular domain identical to the MUC1 form. However, the MUC/SEC form lacks the hydrophobic region that anchors the protein into the membrane and hence is secreted from the cell. The MUC1/SEC form has been shown to be present in the serum of breast cancer patients [20].

MUC1/Y isoform: This form is similar to the MUC1 form in that it also contains the transmembrane and cytoplasmic domains. However, this form is devoid of the central tandem-repeats (therefore lacks the characteristic mucin domain) and their flanking sequences. The MUC1/Y protein has been found to be expressed in various secretory epithelial tumors, but is not detectable in the adjacent normal tissue and has been shown to play role in signal transduction and increase tumorigenic potential [21, 22]. It has been demonstrated that the secreted MUC1/SEC form is the cognate binding protein for the MUC1/Y form that functions as a receptor [22].

MUC1/ZD isoform: This isoform lacks the tandem-repeat region as well and is generated by utilizing the same splice donor site as MUC1/Y but the splice acceptor site

for MUC1/ZD form is located 19 nucleotides downstream to that used by MUC1/Y. The alternate splice acceptor (*S.A.*) site used by MUC1/ZD results in a +1 reading frameshift and hence MUC1/ZD contains a completely new C-terminal protein sequence unique from all the other MUC1 proteins [23]. MUC1/ZD is expressed both in cancer tissue and in epithelial cells of the skin layers and the sebaceous glands. The MUC1/ZD protein exists as an oligomer made of many individual monomer units held by disulphide bonds. The biological functions of this isoform, however, are not yet known.

MUC1/X isoform: This form also lacks the tandem repeat array and except for the 18 amino acid insertion in the extracellular domain, resembles the MUC1/Y isoform [24]. This isoform is a result of splicing that involves the same splice donor site as MUC1/Y but utilizes an alternate *S.A.* that is located 54 nucleotides upstream to that used by MUC1/Y.

MUC1 variants A, B, C, D: These variants all have tandem repeat domain and have been identified in relation to ovarian cancers. These differ only in positions they use for splice acceptor sites. Variants A and D have been associated with malignancy of ovarian samples [25]. So far these variants have not been identified in any of the breast cancers.

Biosynthesis and Processing of MUC1

The pathway that converts the immature form of MUC1 to the mature one is an important basis for the study discussed in chapter three and is described below.

MUC1 protein is synthesized as one large precursor protein in the endoplasmic reticulum(ER). The molecular weight of the first detectable precursor has been shown to be reduced by 20 kDa within 4 minutes due to the proteolytic cleavage that occurs in the ER [26]. This cleavage separates the molecule into two protein products that are held by non-covalent, but strong and stable interactions. The epithelial MUC1 contains two discrete signals, one in the N terminus of the extracellular domain and one at the junction of the cytoplasmic and transmembrane domains that are necessary for targeting to the apical surface[27].

The N-glycosylation occurs by the co-translational transfer of mannose-rich glycans while the protein is still in the ER. After the proteolytic cleavage of the precursor in the ER, the processing of N-glycans to complex-type oligosaccharides takes place in the compartments of the Golgi. The O-glycosylation of MUC1 starts in the *cis* Golgi with addition of N-acetyl galactosamine to serine and threonine catalyzed by a family of N-acetylgalactosaminyltransferases. The process of glycosylation continues all along the transfer to *trans* Golgi with a corresponding increase in the molecular weight. Maturation of MUC1, that involves addition of sialic acid residues, is achieved via clathrin-mediated endocytosis that takes place by repeated re-internalization of the

membrane-exposed MUC1. It has been demonstrated that the cell surface associated MUC1 forms are constitutively internalized (0.9% of surface fraction/min) and recycled. Both the premature and the cell surface associated MUC1 molecules undergo constitutive internalization and recycling until they are either secreted into the medium by a second proteolytic event (mediated by TACE/ADAM 17 metalloprotease) or transported to lysosomes [28, 29]. During this entire process that involves the multiple rounds of recycling from cell surface to Golgi, the two subunits remain associated with each other.

Glycosylation of MUC1 differs from a normal cell to the cancer cell. For example, in the breast cancer cell line T47D, all the five sites in all of the repeats have been shown to be glycosylated (reviewed in [30]). However, the key difference is that the O-glycans in the normal mammary gland are core II based with an extended carbohydrate chain of up to 16 or more monosaccharide units, whereas, in the breast cancer cells are Core I type and are hence short.

The relevance of transgenic mouse models in characterizing the role of MUC1 expression in breast cancer, discussed in chapters four and five, is based on the homology of human MUC1 to the mouse mucin. According to the human Genome Mapping Nomenclature, the proper designation for the human genome term is “MUC1” while the mouse homologue is “Muc1”. The following section highlights the parallels of the same.

Comparison of Human and Mouse Mucin 1

The mouse Muc1 gene is very similar to the human MUC1 with a few exceptions. The mouse Muc1 gene is located on the chromosome 3 in the region that is syntenic with the region on human chromosome 1 where MUC1 is located [31]. The genomic structure of mouse homologue is similar to human with all the exon/intron boundaries conserved. The promoter region upstream of TATAA-box is 100% homologous between human and mouse and may function in the epithelial-specific gene expression [32]. The number of tandem repeats that encode 20-21 amino acids is constant and equals 16 and hence there is no polymorphism [33]. The homology in the tandem repeat region is only 34% although the amino acid composition is almost similar. However, there is very high homology of 87% in the transmembrane and cytoplasmic domains implicating the functional significance of these domains [33].

Phylogeny of MUC1

Most of the studies on mucins are focused on mammalian species and hence the data for non-mammalian species is very limited. However, genes encoding mucin-type molecules have been identified in lower eukaryotes such as *Leishmania major* and *Trypanosoma cruzi* [34],[35]. In *T. cruzi*, the mucin-like glycoproteins have been shown to play important role in the interaction of the mammalian cell during the invasion process. A mucin-type glycoprotein called mucin-D has been identified in the embryonic cells of *Drosophila melanogaster* [36] and is involved in molting events.

Recent phylogenetic analyses by Duraisamy *et al.*, has revealed that the human MUC1 homologs exist in diverse mammals, namely gibbon ape (*Hylobates lar*), macaca monkey (*Macaca mulatta*), cow (*Bos taurus*), pig (*Sus scrofa*), dog (*Canis familiaris*), rabbit (*Oryctolagus cuniculus*), guinea pig (*Cavia cutleri*), golden hamster (*Mesocricetus auratus*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*). Sequences containing the SEA domain and a shorter region having similarity to the MUC1 cytoplasmic tail were also found in bird (chicken, *Gallus gallus*), fish (Fugu, *Takifugu rubripes*) and worm (*Caenorhabditis elegans* and *Caenorhabditis briggsae*). The putative MUC1 homolog in *C. elegans* is closely related to MUC1. However, the domain composition of the *C. elegans* sequence shows the presence of four EGF-like domains in the N-terminal region, a TIL (Trypsin Inhibitor Like) domain and four DoH (Dopamine β -Hydroxylase) domains which are not found in human MUC1, indicating that the similarity of human MUC1 to the *C. elegans* protein is due to convergent evolution from different ancestors. Moreover, in yeast, a mucin-like protein, designated Muc1, which is essential for pseudohyphal differentiation does not contain a SEA domain or a transmembrane region. In addition, other than limited sequence similarity in the low complexity repeat region, yeast Muc1 fails to align with human MUC1. Therefore, this report indicates that MUC1 homologs are exclusively found in mammals [1].

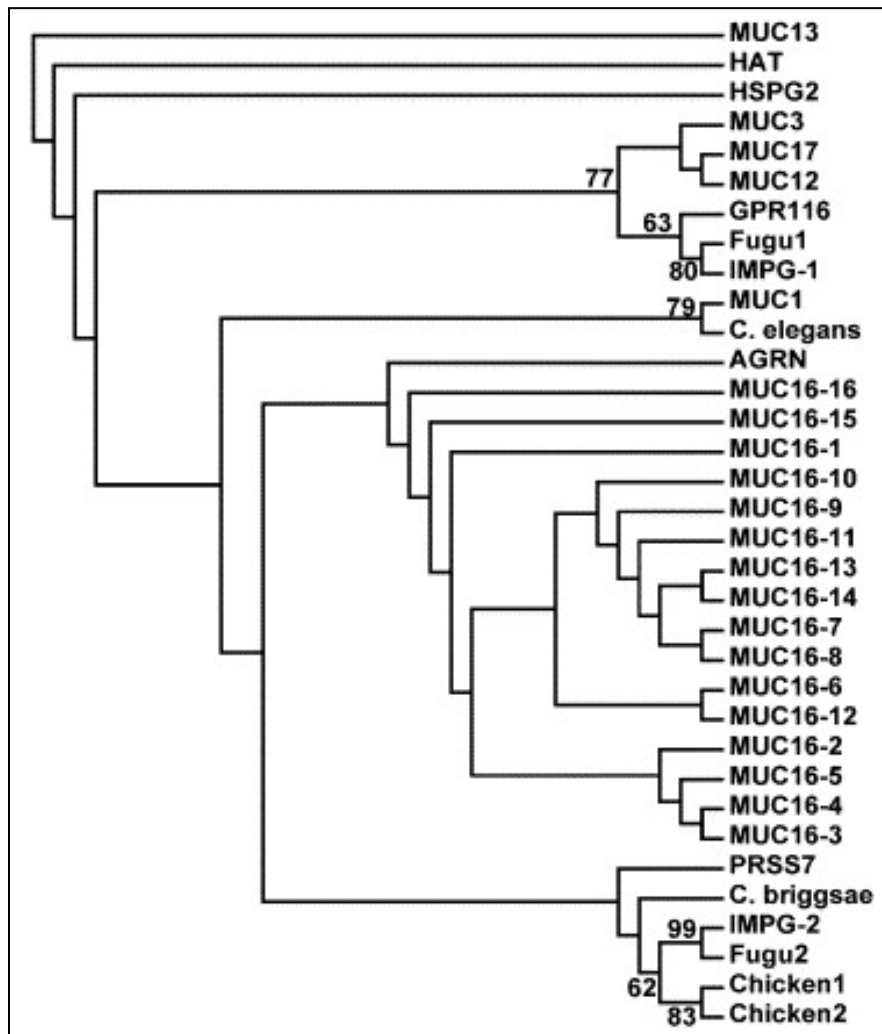


Figure 1.5: Schematic of phylogeny of MUC1

This figure is adapted from [1]

Expression profile of mucins in various normal tissues

Muc1 protein is first detected in the mouse embryonic stomach, pancreas and lung at gestational day 12. In all these tissues, Muc1 is expressed on the apical surface of the luminal epithelial cells. In addition, the expression of Muc1 correlates well with the differentiation status of the stomach, pancreas, lung, trachea, and kidney, salivary and mammary glands. Furthermore, the expression of Muc1 in the mammary gland is induced by treatment with insulin, prolactin and hydrocortisone hormones (reviewed in [9]). The expression profile of different mucins in the normal tissues is summarized in the Table 1.1 adapted from [2]. It is obvious from this table that different types of mucins are expressed in various tissues. The MUC1 core protein expressed in various tissue types is identical, but the mature form varies vastly in the molecular weight due to tissue-specific alterations in the glycosylation pattern. For example, the molecular weight of MUC1 in mammary gland ranges between 250 and 500 kDa while it exceeds 1000 kDa in pancreas, and in colon carcinoma cells it ranges from 600-800 kDa (reviewed in [9]).

Testis	Orange								Blue					Blue	Blue			
Maturing sperm	Orange																	
Urothelium (bladder)	Orange	Blue	Blue	Blue														
Uterus	Orange			Blue		Blue		Blue	Blue	Blue				Blue				
Vagina	Orange			Blue														
Haematopoietic	Orange													Blue	Blue			Blue
Bonemarrowfibroblasts																		Blue
maturing erythrocytes	Orange																	
Dendritic cells	Orange																	Blue
Granulocytes	Orange																	
Leukocyte															Blue			
Lymph node	Orange													Blue	Blue			
Parathyroid gland																		Blue
Peripheral leukocytes														Blue	Blue			
Plasma cells	Orange																	
Spleen														Blue	Blue			
T cell	Orange																	Blue
Thyroid	Orange					Blue												
Thymus				Blue						Blue					Blue			Blue
Tonsil															Blue			
Integument																		
Hair follicle																		Blue
Sweat glands	Orange																	
Neural																		
Cerebellar cortex																		Blue
Ganglion cell																		Blue
Perineurial cells	Orange																	Blue
Schwann cells																		Blue
Ocular and auditory																		
Eustachian tube	Orange			Blue	Blue	Blue												
Eye																		
Conjunctival	Orange			Blue	Blue									Blue	Blue	Blue	Blue	Blue
Cornea epithelium	Orange			Blue											Blue			
Lens epithelium																		Blue
Lacrimal gland	Orange			Blue		Blue		Blue										
Middle ear																		
Nasolacrimal duct	Orange	Blue			Blue	Blue	Blue											
Respiratory																		
Larynx	Orange																	
Lung	Orange	Blue	Blue	Blue	Blue	Blue	Blue	Blue		Blue				Blue	Blue			
Bronchi	Orange	Blue	Blue	Blue	Blue	Blue	Blue	Blue										
Nasal passage	Orange	Blue		Blue	Blue	Blue	Blue	Blue										
Sinus																		
Ethmoid					Blue	Blue	Blue	Blue										
Maxillary			Blue															
Trachea	Orange	Blue		Blue	Blue	Blue	Blue	Blue	Blue					Blue				
Musculoskeletal																		
Skeletal muscle																		Blue

Table 1.1(contd.): Expression profile of mucins in normal tissues [2]

Normal functions of Mucins

Prior to the discovery of roles of mucins in transformation, numerous studies were performed addressing the roles of mucins in the normal tissues. The functions of mucins in normal cells are depicted in the Figure 1.6 adapted from [2] and are summarized as follows:

a) The membrane-bound mucins, due to the presence of an extracellular domain and also a cytoplasmic domain that interacts with cytoskeleton, play a role in outside in signaling by functioning as a molecular sensor and relay the information about the morphological condition and the differentiation status. Due to the extensive glycosylation, MUC1 is negatively charged and hence cells overexpressing high levels of MUC1 potentially repel each other, thus resulting in decreased aggregation of cells [37]. The repelling property of MUC1 has also been shown to inhibit the integrin mediated cell adhesion to extracellular matrix [38]. MUC1 not only can decrease aggregation of cells, but also enhance the cell interactions in the instances where it can function as a ligand to the proteins such as ICAM1 expressed by the endothelial cells [39].

b) The secretory epithelial cells such as those found in the aerodigestive tract are constantly exposed to very harsh environments such as acid pH, salt, bacteria, viruses, food, enzymes etc. In such cell types, especially the secreted gel-forming mucins serve as first lines of defense by providing protection of the epithelial tissues by the mucociliary clearance of the cellular debris and the pathogens. Furthermore, it has been demonstrated that in the cystic fibrosis mice lacking Muc1, there is a decrease in the intestinal mucus

gel implicating the role of Muc1 in either the gel formation or in the retention of the gel at the mucosal surface [40].

c) Because of the rigid rod-like structure, the MUC1 molecule extends more than 100-200 nm above the cell surface, which is 5-10 fold the length of most of the molecules residing in the membrane. As a consequence of the dense array of glycosyl chains on the tandem repeat domain, the MUC1 molecules contribute to a high local concentration of specific molecular structures and thus provide high stoichiometric power to a restricted region.

d) Due to the steric hindrance from the dense glycosylation layer, the large molecules cannot pass through the sieve-like mucus glycoprotein gel and hence cannot access the cell surface, while the smaller molecules can potentially pass through the same.

e) Mucins are highly hygroscopic and hence play a role in lubrication of the cell surface

f) The posttranslational modifications such as sulphation and sialylation result in negatively charged moieties which can potentially role in ion-exchange functions.

g) Sequestration of growth factors, cytokines, chemokines and other compounds by mucins aids in maintenance of a high local concentrations for interactions with the cells in transit through the mucus layer.

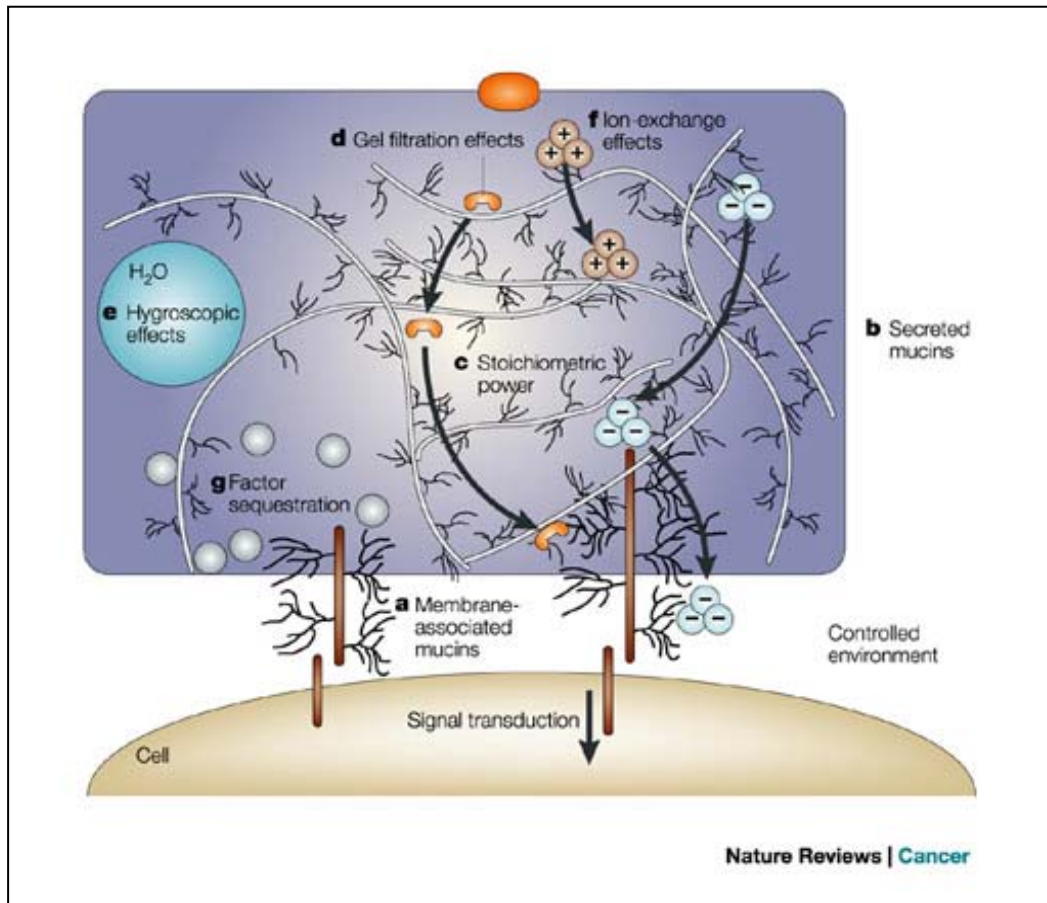


Figure 1.6: Schematic of normal functions of mucins

This figure has been adapted from [2]

Expression of Mucins in Cancer

Multiple researchers have investigated the expression pattern of various types of mucins and demonstrated that the overexpression or inappropriate expression or expression of aberrant forms of mucins contribute to the pathogenesis of cancers. The expression profile of mucins in various malignancies is summarized in the Table 1.2. (reviewed in [2]).

Mucins in breast cancer

Ever since the discovery of MUC1, numerous studies have demonstrated that MUC1 is overexpressed both at the mRNA and protein levels in multiple cancers including breast cancer [41] [42] [43]. MUC1 has been used as a tumor marker for many years and the levels of MUC1 that is shed into the serum of breast cancer patients seems to correlate well with the tumor load of the patient. Hence, assays like the CA-15-3 are used to detect the amount of circulating MUC1 [44]. The redistribution of MUC1 all over the cell surface instead of restriction to the apical surface can be observed in the lymph node metastases of breast carcinomas [45].

It is important to note that although the mucin MUC1 is significantly elevated; many studies revealed that other mucins are involved in breast cancer as well.

Malignancy	Mucin																	
	1	2	3	4	5	5B	6	7	8	9	11	12	13	15	16	17	18	
Gastrointestinal																		
Biliary-duct carcinoma																		
Breast carcinoma																		
ampulla of Vater																		
Cholangiocarcinoma																		
Colorectal carcinoma																		
Oesophageal squamous																		
Gall-bladder carcinoma																		
Gastric carcinoma																		
Gastric MALT																		
Hepatocellular carcinoma																		
cholangiocarcinoma																		
Laryngeal carcinoma																		
Pancreatic carcinoma																		
Salivary-gland carcinoma																		
Genitourinary																		
Bladder carcinoma																		
Cervical carcinoma																		
Choriocarcinoma																		
Endometrial carcinoma																		
Germinoma																		
Ovarian carcinoma																		
trophoblastic tumour																		
Prostate carcinoma																		
Renal carcinoma																		
Haematopoietic																		
AML																		
ALL (B and T cell)																		
Anaplastic large cell L																		
B-cell lymphoma																		
Multiple myeloma																		
T-cell leukemia																		
Thyroid carcinoma																		
Integument																		
Adnexal tumours																		
Melanoma																		
Squamous cell skin																		
Neural malignancies																		
Meningioma																		
Schwannoma																		
Respiratory																		
Lung carcinoma																		
Mesothelioma																		
Sarcoma																		
Angiosarcoma																		
Kaposi's sarcoma																		

Table 1.2: Expression profile of mucins in various malignancies [2]

A recent report focused on a detailed examination of expression profiles and prognostic significance of different (MUCs 1,2,3,4, 5AC and 6) in 1447 cases of invasive breast carcinoma [42]. This study revealed that MUC1 was expressed in 91% of the cases. The interesting finding from this study was that the important factor that determined poor outcome was not the level of MUC1 protein but where it was localized: Aberrant cytoplasmic and circumferential membranous localization correlated with poor outcome as compared to apical membrane localization.

MUC2 is a secretory mucin that is abundantly expressed by intestinal and airway epithelia. However, MUC2 expression can be also be detected in all the mucinous carcinomas derived from various organs including breast, colon and prostate and might be a prognostic indicator. Mucinous carcinoma of the breast usually shows less frequent lymph node metastases and more favorable outcome as compared to the aggressive invasive ductal carcinoma of the breast. It has been shown that most of the mucinous carcinomas exhibit MUC1+ and MUC2+ expression pattern whereas the invasive ductal carcinomas are MUC1+ and MUC2-[46]. Hence it has been suggested that because of the dense gel forming characteristic of MUC2, its expression could in fact be associated with inhibition of cell invasion. In the study mentioned above, MUC2 was detected only in 8.3% of the total cases.

MUC3 expression was detected in 91% of the cases and was associated with increased local recurrence and lymph node stage. The membranous expression of MUC3 was associated with poor prognosis and negative ER expression.

MUC4 provides a protective role to the epithelial cells in the normal conditions. It has been recently proposed that MUC4, due to the presence of EGF-like domains in the extracellular domain, can serve as a ligand for the ErbB2 receptor and can potentially modulate signaling induced by ErbB2 activation. Although some studies implicated MUC4 expression as a prognostic factor [47] in breast cancer, this study showed that MUC4 expression though detected in breast cancer is not associated with prognosis.

MUC5AC is a mucin expressed abundantly on the mucosal layer of the cardia, fundus and antrum of the stomach whereas MUC6 is detected in the pyloric glands. In the above study, MUC5AC and MUC6 were expressed only in 37% and 20% of the cases respectively. MUC6, similar to MUC2, was expressed in mucinous carcinomas.

In conclusion, though many mucins are expressed in breast cancer, only MUC1 and MUC3 are potential indicators of prognosis with MUC1 being the most significant mucin and hence is the focus of the current study.

Signaling by mucins

Currently, there is not a lot known about the contributions of other membrane associated mucins as they are not extensively studied. The cytoplasmic tail of MUC1 consists of 72 amino acids, while the putative size of the cytoplasmic tail of other mucins varies from 22 to 80 residues. It has been shown that the phosphorylation status of cytoplasmic tail of MUC1 correlates with increased oncogenic potential of tumor cells but the similar data is not available for the other mucins.

The common feature of the cytoplasmic region in all the membrane associated mucins (1,3,12, 15, 16 and 17) is the presence of a positively charged lysine-arginine rich motif which possibly serves as a spatial delimiting sequence preventing further insertion of the cytoplasmic domain into the membrane (reviewed in [4]). In addition, this domain also could play a role in the localization of mucins to the nucleus, although only MUC1 has been reported to be localized to the nucleus so far. Furthermore, the lysine-arginine rich motif can also be a site for enzymatic cleavage and allow the cytoplasmic region to be localized to other organelles within the cell in response to a stimulus. Indeed, the cytoplasmic tail of MUC1 has been shown to be localized to the mitochondria in response to genotoxic stress.

The other conserved motif in the cytoplasmic region of mucins is the YXX (L/M/V/I/F) that has been shown to be critical for the clathrin-mediated endocytosis of MUC1 [48]. Similar motifs can be found in MUC3, 12, and 17.

Cytoplasmic domain of MUC1

The significance of cytoplasmic tail of MUC1 in transformation comes from the studies that overexpressed the human MUC1 in mice. These studies demonstrated that only the mice that overexpress full length MUC1 develop mammary tumors, while the mice that overexpress MUC1 lacking the cytoplasmic tail region do not develop any mammary tumors [49]. The sequence of the cytoplasmic domain together with some of the proteins it binds to is depicted in the Figure 1.7.

Signaling by MUC1

The cytoplasmic sequence of MUC1 is very unique as it is very short and yet can bind many signaling molecules in a context-dependent manner. Although the function of MUC1 as a scaffolding protein has not yet been established, it is clear that it brings together many molecules together and mediates many critical events in cancer progression including increase in cell proliferation, increase in invasion, inhibition of apoptosis, etc. The different signaling pathways mediated by MUC1 are depicted in the Figure 1.8.

Although MUC1 can mediate many signaling events, the molecule of particular interest to us is the EGFR for this study and the significance of EGFR family of receptor tyrosine kinases is described in the following section.

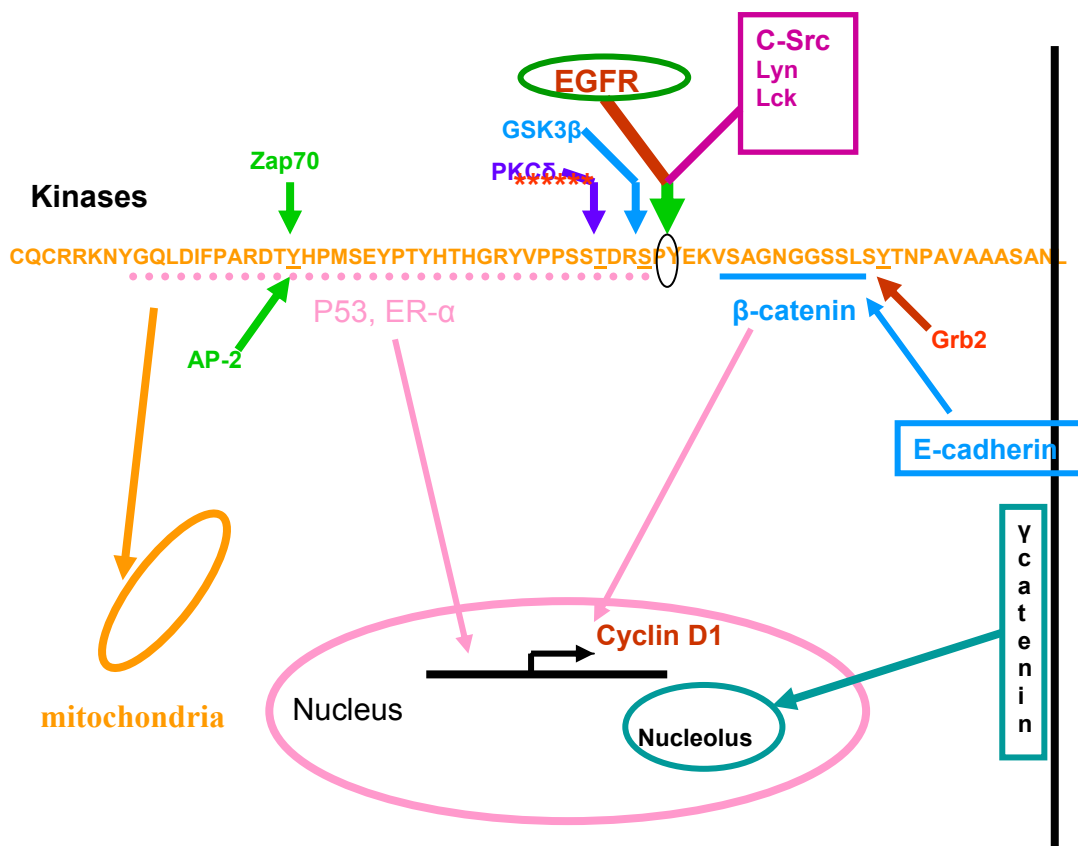


Figure 1.7: Schematic of cytoplasmic sequence of MUC1 with interacting proteins. The cytoplasmic tail of MUC1 consists of 72 amino acids, with only tyrosines being 7 of them. The kinases that are known to phosphorylate MUC1 are denoted above the sequence. The other molecules that bind to MUC1 that are not Kinases are represented below the sequence. Although MUC1 is a membrane protein, it can translocate to mitochondria in response to genotoxic stress, and also translocate to nucleus and nucleolus as well.

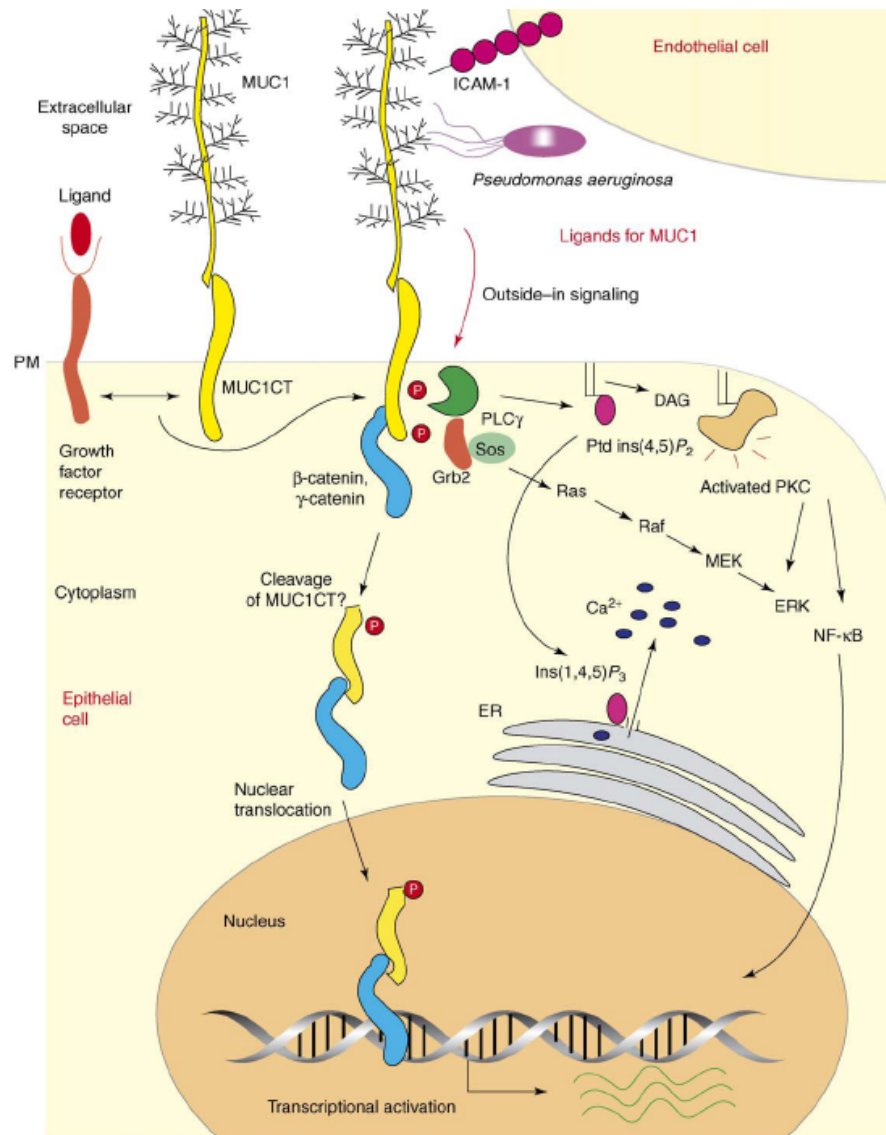


Figure 1.8: Schematic of signaling pathways mediated by MUC1. The above figure depicts the various signaling pathways mediated by MUC1 including the outside-in signaling in response to binding of pathogens, activation of ERK MAPkinase in response to EGFR activation and also activates PLC γ mediated events. In addition, MUC1 also binds to β -catenin, undergoes cleavage and regulates the transcription of cyclinD1. Not shown here is also the ability of MUC1 to translocate to mitochondria and inhibit cytochrome C release and regulate apoptosis in response to genotoxic stress (adapted from[4])

EGFR/ERBB Receptor Tyrosine Kinases

The epidermal family of receptor (EGFR) tyrosine kinases are one of the most extensively studied proteins for their myriad roles not only in normal physiological processes but also in the development of various malignancies. Ever since the discovery and characterization of the first ligand, EGF, in 1962, by Dr. Stanley Cohen who received the Nobel prize for his seminal discovery, there have been hundreds of thousands of studies reporting the identification of the receptors, the ligands that bind and other detailed characterization of many aspects. While an attempt to cover all the features related to their structure, ligands, mode of activation and the signaling pathways they mediate is beyond the scope of any single review, following is the summary of only the major aspects pertinent to the current study.

EGFR (also known as ErbB1/HER1) is the prototype of this receptor family and in fact, the term “ErbB” is designated based on the homology of these proteins to the product of v-erbB oncogene from the avian erythroblastosis virus that expresses a truncated form of EGFR [50]. This receptor family also includes three other members, namely, ErbB2/HER2/Neu, ErbB3/HER3 and ErbB4/HER4. All the four receptors are characterized by the presence of the following regions: Extracellular cysteine-rich domain with 4 subdomains (I, II, III, and IV), transmembrane domain, a small juxtamembrane domain preceding the Kinase domain, and a C-terminal domain with the potential docking sites for various phospho-tyrosine binding proteins.

Architecture of EGFR

The EGFR protein is synthesized initially as a 1210-residue polypeptide which then undergoes a cleavage at the N-terminal sequence, and finally gets inserted into the cell membrane as a 1186 amino acid containing protein [51]. More than 20% of the mass of EGFR is derived from the N-linked glycosylation which is important for targeting the protein to the cell surface [52]. Once at the cell surface, EGFR together with the other receptors in this family serve as critical mediators, transducing signals between the extracellular milieu to the inside of the cell and regulate diverse biological processes.

Ligands

Thus far, 15 polypeptide extracellular ligands, all containing a conserved EGF domain, have been identified. They include amphiregulin, betacellulin, biregulin, EGF, epiregulin, HB-EGF, heregulin α/β , neuregulin (NRG) 1 $\alpha/1\beta/2\alpha/2\beta/3/4$, and transforming growth factor alpha (TGF α) (reviewed in [53]). All the ErbB ligands initially exist as membrane-bound precursors which later get proteolytically cleaved to yield soluble mature ligands. It has been shown that the proteolytic cleavage is not necessary for the activation of ligand and hence the ligands can activate the receptors both in paracrine and juxtacrine manner [54]. Although all of these ligands activate ErbB receptors, the significance of EGF and TGF α have been extensively studied for their roles in various aspects of transformation and hence will be addressed in this study.

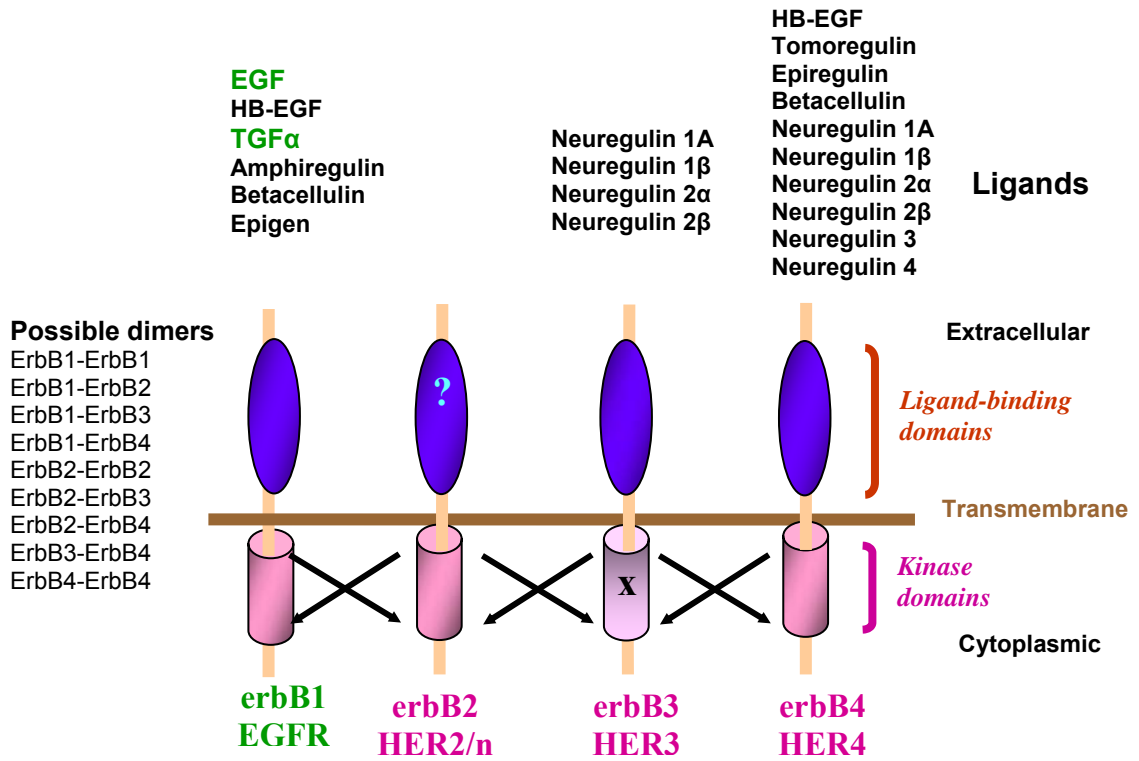


Figure 1.9: Schematic of different ligands and possible ErbB receptor heterodimers

Mechanism of activation

Prior to ligand binding, the receptors are maintained in an inactive state with respect to the kinase activity. The interaction between the various domains in the extracellular domains dictates the activation status of the receptor. The extracellular domain is composed of four distinct domains that include two large leucine-rich domains (I and III) that bind the ligand and two cysteine-rich domains (II and IV). These domains are present in the order I-II-III-IV (other nomenclature that is also used for these domains is L1-CR1-L2-CR2). In the absence of the ligand, the receptor is maintained in an auto-inhibited stage by the interaction between the cysteine rich domains II and IV which restricts the orientation of the domains I and III. However, binding of the ligand to the domains I and III results in a change in the conformation and relieves the autoinhibition of the receptors which leads to the formation of homo or heterodimers (reviewed in [55]).

Ligand binding to the ErbB receptors results in the formation of homo/heterodimers and consequent induction of the kinase activity. It is important to note that no cognate ligand has been identified so far that can bind ErbB2 and in addition, ErbB3 lacks kinase activity. Due to these reasons, the possible combinations of dimers that can form are 9 and are represented in the Figure 1.9. The activation of kinase domain thereafter leads to autophosphorylation of the tyrosine residues which then serve as docking sites to several downstream molecules. The cytoplasmic domain of the four receptors is not entirely identical and hence the molecules recruited to them also differ (Figure 1.10). One of the

molecules that binds EGFR upon activation is Cbl, a ubiquitin ligase that targets it for degradation. It is important to note that the downregulation of EGFR is one of the key aspects that determine the strength of EGFR signaling. While the Cbl binding to EGFR and the subsequent internalization and trafficking to lysosomes results in degradation of the receptor, the inhibition of ubiquitination can lead to a majority of the recycling of the receptor back to the surface. The recycling of EGFR back to the surface makes it available for further binding to the ligands and enhances signaling.

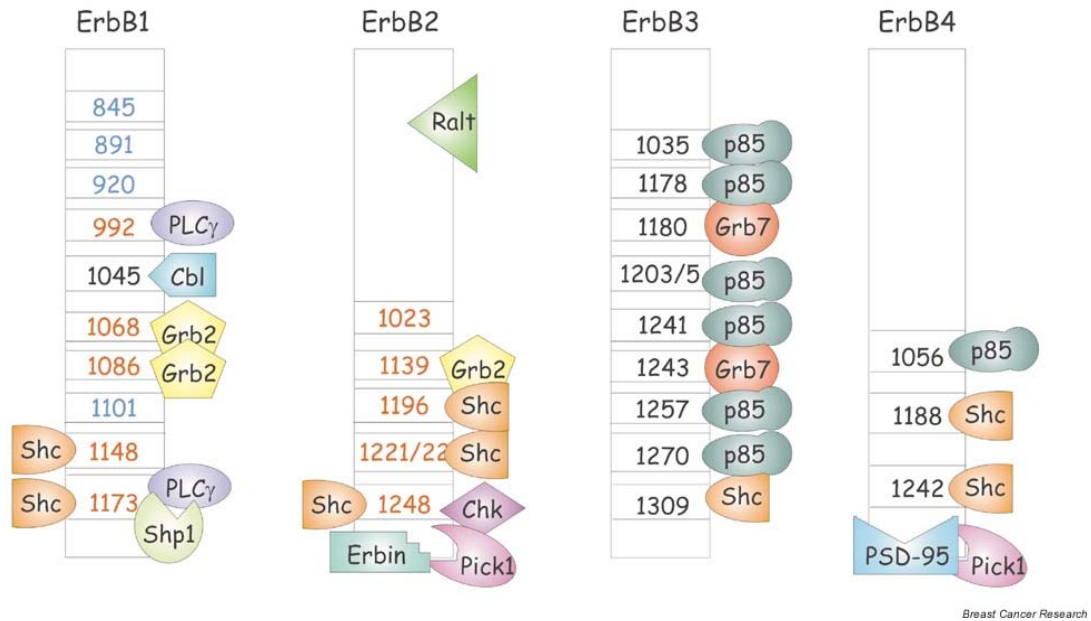


Figure 1.10: Schematic of the cytoplasmic domain of different ErbB receptors together with the proteins that bind to the phosphorylated Tyrosines. This figure is adapted from [3]

Transactivation

Several studies have now established that the ErbB receptors are not only activated by the traditional signaling initiated by the 15 ligands mentioned above, but many other signaling pathways impinge on these receptors as well. For example, many non-physiological stimuli such as UV and gamma radiation, membrane depolarizing agents and several oxidants can ultimately involve the activation of the ErbB receptors. Furthermore, the ErbB receptors can be activated by signals emanating from the G-protein coupled receptors, cytokine receptors, Wnts growth factors, etc (reviewed in [56]). Of all the molecules that can result in modulation of ErbB receptors, the protein of interest for this study is MUC1.

Emergence of a complicated network

The fact that there are about 15 different ligands, 9 possible dimers, many different docking sites in the cytoplasmic domain and several modes of transactivation, results in a very complicated network of signaling events mediated by these receptors. Consequently, it is not surprising that the ErbB receptors can mediate a myriad of pathways by integrating numerous stimuli, both from outside the cell, as well as inside, and are involved in regulation of diverse fundamental biological processes, both during normal development, as well as in transformation.

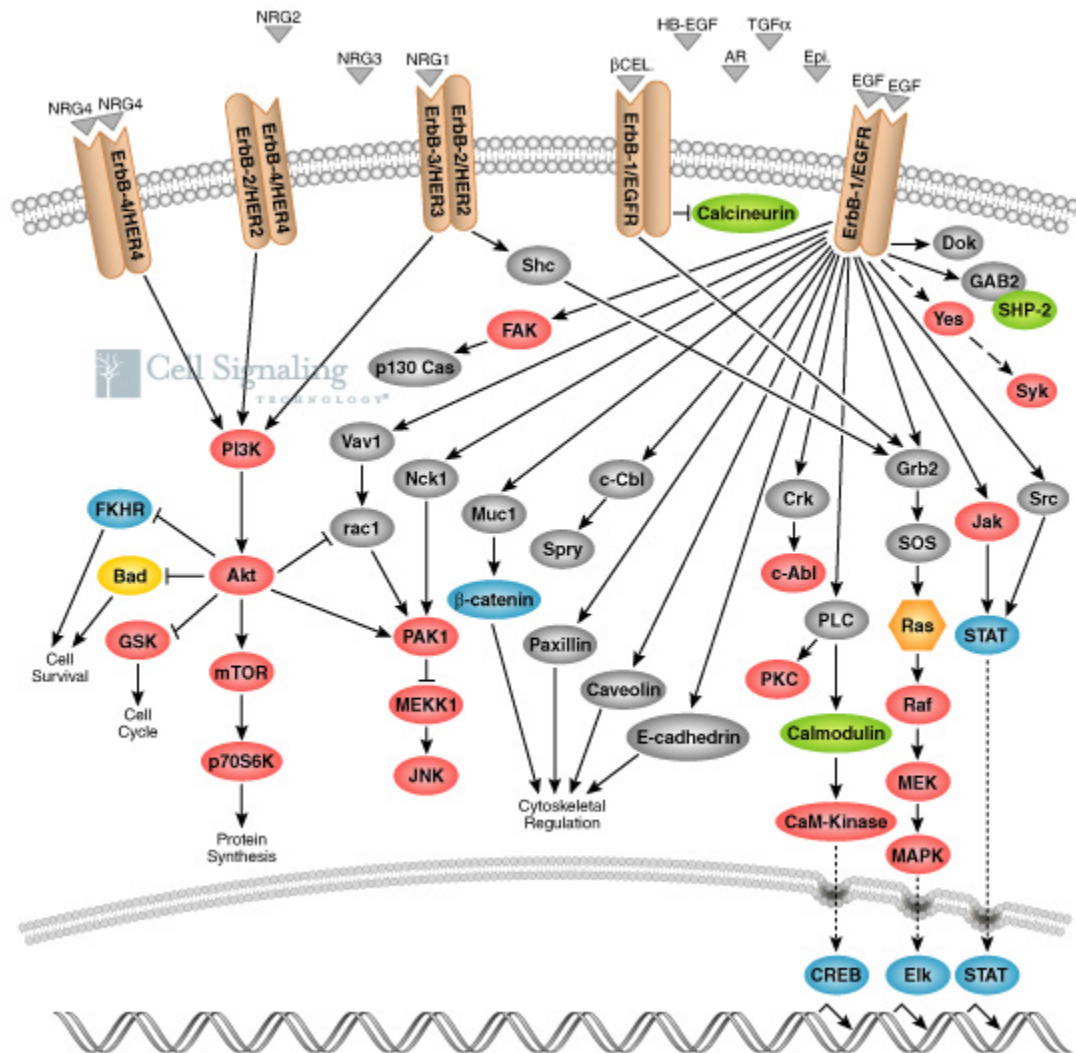


Figure 1.11: Schematic of signaling pathways mediated by EGFR. This figure illustrates the multiple number of signaling pathways initiated by EGFR activation and is adapted from the Cell Signaling.

The activation of various signaling pathways initiated by EGFR homo or hetero dimmers ultimately result in increase of cell proliferation, inhibition of apoptosis, angiogenesis, etc. The ErbB receptors can also be activated in the absence of growth factors due to constitutive activation of the receptors and the growth factors that activate the ErbB receptors are produced by the cancer cells. Therefore, the ErbB receptors contribute to a majority of the hallmarks of cancer and have been the therapeutic targets for various malignancies and many drugs have been designed to specifically inactivate these receptors in the cancer cell. The fact that these drugs are effective only in a fraction of patients, demands the investigation of other molecules that can regulate these receptors and the contribution of the glycoprotein MUC1 towards the regulation of EGFR is the focus of the current study.

Statement of the problem

Breast cancer is the most frequently diagnosed cancers in women worldwide. According to the estimates by the National Breast Cancer Coalition, in the U.S alone, about 275,000 women will be diagnosed with breast cancer in 2006, and 40,000 women are expected to die from it this year. Hence the need for breast cancer therapies cannot be understated.

Research in the past decade has demonstrated that the ErbB/EGFR family of receptor tyrosine kinases are frequently deregulated in many cancers, including breast cancer, and serves as major prognostic as well as diagnostic indicators. In line with this, many drugs have been developed that specifically target these receptors with the goal of treating breast cancer. In fact, Herceptin, which was approved by FDA in 1998, is the first target-selective drug raised against an oncogenic cell surface receptor ErbB2 and therefore represents the first paradigm of a new era of anti-cancer therapy. However, this drug is effective only in a fraction of all the patients that overexpress erbB2, implying that other molecules are involved as well, and hence a single drug is not very effective. Accordingly, a combination of the drugs targeting both EGFR as well as ErbB2 has been found to be more efficient than a single drug alone. However, even the combination therapy does not suffice to treat breast cancer. Hence, the quest for other molecules that regulate the major players in the breast cancer progression is ongoing. Given that the ErbB receptors modulate a myriad of signaling pathways, focus on the molecules that plausibly regulate the key aspects of these receptors is absolutely indispensable. Recent

studies have unraveled the prognostic significance of a mucin called MUC1 that is overexpressed in many epithelial cancers. Indeed, estimates indicate that MUC1 is overexpressed in about 90% of the breast cancer patients. The goal of the present study is to decipher the role of MUC1 expression on EGFR signaling in breast cancer and is based on the following previously published findings:

The proof that EGFR can function as an oncogene comes from the studies that report that the overexpression of EGFR in fibroblasts confers growth in soft agar, and in addition, results in the induction of tumors in nude mice [57] [58]. About 16 years after the demonstration of EGFR as an oncogene, similar studies using the rat 3Y1 fibroblasts and transgenic expression of full length MUC1 in the mice uncovered the role of MUC1 in transformation as well [59] [49]. At the time the studies addressed in this dissertation were undertaken, the following was known about the relationship between EGFR and MUC1:

- i) MUC1 and the erbB family of receptor tyrosine kinases biochemically interact in the MDA-MB-468 human breast cancer cell line as well as in transgenic mice overexpressing MUC1 (MMTV-MUC1) [60]. Importantly, experiments in the MMTV-MUC1 transgenic model have shown that this interaction results in the potentiation of EGF-dependent signaling pathways. Examination of the Ras/MAP Kinase pathway in these transgenic mice demonstrated that the overexpression of MUC1 vastly increases EGF-dependent p42/44 ERK activation during lactation [60].
- ii) EGFR associates constitutively with MUC1 at the cell membrane in the human ZR-75-1 breast carcinoma cells. In addition, activation of EGFR upon treatment with the ligand EGF, results in the direct tyrosine phosphorylation of MUC1 at the YEKV motif in the

cytoplasmic tail. Furthermore, phosphorylation of MUC1 by the activated EGFR leads to the binding of c-SRC and β -catenin to MUC1 [61].

Therefore, in summary, MUC1 is a substrate for activated EGFR and their biochemical interactions can potentiate EGFR-dependent signaling pathways that ultimately regulate cell proliferation, apoptosis, invasion etc, all of which are deregulated in cancers. Based on these studies, we hypothesize that the biochemical interactions between MUC1 and EGFR play a pivotal role in the mammary transformation.

To specifically test this hypothesis and to determine the relationship between these two proteins, and for the findings to be relevant to humans, we utilized both transgenic mouse models as well as human mammary epithelial cell lines. The mouse models were chosen as various studies have demonstrated that there are profound similarities between the functions of the mammary gland in humans and the mouse model. As a consequence, mammary cancer in rodents show a pathogenesis very much similar to that in humans and hence offer invaluable tools to study the development of cancer *in vivo*. Furthermore, the breast cancer cell lines provide more amenable tools to dissect the pathways further by allowing for more biochemical manipulations.

The objective of this dissertation is to determine the role of MUC1 expression in EGFR signaling in breast cancer by asking the following fundamental questions.

- 1) How does MUC1 expression enhance EGFR dependent signaling pathways?
- 2) Does removal of Muc1 expression have any effect on mammary tumor progression induced by EGFR activation?
- 3) Does MUC1 expression alter EGFR-mediated invasion and metastases?
- 4) What is the role of EGFR activation in MUC1-induced mammary carcinoma?

The following chapters cover the investigation of the above questions and the findings.

II. MATERIALS AND METHODS

Cell lines

BT20 and MDA-MB-231 human breast cancer cells and MCF10A immortalized human mammary epithelial cells were purchased from ATCC and maintained in RPMI (Gibco) with 10% FBS (Biomed) and 1.0 % Penicillin-streptomycin (Gibco) in 5.0% CO₂ at 37°C. Growth medium for MCF10A cells was supplemented with 10ng/ml cholera toxin (Sigma), 0.5µg/ml hydrocortisone (Sigma) and 5ng/ml EGF (Invitrogen). Cells were grown to 80% confluency for use.

Growth Factors, Inhibitors and cDNA constructs

ErbB1 kinase inhibitor (AG1478) was obtained from (Sigma). The MUC1 cDNA was a kind gift from M. A. Hollingsworth at the Eppley Cancer Institute, University of Nebraska Medical Center. The MUC1 cDNA was subcloned into the pCMV-DNA3.1 vector (Invitrogen) using standard techniques and constructs were transfected into MDA-MB-231 cells using Lipofectamine 2000, and selected with 1mg/ml G418.

Antibodies

The list of antibodies and their source are summarized in the Table 2.1.

NAME OF THE ANTIBODY	ANIMAL	COMPANY	CATALOG #
AKT	Rabbit	Cell signaling	9272
PHOSPHO-AKT (Ser 473)	Rabbit	Cell signaling	9271
β -actin	Mouse	Sigma	A 5441
CT2/ MUC-1 Ab-5 (MUC1 cytoplasmic domain of both mouse and human)	Hamster	Neomarkers	HM-1630-P
Cyclin D1 M-20	Rabbit	SC	SC-718
DF3 (MUC1 extracellular domain)	Mouse	DAKO	M 3518
EGFR -1005	Rabbit	Santacruz	SC-03
EGFR – Ab-17	Rabbit	Neomarkers	RB-1417-P1
EGFR – Ab-1	Mouse	Neomarkers	MS-268-P1
Phospho EGFR-992	Rabbit	Cell signaling	2235
Phospho EGFR-1045	Rabbit	Cell signaling	2237
Phospho EGFR-1068	Rabbit	Cell signaling	2234
ERK 1,2 MAP KINASE	Rabbit	Cell signaling	9102
Phospho ERK 1,2 MAP Kinase	Mouse	Sigma	M 8159
Phospho-p38 MAP Kinase	Rabbit	Santacruz	SC-17852-R
Phospho-Tyrosine-HRP (PY-99)	Mouse	SC	SC-7020
Ubiquitin (p4d1)	Mouse	Cell signaling	3936
NCL-Ubiquitin	Rabbit	Novocastra	NCL-UBIQ
SECONDARY ANTIBODIES			
Anti-Hamster-HRP		Jackson Immunoresearch	127-035-160
Anti-Rabbit-HRP		Fisher	PI31458
Anti-Mouse-HRP		Pierce	31444
Anti-Streptavidin-HRP		Pierce	21126
Anti-hamster FITC antibody		Jackson Immunoresearch	127-095-160
Texas-Red-Hamster IgG		Jackson Labs	127-075-160
Alexa Fluor 488 anti-mouse		Invitrogen	A21202
Alexa Fluor 594 anti-mouse		Invitrogen	A21203
Alex Fluor anti-rabbit 488		Invitrogen	A-21206
Alexa flour anti-rabbit 594		Invitrogen	A21207
Normal rabbit IgG		Santacruz	SC-2027

Table 2.1: List of antibodies used and their source

RNAi

RNAi was performed by either transient siRNA treatment (Qiagen or Dharmacon) or stable selection of pSUPER hairpin loop vectors (OligoEngine).

siRNA: MUC1 specific siRNA was generated to the following target sequence of the MUC1 extracellular domain: ³¹⁰⁴AAGACTGATGCCAGTAGCACT [11], and a non-silencing siRNA was designed to the following target sequence, which lacks homology to any known mammalian gene: AATTCTCCGAACGTGTCACGT (Qiagen). Transfections were performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's suggestions. For MCF10A cells, the transfection was performed once and the cells were lysed on day 3 post-transfection. BT20 cells required a double transfection for complete knockdown. The cells were transfected on day 1 then transfected again on day 3. BT20 lysates were collected on day 5. Knockdown was verified by immunoblotting for protein expression.

Additional controls were utilized to verify the effects of the Qiagen manufactured siRNA by testing MUC1 siRNA oligos from a second company (Dharmacon, *SMART*pool of 4 siRNA oligos) on these assays. These target sequences were as follows [and lie in the extracellular (#1-3) and cytoplasmic domain (#4)]:
 (#1)³²⁷⁰ACCAAGAGCTGCAGAGAGA, (#2)³³⁶³GATCTGTGGTGGTACAATT,

(#3)³⁴⁶⁵GATATAACCTGACGATCTC, (#4)³⁷⁴⁶GATCGTAGCCCCTATGAGA [11].

Those studies were performed following the manufacturer's instructions.

pSuper: pSuper vectors were purchased from (OligoEngine), using the companies software to generate both MUC1 and control RNAi hairpin loops. The following extracellular target sequence was used for the MUC1 hairpin loop: ³⁰⁷³TACTCCTACCACCCTTGCC [11]. Cells were transfected using Lipofectamine 2000 (Invitrogen), selected using G418 (Invitrogen) and were continually grown under 250 µg/ml during experiments.

Endocytosis assays

Cells were serum starved overnight, then incubated with 20ng/ml receptor grade EGF (Invitrogen) for 10' on ice. Unbound EGF was then removed by washing 2X with PBS at 4°C. Cells in serum-free medium were incubated at 37°C for the indicated time points and lysed [20 mM HEPES pH 7.5, 150 mM NaCl, 2mM EDTA pH 8.0, 2mM EGTA pH 8.0 and 1.0% Triton X-100, 2.0 mM Sodium orthovanadate, 50.0 µM ammonium molybdate and 10.0 mM sodium fluoride and Complete protease inhibitors (Roche)]. Cell lysates were vortexed briefly, centrifuged and the supernatant stored at -80°C. Protein concentrations were determined by BCA assay (Pierce).

Biotinylation assays

BT20 or MDA-MB-231 (C or CM) cells were grown serum-free overnight, then incubated at 4°C with 4 ml of 0.3mg/ml Sulfo-NHS-SS-biotin (Pierce) for 30' to analyze receptor internalization (Figure 3A). The biotinylation reaction was stopped by washing 3X with ice cold quenching buffer (10mM Tris pH 7.4, 154mM NaCl). Cells were then washed with ice cold PBS and treated with EGF as described in the endocytosis assay. For analysis of surface retention (Figure 4A), cells were first treated with EGF and incubated for 5 minutes at 37°C to promote internalization, then treated with biotin and lysed as described above. Lysates were precipitated with streptavidin coated agarose beads, washed and resuspended in SDS-PAGE buffer (0.4M Tris, 0.2mM EDTA, 25% glycerol, 10% β -mercaptoethanol and 0.4% bromophenol blue) for immunoblotting.

Immunofluorescence analysis

Cells were grown on glass coverslips and serum starved overnight. Cells were treated with EGF as described for the endocytosis experiment, washed with 0.02% NaN₃ /PBS, and then fixed with a 1:1 mixture of ice cold methanol-acetone. Cells were blocked with 20% FBS/0.02% NaN₃ /PBS, incubated with the indicated antibody overnight at 4°C, washed, and then incubated with Alexa fluor-conjugated secondary antibodies. Cells were then mounted with Slowfade Antifade reagent with DAPI (Molecular Probes) and visualized using a Leica DMBL 100s system with MagnaFire software.

Immunoprecipitation and immunoblotting

Protein lysates were incubated with their respective antibodies in TNEN buffer (50mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA pH 8.0 and 0.5% Igepal CA 630, 2.0 mM Sodium orthovanadate, 50.0 μ M ammonium molybdate and 10.0 mM sodium fluoride and Complete protease inhibitors) and rProtein G agarose beads (Invitrogen). Precipitates were washed with TNEN buffer 3X, and proteins were resuspended in 2X SDS PAGE buffer. Proteins were then separated by SDS-PAGE and transferred onto PVDF membrane (Millipore). The membrane was blocked with either 5% nonfat milk in PBS (0.1% Tween20) or 3% BSA in TBS (0.1% Tween20) and immunoblotted. The membrane was then treated with Super Signal West Pico Chemiluminescent Substrate (Pierce), visualized on Imagetech-B film (American X-ray supply Inc), and developed with a Konica SRX-101C.

Note: For ubiquitination experiments, TNEN buffer also contained 10mM N-ethylmaleimide (Pierce) to inhibit deubiquitination.

Recycling Assay

MDA-MB-231 cells transfected with MUC1 or with PcdNA3 alone were plated in triplicates and treated with 10ng/ml EGF at 4°C for 10'. Unbound EGF was removed by washing (2X) with cold PBS, cells were incubated at 37°C for 10', 60', or 90',

trypsinized and blocked with 5% BSA for 1h on ice. The cells were then incubated with R-phytoerythrin (R-PE) conjugated erbB1 antibody (BD Pharmingen San Diego CA) on ice. Fluorescence intensity was then immediately determined on a FACScan flow cytometer (BD biosciences, San Jose CA) and the results were analyzed using a CellQuestPro 4.0 software (BD biosciences, San Jose CA) using standard methodology followed by the Arizona Cancer Center Flow Cytometry Shared Service (FCSS). The geometric mean of stained cells was corrected against non stained cells to eliminate any autofluorescence and the results were plotted as a % difference where the 10' time point was considered the time point of origin at which no recycling occurs. $[(TP-T10')/T10' * 100]$

Mice

A list of all the mice used for the current study is summarized in the Table 2.2. All the mice were maintained under conditions specified by AALAC and the Institutional Animal Care and Use Committee (Tucson, AZ, USA).

Genotyping of the Mice

Following is the sequence of the primers used for determining the genotype of mice. The details for the waved-2 PCR can be found in chapter six.

PCR for TGF α transgene (MMTV-Wnt1 mice)

TGF α F = CAGTATTGTTTCCATGGGACCTGC

TGF α R = TCTCGTGTCTGCAGACGAGGGCAC

PCR for MUC1 transgene (MMTV-MUC1 mice)

SV40R3 = ATTATGGAGGAGTAGAATGTTGAGAG

MMF5' = CAGTCTGATATCGGGGCTGGGGTGCCAGGC

PCR for Wnt1 transgene (MMTV-Wnt1 mice)

IMR513 = GGA CTTGCTTCTCTTCATAGCC

IMR514 = CCACACAGGCATAGAGTGTCTGC

PCR for Muc1 knock out mice

This PCR involves detection of the wild-type allele as well as the knock-out allele and hence consists of reactions that involve two sets of PCR reactions with the primers as follows:

Wild- type

Muc1 α = CCTCACACACGGAGCGCCAGC

Muc13' = TCCCCCTGGCACATACTGGG

Knock-out

Muc1 α = CCTCACACACGGAGCGCCAGC

LacZ 3' = TTCTGGTGCCGAAACCAGGC

PCR for waved-2 allele (*waved-2* mice)

Wa-2 F = CCCAGAAAGGGATATGCG

Wa-2 R = GCAACCGTAGGGCATGAG

NAME	GENETIC BACKGROUND	SOURCE
WAP-TGF α	C57/BL6	Dr. David Lee, UNC Chapel Hill
Muc1 knock out	C57/BL6	Dr. Sandra Gendler, Mayo, Scottsdale
MMTV-Wnt1	FVB	Jackson Laboratories
MMTV-MUC1	FVB	Dr. Sandra Gendler, Mayo, Scottsdale
Waved-2	FVB	Dr. Noreen Luetkeke, USF, Tampa
WILD TYPE	C57/BL6	Jackson Laboratories

Table 2.2: List of Mice used in the current study

Whole Mounts

The thoracic and inguinal mammary glands were collected and the whole mounts were prepared by stretching the glands onto a glass slide and air dried for 2-3 hours. The glands were fixed in 1:3 glacial acetic acid:100% ethanol for 1 hour at room temperature and subsequently washed in 95% ethanol for 15 minutes and in 100% ethanol for another 15 minutes. Defatting of the mammary glands was performed by using acetone and incubating for four days with changes of acetone every day. The glands were rehydrated by washing in 100% ethanol, then 95% ethanol and finally followed by 70% ethanol. The slides with whole mounts were rinsed in distilled water and stained in 0.2% Carmine/0.5% aluminum potassium sulfate solution overnight. Destaining was performed by washing the glands for 15 minutes in 70% ethanol, 95% ethanol, 100% ethanol, again in 95% ethanol and followed by 70% ethanol. Images of the whole mounts were captured using a Leica MZFCIII dissection scope (Bannockburn, IL, USA) with an Optronics MagnaFire camera (Goleta, CA, USA) at various magnifications and then the slides were stored in glycerol.

Quantification of Western Blots and Statistical Analysis

Immunoblots were scanned in as TIFF images and the density of the bands were analyzed by Scion Image (NIH, Bethesda, MD, USA). The density values for CyclinD1, EGFR, and Y992 EGFR were averaged for Muc1^{-/-} and Muc1^{+/+}. Statistical significance

($p \leq 0.05$) was determined by an analysis of variance test between Muc1^{-/-} and Muc1^{+/+} values.

Immunofluorescence

Inguinal mammary glands were fixed in methacarn for two hours, changed to 70% ethanol and sectioned by the Tissue Acquisition and Cell Molecular Analysis shared service at the Arizona Cancer Center (Tucson, AZ, USA). The slides were rehydrated and immunostained with primary antibodies at 1:100 dilutions and secondary antibody at 1:200 concentration. The primary antibodies used were the same applied in the immunoblotting protocol. The secondary antibodies used were Texas Red anti-hamster IgG (Jackson ImmunoResearch, West Grove, PA, USA) and Invitrogen's Alexa Fluor 488 anti-rabbit IgG (Eugene, OR, USA). The slides were analyzed using a Leica DMLB compound microscope (Bannockburn, IL, USA).

Quantification of Hyperplastic tissue

Mammary glands were whole mounted and hyperplastic foci were counted under a Leica dissection microscope, including only foci of ~1.0 mm in size.

Invasion Assay

A collagen gel matrix [0.9 mg/mL Type I rat tail collagen (BD Biosciences), 83.0% M-199 medium (Life Technologies), 0.18% NaHCO₃ was poured into 24-well plates. Costar Transwell inserts (Corning, Cambridge, MA, 8- μ m pore) were placed into the wells and gels were allowed to polymerize. Gels were then hydrated with DMEM (Life Technologies) supplemented with 20% fetal bovine serum (Biomedica) overnight. Cells were labeled with a 5.0 μ L/mL Vybrant DiO solution (Molecular Probes, Eugene, OR) in serum-free DMEM for 30 minutes. Cells were washed twice in serum-free medium, trypsinized, counted, and placed in the upper chamber of the transwell insert and allowed to invade for the indicated time points. After invasion, the transwell inserts were removed from the plate and the quantity of invading cells into the gel matrix was determined by reading the fluorescence of DiO in a fluorescence plate reader at 485/538 nm (Spectramax Gemini, Molecular Devices, Sunnyvale, CA).

III. MUC1 IS A NOVEL REGULATOR OF ERBB1 SIGNALING AND TRAFFICKING IN BREAST CANCER

Note: The experiments described in this chapter (with the exceptions of Figure 3.2 and Figure 3.4) have been published in [62]. In addition, note that the experiments depicted in the figures 3.5-3.7 and 3.9 have been performed by another graduate student Rachid El Bejjani.

Introduction

The erbB receptor family of tyrosine kinases are frequently deregulated in cancer, and commonly amplified and/or overexpressed in invasive carcinoma (reviewed in [63]). Ligand-induced receptor homo- or hetero-dimerization results in the activation of tyrosine kinase domain and transphosphorylation of tyrosine residues in the cytoplasmic domain. This leads to the recruitment of a variety of effector proteins and activation of further signal transduction pathways that ultimately resulting in proliferation, inhibition of apoptosis, cell migration etc. [64-67]. It is important to note that these signaling pathways occur both in normal as well as transformed cells. The key difference is that there are various proteins that contribute to deregulation of these signaling pathways and hence occur in an abnormal/uncontrollable manner.

In addition to activation of signaling cascades at the cell surface, erbB1 also maintains signaling complexes during endocytosis [68]. Upon ligand binding, erbB1 becomes

bound by the ubiquitin ligase, *cbl*, and is recruited into clathrin-coated vesicles and internalized [68, 69]. These early endosomes traffic through the cell, eventually maturing into late endosomes and finally delivering their cargo to the lysosome, where the activated receptor is degraded. ErbB1 continues to activate signaling pathways during endosomal trafficking, including Ras and Akt [70, 71]. Receptors not targeted for lysosomal degradation are sent to the recycling endosome after releasing their ligand, where they return to the cell surface. It is important to note that degradation of activated receptor is critical to normal regulation of erbB1, and the loss of erbB1 degradation machinery results in transformation [72, 73].

The function of erbB receptors can be modulated by the coexpression of non-erbB transmembrane proteins. One protein shown to modulate the function of erbB1/EGFR is the proto-oncogene MUC1. It has been established in both human breast cancer cell lines and transgenic mice overexpressing MUC1 (MMTV-MUC1) that MUC1 and the erbB family of receptor tyrosine kinases biochemically interact [60, 61]. Importantly, experiments in the MMTV-MUC1 transgenic model have shown that this interaction results in the potentiation of EGF-dependent signaling pathways. Examination of the Ras/MAP Kinase pathway in these transgenic mice demonstrated that overexpression of MUC1 vastly increases EGF-dependent p42/44 ERK activation during lactation [60].

The objective of the study described in this chapter is to analyze the effects of MUC1 expression on erbB1 stability and signaling and decipher a mechanism by which MUC1 potentiates erbB1 signaling in breast cancer.

Results

MUC1 expression prolongs EGF-dependent erbB1 phosphorylation

Regulation of erbB1 receptor signaling and its role in transformation are dependent upon the expression levels and duration of activation. Therefore we sought to determine if MUC1 plays a role in either or both of these events by altering the levels of MUC1 expression in breast epithelial cell lines. Three cell lines were chosen for our study, two breast cancer cell lines, BT20 and MDA-MB-231, and one immortalized breast epithelial line, MCF10A. Both BT20 and MCF10A cells express significant levels of MUC1 and erbB1 and were used to analyze the effects of MUC1 knockdown on erbB1 expression in transformed and immortalized conditions, respectively. Parental MDA-MB-231 cells express low levels of MUC1, but similar levels of erbB1 to BT20 cells, and were used to determine the effects of MUC1 overexpression on erbB1 expression and function.

Using RNAi (siRNA), we transiently reduced the expression of MUC1 in BT20 cells (Figure 3.1A, panel 3). We verified the specificity of our siRNA by demonstrating that the expression of an unrelated protein, β -actin, was unaffected (Figure 3.1A, panel 4).

Furthermore, we verified that these effects were specific to the MUC1 siRNA by using an unrelated control siRNA that had no effect on MUC1 protein expression (Figure 3.1A, panel 3). The control siRNA sequence was chosen based on its known non-homology to any mammalian genes, and did not result in the alteration in the expression of MUC1, erbB1, or β -actin (see figures 3.1 -4). Finally, we also used 4 additional MUC1 siRNA oligos that gave similar results to those described here to verify specificity (data not shown).

Ligand-bound erbB1 typically dimerizes, becomes internalized into early, then late endosomes, and is either degraded or recycled to the cell surface [68, 69, 74, 75]. To induce ligand-dependent degradation of erbB receptors, cells transfected with siRNA to MUC1 (BT20-M) or a non-silencing control (BT20-C) were treated with EGF, and endocytosis was allowed to proceed. At times corresponding to receptor activation and internalization (5') and receptor trafficking (15') and degradation (30' or greater), cells were lysed and levels of phosphorylated receptor were analyzed. Cells that were grown in the presence or absence of serum (but not treated with EGF) were used as controls to distinguish the effects of the growth factors present in serum.

We found that while the total levels of tyrosine-phosphorylated receptor (Figure 3.1A, top panel) are unchanged in BT20-M at early time points (5' and 15'), the same cells showed a significant decrease in phosphorylated erbB receptor at 30'. We continued to monitor the effect of MUC1 expression on erbB stability for 60' and 120' and found that

the presence of MUC1 prevented the degradation of phosphorylated erbB receptor for these extended time points. Additionally, we observed decreased levels of total erbB receptor in the same treatment groups indicating total protein loss occurred, instead of loss of receptor phosphorylation (Figure 3.1A, panel 2).

To determine if this effect was dependent upon active erbB1 kinase, BT20 cells (not treated with RNAi) were treated with the erbB1 kinase inhibitor AG1478 (10 μ M) 2 hours prior to EGF treatment [76, 77]. In these cells, the detection of phosphorylated erbB receptor was ablated by treatment with the kinase inhibitor (Figure 3.1B). This demonstrated that the observed phosphorylation was dependent upon the kinase activity of erbB1 receptor.

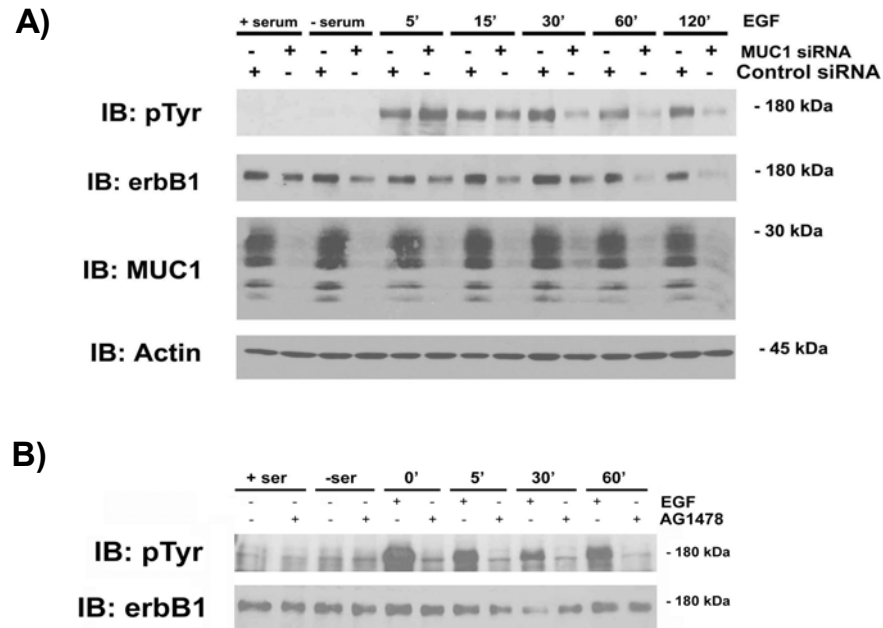


Figure 3.1: MUC1 inhibits the degradation of EGF-stimulated erbB1.

(A). BT20 cells were transfected with either MUC1 siRNA or control (nonsilencing) siRNA, and either left in normal growth conditions (+serum), serum starved overnight (-serum), or serum starved overnight and treated with 20 ng/ml EGF for the indicated times before lysis. Protein lysates (25 μ g) were separated and immunoblotted with antibodies to detect phosphotyrosine, erbB1, MUC1 or β -actin. **(B)** BT20 cells were serum-starved overnight, treated with 10 μ M AG1478 for 2 h before treatment with 20 ng/ml EGF for 10 min on ice. Cells were then washed with PBS and incubated at 37°C for the indicated times and lysed. Proteins were then immunoblotted with either anti-erbB1 or anti-phosphotyrosine antibodies. Note: Human MUC1 separates into multiple, differentially glycosylated and phosphorylated sizes (and the cytoplasmic tail that reacts to CT2 is ~14–35 kDa)

MUC1 expression dependent prolongation of phosphorylation can be detected in various tyrosine residues in the cytoplasmic domain of erbB1

Ligand binding to erbB1 induces the formation of homo or heterodimers, followed by the activation of kinase domain and subsequently results in the autotransphosphorylation of specific tyrosine residues within the cytoplasmic tail. These phosphorylated tyrosine residues then serve as docking sites for many downstream molecules that ultimately trigger diverse signaling cascades. The final biological response depends on the specific signaling pathways that are activated. Hence, we investigated if the expression of MUC1 altered the phosphorylation status of all the tyrosine residues in the cytoplasmic tail of *erbB1* in general, or if it is unique to certain tyrosine residues. We performed the endocytosis assay in the BT-20 breast cancer cells and immunoblotted for various phosphor-erbB1 antibodies at different time points as indicated in Figure 3.2. The tyrosines tested include Y992 (an autophosphorylation site that activated PLC γ), Y1068 (a docking site for the adapter molecule Grb2) and Y1045 (the phosphorylated site which leads to binding of the E3 ubiquitin ligase, *cbl*). The results demonstrate that in the BT-20 breast cancer cell line, Y992 is constitutively phosphorylated, while the phosphorylation of Y1045 and Y1068 is induced upon EGF treatment. In addition, the phosphorylation levels of Y1068 and Y1045 are similar at the earlier time points (5') after EGF treatment both in the presence and absence of MUC1, while at a later time point (30' and 60'), the expression of MUC1 correlates with prolonged phosphorylation.

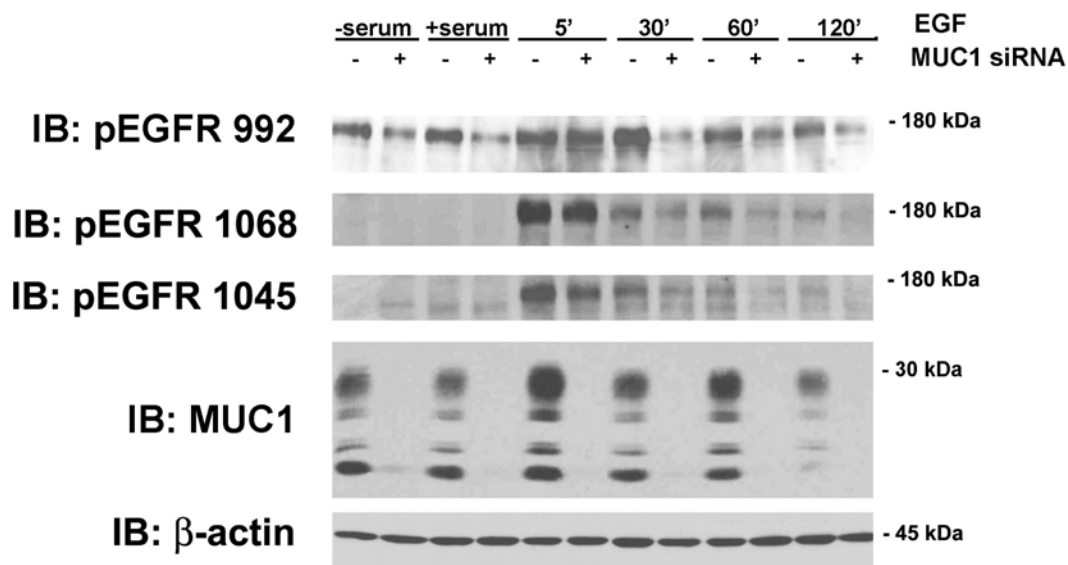


Figure 3.2: MUC1 expression prolongs erbB1 phosphorylation on various tyrosine residues. BT20 cells were transfected with either MUC1 siRNA or control (nonsilencing) siRNA, and either left in normal growth conditions (+serum), serum starved overnight (-serum), or serum starved overnight and treated with 20 ng/ml EGF for the indicated times before lysis. Protein lysates (25 μ g) were separated and immunoblotted with antibodies to detect the erbB1 phospho-specific tyrosines as indicated on the left.

Stable alteration of MUC1 expression affects erbb1 degradation

As cells transfected with MUC1 siRNA showed a loss of erbb1 over time, we next determined the long-term effect of changes in MUC1 expression through the transfection and stable selection of either a RNAi hairpin loop vector (pSuper or pSuper-MUC1) or a CMV-MUC1 overexpression construct. To perform these experiments, we utilized either the MCF10A cell line (to knockdown MUC1 expression) or the MDA-MB-231 cell line (to overexpress MUC1). The MDA-MB-231 cells endogenously express very low levels of MUC1 compared to MCF10A cells, but similar levels of erbb1 (Figure 3.3 B).

We first examined if overexpression of MUC1 would stabilize erbb1 expression upon EGF treatment (have the opposite effect to MUC1 knockdown observed in Figure 3.1A). We stably transfected the MDA-MB-231 cell line with either a CMV-MUC1 (CM) or CMV (C) expression construct [78], which resulted in increased expression of MUC1 (Figure 3.3A, panel 3). We found that overexpression of MUC1 in these cells stabilized phospho-erbb1 expression in response to EGF treatment as early as 5' after ligand treatment, when phospho-erbb1 begins to be lost in these cells (Figure 3.3A, panel 1). This inhibition of degradation continued on and became more pronounced at 30'. Furthermore, after EGF treatment we observed a decrease in the degradation of total erbb1 levels in the presence of MUC1, similar to our observations with the BT20 RNAi experiment (compare Figure 3.3A, panel 2 to Figure 3.1A, panel 2). Note that in the

absence of serum total levels of erbB1 were unaffected by MUC1 overexpression (Figure 3.3A).

Finally, we found that stable knockdown of MUC1 expression altered the EGF-induced degradation of phosphorylated erbB1 in MCF10A breast epithelial cells. We used the commercially-available pSuper vectors to express either MUC1 or control sequences that form hairpin loops upon transcription [79]. These hairpins are then processed by the cells endogenous machinery to create dsRNA, inducing RNAi-mediated degradation of target sequences. MCF10A cells were transfected with either the pSuper-MUC1 (pSM) or pSuper-Control (pS) vector and selected with neomycin. Stable selection resulted in a significant loss of MUC1 expression in the pSM, while not affecting MUC1 expression in the pS cells (Figure 3.3C, panel 3). This loss of MUC1 correlates with a significant loss of erbB phosphorylation in response to EGF treatment at the 5' time point, although total erbB1 expression is minimally affected at this time. After 45' of treatment with EGF, total erbB1 expression is lost in the absence of MUC1 expression, while erbB1 is still detected in MUC1 expressing cells.

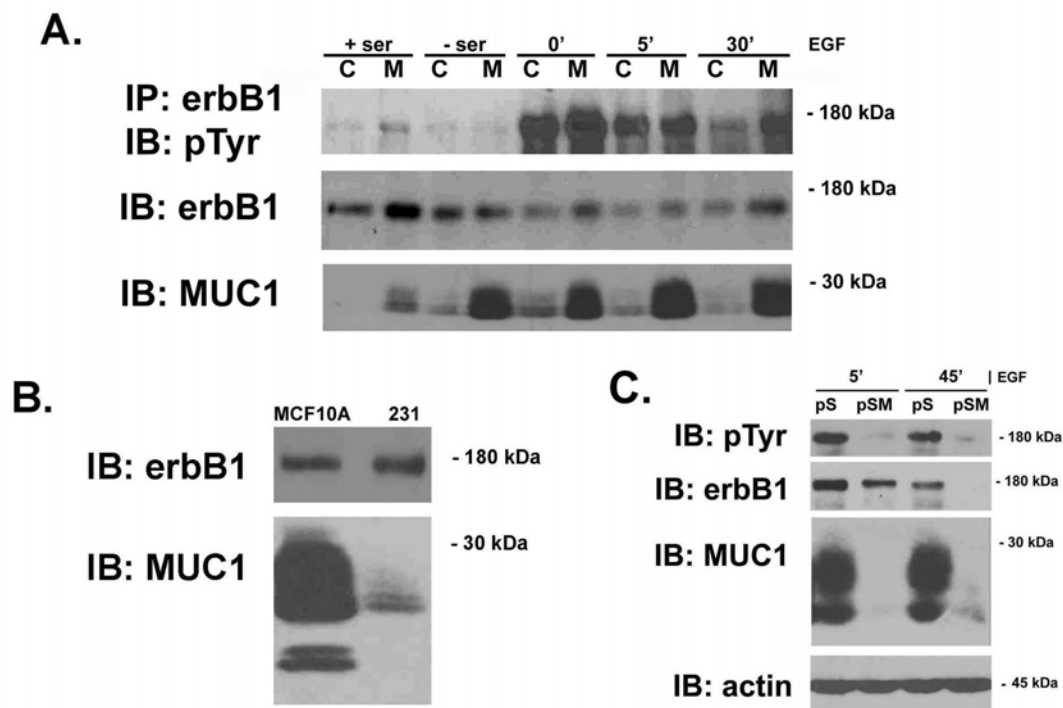


Figure 3.3: Stable alteration of MUC1 expression affects erbB1 degradation. (A) MDA-MB-231 cells were transfected with either CMV-MUC1 (CM) or parental CMV (C) driven vectors, then stably selected with neomycin. Cell lines were serum starved (-ser) overnight, then treated with 20 ng/ml EGF for 10 min at 4°C and washed with PBS to remove unbound ligand. Cells were then incubated for the indicated times at 37°C and lysed. Protein lysates were immunoprecipitated (IP) and separated by SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine, anti-erbB1 or anti-MUC1 antibodies. (B) Protein lysates from parental MCF10A and MDA-MB-231 were made, and 40 μ g were separated by SDS-PAGE. Relative levels of erbB1 and MUC1 expression in each cell line were determined by immunoblotting with anti-erbB1 and anti-MUC1 antibodies, respectively. (C) MCF10A cells were transfected with either pSuper-Control (pS) or pSuper-MUC1 (pSM) RNAi constructs, then stably selected with neomycin. Cells were then treated and immunoblotted as described above in (A)

MUC1 enhances EGF-dependent Akt activation in an endocytosis-dependent manner

We next investigated the significance of MUC1 expression in EGF-dependent signaling cascades. It has been demonstrated that MUC1 overexpression results in an inhibition of apoptosis in transgenic animals and it is well known that activation of the Akt pathway results in the inhibition of apoptosis [80] [49]. Furthermore, recent evidence indicates that Akt is preferentially activated by internalized erbB1 receptor, as opposed to erbB1 found on the cell surface [71].

To determine how MUC1 affects EGFR signaling pathways, we knocked down the expression of MUC1 in BT20 cells using MUC1 siRNA or control siRNA (Figure 3.4A). We found that the reduction of MUC1 expression resulted in reduced phospho-Akt in cells after EGF treatment (Figure 3.4A). We also analyzed the ability of MUC1 to affect p42/44 ERK activation. Although EGF does induce ERK activation in both cell lines, MUC1 expression did not alter its activation (Figure 3.4A). This indicates that the ability of MUC1 to alter erbB signaling is pathway-specific, and may indicate distinct internal vesicle sorting.

We also examined activation of Akt in the immortalized MCF10A cell line and found similar results, with MUC1 expression altering the extent of Akt activation upon EGF stimulation (Figure 3.4B). Additionally, we found that, as with BT20 cells, MUC1

specifically altered the Akt pathway but not EGF-dependent activation of the p42/p44 (ERK) MAPKinase pathway (Figure 3.4B). These results indicate that MUC1 expression affects erbB1 receptor signaling in breast epithelial cells, regardless of the state of transformation. Also, MUC1 affects discrete erbB1 signal transduction pathways and is not a global modulator of erbB1 signaling.

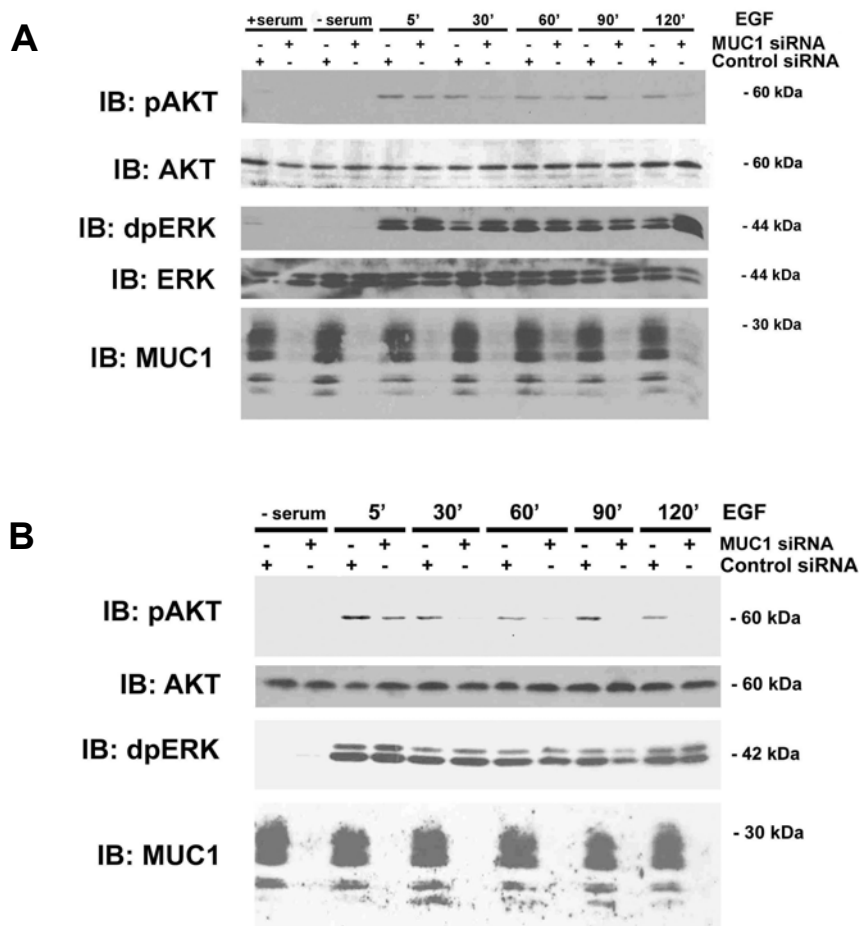


Figure 3.4: MUC1 expression prolongs endocytosis dependent Akt, but not dual phosphorylated ERK1,2 (dpERK) activation. A) BT-20 breast cancer cell line and B) MCF10A immortalized mammary epithelial cell line were subjected to the endocytosis assay for the time points indicated. The protein lysates were probed with antibodies to phospho-AKT, dpERK, total AKT and ERK1,2.

MUC1 expression affects levels of plasma membrane localized erbB1

To determine if the pool of erbB1 that is affected by MUC1 was surface or cytoplasmically derived, we biotinylated cell surface proteins (with cell-impermeable biotin) and performed endocytosis assays to determine the fate of surface-associated erbB1. BT20 cells (either control or treated with MUC1 siRNA) were treated with biotin to label all surface proteins, the biotin reaction was quenched, cells were treated with EGF to induce endocytosis, and biotinylated protein was precipitated with streptavidin beads (Figure 3.5A, top panel). We found that a reduction of MUC1 expression by RNAi resulted in reduced detection of surface biotinylated erbB1 at the 30' and 60' timepoints. Alternatively, surface biotinylated erbB1 continued to be detected at these timepoints in the presence of MUC1 expression.

Densitometry analysis of 3 separate experiments demonstrated an average of ~50% reduction in erbB1 receptor expression in cells treated with MUC1 RNAi compared to controls after 30', which increased to ~80% after 60' (Figure 3.5B). Importantly, these data demonstrate that the pool of erbB1 receptors that is affected by MUC1 expression resides at the membrane. Additionally, as we detected total surface erbB1 and its degradation, these data clarify that we detected the degradation of erbB1 protein, and not merely a loss of erbB1 phosphorylation.

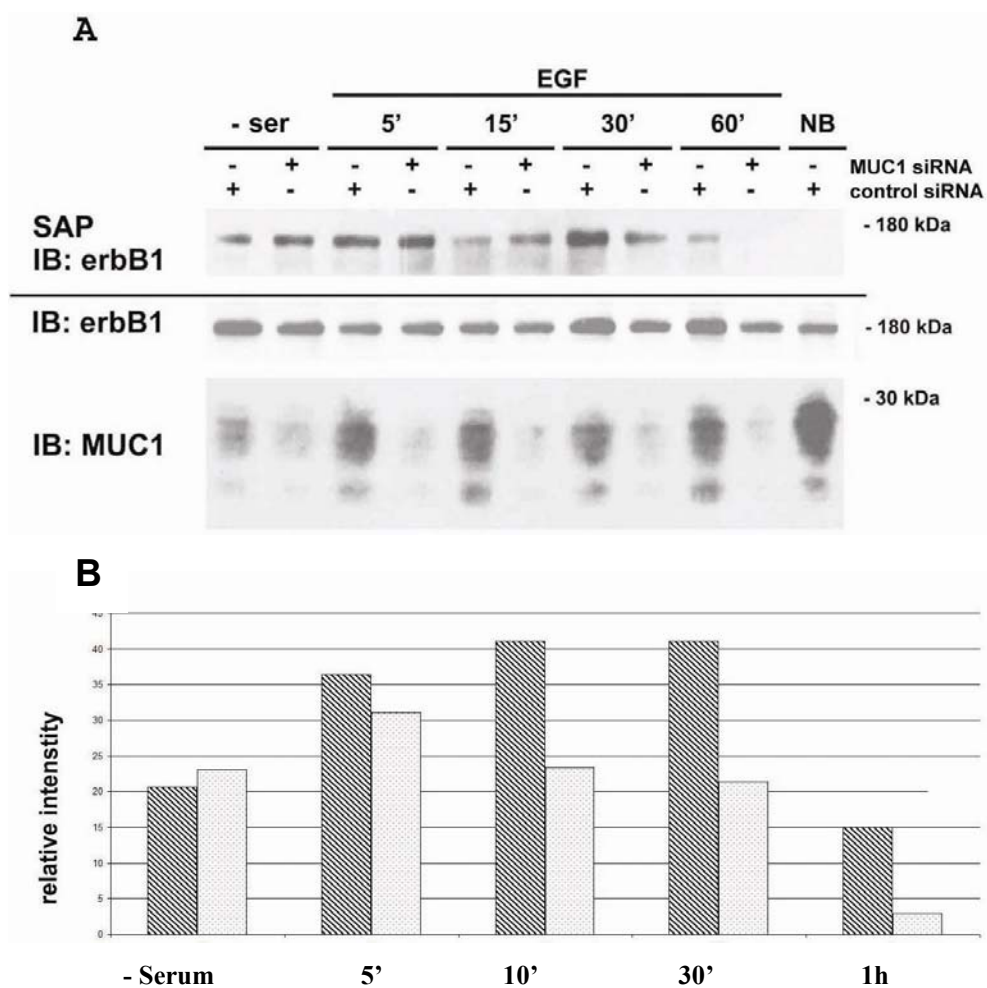


Figure 3.5*: MUC1 expression alters cell-surface erbB1. (A) BT20 cells were transfected with siRNA. After knock down, cells were biotinylated at 4°C to label cell surface receptors, and the reaction was quenched with a Tris buffer. After washing, cells were treated with EGF for the indicated time points, lysed and protein was precipitated using streptavidin beads (SAP). Proteins were then immunoblotted with anti-erbB1. Total cell lysates were also separated to demonstrate total cellular levels of protein and immunoblotted with anti-erbB1 or anti-MUC1, bottom two panels. NB=Non-biotinylated control. (B) Quantification of the differences observed in a. Densitometry from three separate experiments was obtained using the Scion Image software, corrected against background and averaged. Striped bars represent control-RNAi treated cells and dotted bars represent MUC1-RNAi-treated cells. (*This figure was contributed by Rachid El Bejjani)

We next determined where MUC1 and erbB1 localized in the cell before and after treatment with EGF. To do this, we examined localization of MUC1 and erbB1 in serum starved or EGF treated BT20 cells (without siRNA treatment). As expected, MUC1 and erbB1 colocalized at the cell surface prior to treatment with ligand (Figure 3.6 left panel, arrow). MUC1 and erbB1 colocalized in internal compartments upon treatment with EGF for 30', accompanied by an accumulation at distinct intracellular vesicles (Figure 3.6, right panel, arrow). These results demonstrate that while MUC1 interacts with erbB1 at the cell surface, the two proteins can be observed in cytoplasmic compartments upon ligand treatment.

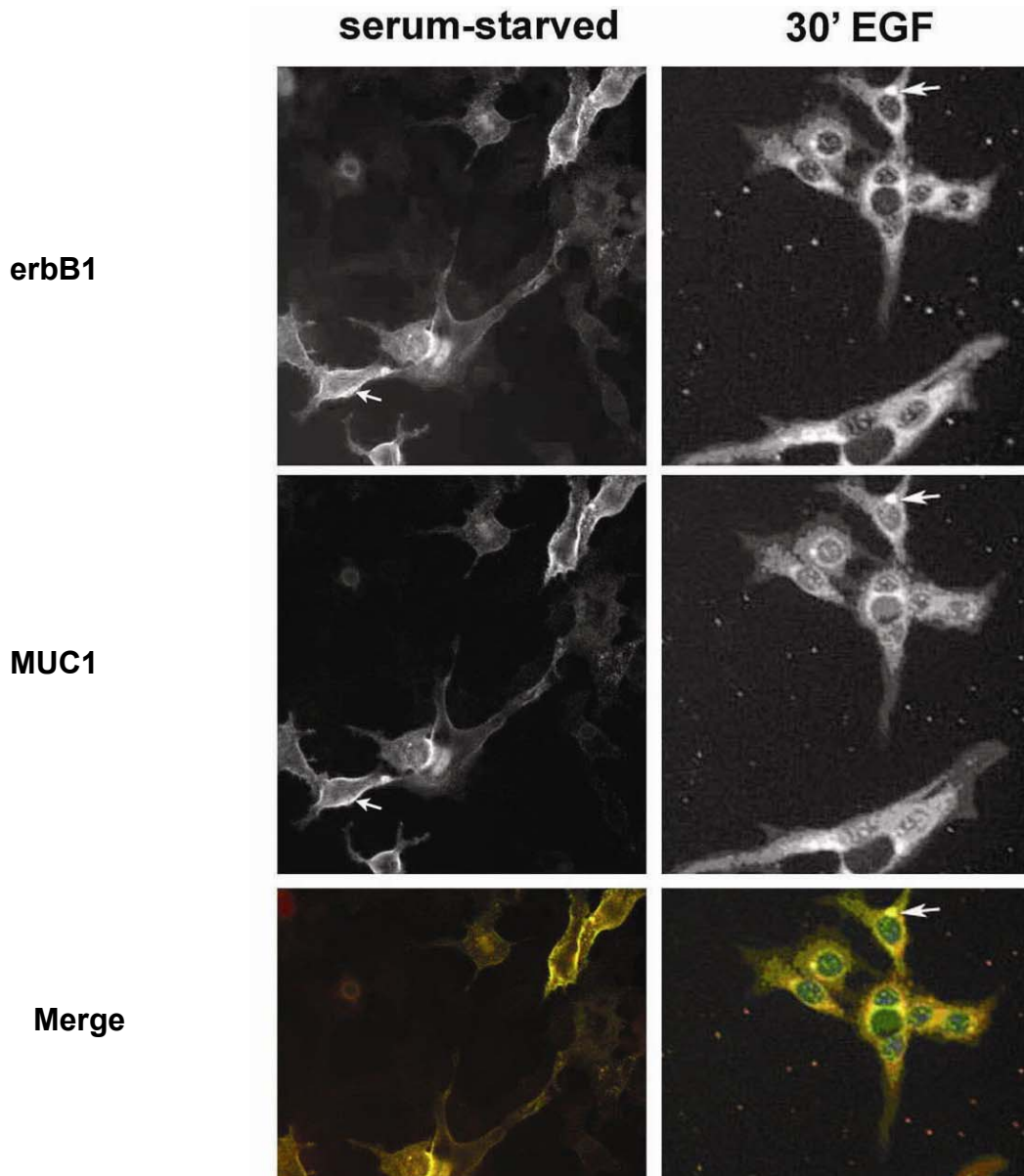


Figure 3.6*: Analysis of erbB1 localization in the presence of MUC1. Left panel: To localize surface erbB1, BT20 cells were serum-starved overnight, labeled with anti-erbB1 (Ab-21) and anti-MUC1 (DF3) Right panel: to analyze internal colocalization during EGF treatment, BT20 cells were serum-starved overnight, then incubated with 20 ng/ml EGF for 5 min, washed and incubated at 37°C for 30 min. Cells were labeled with anti-erbB1 (Ab-1) and anti-MUC1 (CT2). [MUC1=green, erbB1=red] Magnification is 400X. Arrows indicate areas of colocalization. (*This figure was contributed by Rachid El Bejjani)

MUC1 promotes internalization of erbB1 in response to EGF

Since MUC1 expression inhibits the degradation of plasma membrane-localized erbB1, we next examined if MUC1 expression alters erbB1 retention at the membrane. Stably transfected MDA-MB-231 C and CM cells (described in Figure 3.3) were serum-starved, then treated with EGF to induce internalization (Figure 3.7A). 5, 30, 60 and 120' after internalization, cell surface proteins were biotinylated, and precipitated with streptavidin. It is important to emphasize that in this experiment, we biotinylated surface proteins after EGF treatment and internalization, a method that would indicate levels of receptor remaining on the surface after ligand-induced internalization.

Therefore only those proteins that remained on the cell surface after ligand treatment and internalization (or had returned to the surface through recycling) would be detected. We observed significantly less erbB1 on the cell surface in the presence of MUC1 (after ligand treatment and endocytosis proceeded; Figure 3.7A), indicating that MUC1 promotes the loss of erbB1 from the cell surface. Increased internalization of erbB1 was strongly enhanced by MUC1 expression at 5' and 30', with total loss of surface erbB1 observed at 60' regardless of MUC1 expression. Densitometry analysis of 3 separate experiments showed a decrease of surface erbB1 in the presence of MUC1 of ~40% after 5', with total levels of surface erbB1 returning to approximately equal after 2 hours [rel. intensity = 16 (C) vs. 10 (CM) at 5'; and 16 (C) vs. 14 (CM) at 2 hours]. Note that we are comparing relative levels of C to CM at these timepoints.

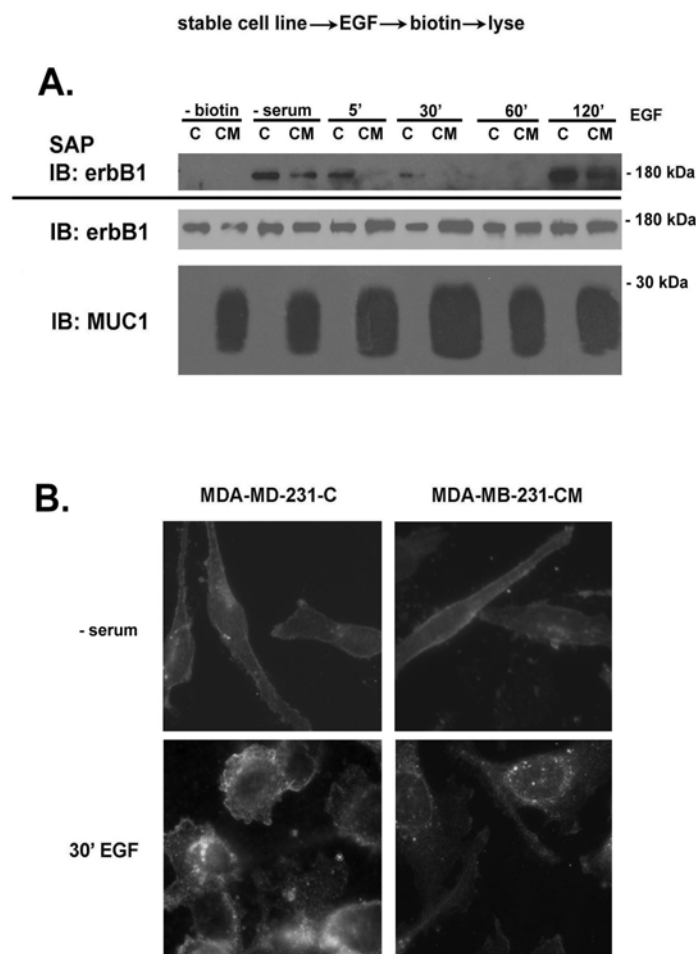


Figure 3.7*: MUC1 expression promotes EGF-dependent internalization. (A) To assess receptor internalization, MDA-MB-231 breast cancer cells stably transfected with MUC1 (CM) or with vector alone (C) were treated with 100 ng/ml EGF at 4°C (to saturate erbB1 receptors) and incubated for the indicated time point before being surface biotinylated and then extracted. Biotinylated proteins were then precipitated with streptavidin (SAP) and immunoblotted with anti-erbB1 (1005). A separate set of protein lysates were treated and prepared as described and analyzed for MUC1 expression using anti-MUC1 (CT2), bottom panel, under separation line. (B) Immunofluorescence of erbB1 in MDA-MB-231 cells expressing either the CMV vector (C) or CMV-MUC1 (CM), serum starved (top panels) or serum starved and treated with 10 ng/ml EGF on ice, washed, then incubated for 30 min at 37°C (bottom panels). Cells were then fixed with methanol/acetone and stained with antibodies against erbB1 (Ab-1), magnification= 630x. (*This figure was contributed by Rachid El Bejjani)

Analysis of erbB1 localization in stably transfected MDA-MB-231 cells (both CM and C) visually recapitulated this observation. In the absence of serum, erbB1 is found at the plasma membrane regardless of MUC1 expression (Figure 3.7B, top panels). In addition, the MUC1 overexpressing cells (CM) display significant internalized erbB1, with very little surface erbB1 visible upon 30' of EGF treatment (Figure 4B, right panel). Alternatively, while the control (C) cells demonstrated internalized erbB1 in response to EGF treatment, there were still significant levels of erbB1 observed on the surface (Figure 3.7B, left panel). These data again indicate that in those cells expressing high levels of MUC1, erbB1 internalization was enhanced. Together, these results demonstrate that, although MUC1 inhibits the degradation of ligand-activated erbB1, it also promotes the internalization of the receptor.

MUC1 expression inhibits the ubiquitination of erbB1

To determine the mechanism of MUC1-mediated inhibition of erbB1 degradation, we examined the ubiquitination of erbB1 in response to EGF treatment. Ligand-bound erbB1 is normally ubiquitinated (ub-erbB1) upon internalization and vesicular trafficking then proceeds [reviewed by [68]]. We treated either MDA-MB-231C or CM cells with serum alone or EGF for 2' at 37°C to induce internalization and ubiquitination. While ub-erbB1 was observed with this treatment in control cells (C), ub-erbB1 was markedly reduced in CM cells (Figure 3.8A). Also, while ub-erbB1 was not observed in the presence of serum at the exposure times required to detect EGF-induced ub-erbB1, we did observe ub-erbB1 in the presence of serum at longer exposure times (Figure 3.8B). This ub-erbB1 was also reduced in the presence of MUC1 expression, indicating that the growth factors present in serum can also induce (albeit much less) ubiquitination of erbB1. These data demonstrate that MUC1 expression alters the ubiquitination of erbB1, indicating a mechanism for the decrease in erbB1 degradation.

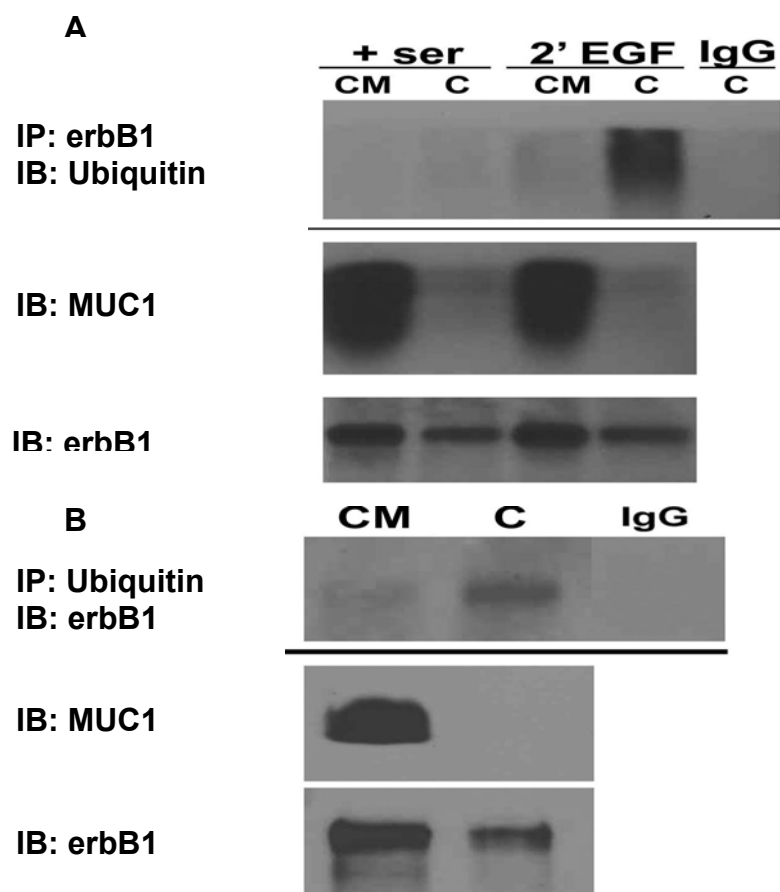


Figure 3.8: MUC1 expression inhibits the ubiquitination of erbB1. (A) MDA-MB-231 cells overexpressing CMV-MUC1 (CM) or the CMV vector (C) were serum starved overnight and treated with 20 ng/ml EGF at 37°C for 2 min or grown in serum and lysed (+ser). Protein lysates (500 µg) were immunoprecipitated with anti-erbB1 antibody (Ab-1) or control mouse IgG and immunoblotted with an anti-ubiquitin antibody. Protein lysates (50 µg) from these samples were also immunoblotted with anti-MUC1 or anti-erbB1 (1005) to show total levels of protein (bottom two panels). (B) MDA-MB-231 cells expressing CM or C were grown in serum and lysed. Protein lysates (300 µg) were immunoprecipitated with anti-ubiquitin antibody (NCL-ubiq) or control rabbit IgG and immunoblotted with anti-erbB1 antibody (1005). Protein lysates (50 µg) from these samples were also immunoblotted with anti-MUC1 or anti-erbB1 (Santa Cruz, 1005) to show total levels of protein (bottom two panels). Note that detection of ubiquitinated erbB1 in (A) was obtained with an exposure time of 30 s (using DuraSignal, Pierce Chemical Co.), while the detection of ubiquitinated erbB1 in (B) was obtained with an overnight exposure (using DuraSignal, Pierce Chemical Co.)

MUC1 expression increases erbB1 recycling

To determine if the decrease in erbB1 ubiquitination was due to altered erbB1 trafficking, we examined erbB1 recycling to the plasma membrane. MDA-MB-231C and CM cells were pulsed with EGF for 10' on ice and unbound EGF was then washed off. Treated cells were then chased by incubating at 37°C for 10, 60 and 90' before being harvested and incubated with an erbB1-PE antibody [81-83].

We detected an ~80% increase in the amount of recycled erbB1 in CM cells compared to C cells 60' after EGF stimulation, and an approximate 40% increase in recycling 90' after stimulation (Figure 3.9). Recycling is shown as the % difference between the average corrected geometric mean at each experimental time point (TP) minus the starting (10') timepoint $[(TP-T10')/T10' * 100]$, and represents the amount of erbB1 that is recycled to the cell surface after each time analyzed. These results indicate that MUC1 overexpression promotes the recycling of erbB1 to the cell surface, providing a mechanism by which MUC1 inhibits erbB1 degradation and promotes erbB1 signaling.

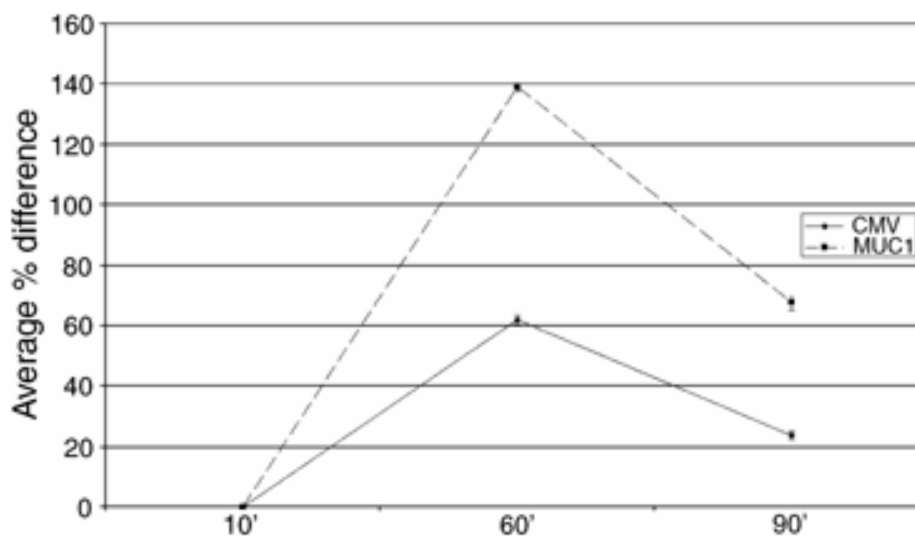


Figure 3.9*: MUC1 overexpression promotes the recycling of erbB1. MDA-MB-231 cells expressing CMV-MUC1 (MUC1/dashed line/squares) or CMV (CMV/solid line/diamond) were pulsed with 10 ng/ml EGF for 10 min on ice and then incubated at 37°C to induce internalization, and cells were harvested at either 60 or 90 min. The mean fluorescence intensity (MFI) was measured by flow cytometry, and the MFI at 10 min after EGF stimulation was considered a reference point at which the activated receptors are internalized but not yet recycled. Recycling is represented as the percentage difference between the MFI at the indicated time point and the MFI at the 10 min reference time point. Percentage recycling = $((TP - T10 \text{ min}) / T10 \text{ min}) \times 100$. (*This figure was contributed by Rachid El Bejjani)

Discussion

We report here that MUC1 expression inhibits the degradation of ligand-activated erbB1 receptor. We show through RNAi-mediated loss of MUC1 expression that MUC1 inhibits the degradation of phosphorylated erbB1 after ligand binding in both BT20 breast cancer cells and MCF10A breast epithelial cells. Overexpression of MUC1 in MDA-MB-231 cells recapitulates this effect by stabilizing ligand-activated erbB receptor. Biotinylation of surface proteins demonstrates that MUC1 promotes the internalization of cell-surface associated erbB1 while protecting it against ligand-activated degradation. Importantly, MUC1 expression inhibits the ubiquitination of erbB1 and enhances its recycling to the plasma membrane.

MUC1 has been previously shown to both interact with erbB receptors [60, 84] and affect EGF-dependent activation of MAP Kinase pathways in lactating mammary glands [60]. The mechanism by which MUC1 accomplishes this task, though, was undetermined. ErbB receptors can be regulated both by their activation [i.e. through ligand binding or as a substrate for *Src* kinase [85]] and through downregulation of the receptor. Work involving the ubiquitin ligase cbl has shown that downregulation of erbB1 is a critical component of its normal function, and degradation of erbB1 is a key component in preventing it to act in an oncogenic manner [72, 73]. Our current data now defines MUC1 as another important regulator of erbB1 receptor degradation. By examining both overexpression constructs and RNAi knockdowns of MUC1, we have

demonstrated a functional role for MUC1 as a regulator of erbB1 signaling. We found that CM cells mimicked pS cells (both expressing high MUC1 and displaying stable erbB1 expression) and C cells, in turn, mimicked pSM cells (both expressing very low MUC1 and displaying accelerated erbB1 degradation in response to ligand). These experiments show that both overexpression of MUC1 in low-MUC1-expressing cells or loss of expression in high-MUC1-expressing cells can modulate the stability of phosphorylated erbB1 expression. While a previous study reported that MUC1 expression affects the transcription of erbB1 [86], we were unable to detect alterations of the steady state level of erbB1 levels in response to MUC1 loss or overexpression. It will be interesting in future work to evaluate the effect of MUC1 expression on the ligand-induced degradation of all members of the erbB family. Given that MUC1 and erbB receptors are commonly overexpressed in breast cancer [reviewed in [63, 87]], the interactions between these proteins may be potent inducers of carcinoma in the appropriate molecular setting.

Our data demonstrate that although MUC1 inhibits ligand-induced degradation of erbB1, it also promotes its internalization. This apparent paradox may be explained by one of two non-exclusive hypotheses. The first is that MUC1 promotes the entry of erbB1 into a recycling pathway instead of an ubiquitination pathway. Our data demonstrate that MUC1 expression does, in fact, inhibit the ubiquitination of erbB1 concurrently with an increase in the recycling of erbB1 to the plasma membrane.

Additionally, MUC1 expression could also be driving erbB1 into an alternate internal trafficking pathway, one that does not lead to protein degradation. This could potentially involve trafficking to the ER or Trans Golgi Network, both of which have been shown to alter the degradation of ligand-activated erbB1 [88, 89]. We are currently examining potential mechanisms of alternate trafficking.

Our data indicate that MUC1 alters ligand-induced internalization and degradation of erbB1. While MUC1 promotes internalization of erbB1, MUC1 also inhibits the degradation of erbB1 that would normally follow. Importantly, we have found that MUC1 is altering the normal ubiquitination of internalized erbB1 and promoting the recycling of erbB1 to the cell surface. This study implicates MUC1 as a potentially critical mediator of erbB1 stability and function, one that may have dramatic implications for erbB1-mediated breast cancer progression.

IV. EPIDERMAL GROWTH FACTOR RECEPTOR DEPENDENT MAMMARY CANCER PROGRESSION IS REGULATED BY MUC1 EXPRESSION

Introduction

The Epidermal Growth Factor Receptor (EGFR) family of tyrosine kinases are frequently deregulated in cancer, and commonly amplified and/or overexpressed in invasive carcinoma [reviewed in [63]]. In addition, the expression of the ligands that activate these receptors is altered in carcinomas as well. Accordingly, studies in the human breast cancer biopsies have shown that the ligand TGF α is over-expressed both at mRNA and protein levels in over 70% of breast cancer patients (reviewed in [90]). Most importantly, it has been reported that the co-expression of EGFR and TGF α predicts worse prognosis in the invasive breast cancer patients [91].

In order to decipher the mechanism by which TGF α overexpression results in EGFR activation induced mammary carcinoma, various transgenic mouse models have been generated, including MT-TGF α (driven by the zinc-inducible metallothionein promoter) MMTV-TGF α (Mouse Mammary Tumor Virus promoter) and WAP- TGF α (Whey Acidic Protein promoter). These models demonstrate that TGF α overexpression induces breast cancer with a variety of histotypes and onset, depending on the strength of the promoter (reviewed in [92]). Out of these models WAP- TGF α , is an excellent model of choice as the tumor progression parallels multiple events known to be involved in human

breast cancer progression. In addition, WAP-TGF α mice display increased incidence and decreased latency of tumors as compared to the MT- TGF α or MMTV- TGF α mouse models (reviewed in [92]). In the WAP-TGF α transgenic model, tumors are generally well-differentiated, continue to express both keratin 18 and whey acidic protein, and also express high levels of cyclin D1 compared to non-transformed contralateral glands. Additionally, these tumors express high levels of Wnt-1 and Wnt3a, indicating a potential role for β -catenin stabilization in tumor progression. Although activation of EGFR is a known promoter of metastatic progression, the WAP-TGF α animals show no evidence of metastasis when generated on a mixed genetic background [93, 94].

The duration and strength of signaling of EGFR and hence its role in cancer progression, is dependent on the fate of intracellular trafficking upon ligand treatment. It has been established that the ligands EGF and TGF α differ in their ability to induce the intracellular trafficking and processing of EGFR [95]. While EGF treatment, in general, results in the lysosomal degradation of both ligand and receptor, binding of TGF α to EGFR leads to recycling of EGFR to the cell surface. However, the role of MUC1 expression in relation to EGFR trafficking has not been investigated by any other labs so far. As described in the chapter five, studies in our laboratory have demonstrated for the first time that MUC1 expression enhances the stability of EGFR by inhibiting the downregulation upon EGF activation. Furthermore, we have established that MUC1 expression inhibits the ubiquitination of EGFR upon treatment with the ligand EGF [62]. These studies prompted us to investigate the effects of Muc1 expression in mammary

transformation induced by the ligand TGF α . In addition, we determined the biochemical associations of Muc1 and EGFR and possible consequences of their interactions in MMTV-Wnt1/MMTV-MUC1 bitransgenic mice.

We report here that the biochemical interactions between Muc1 and EGFR result in a dramatic alteration in tumor onset and development in the WAP-TGF α transgenic mice. Furthermore, we have identified that the expression of Muc1 correlates very strongly with the overexpression of Cyclin D1 levels and provide a plausible mechanism for the dramatic effect of Muc1 expression on mammary transformation.

Results

Muc1 and EGFR interactions are specific to hyperplasia and transformation.

We have recently discovered that MUC1 can inhibit ligand-mediated degradation of EGFR through decreased ubiquitination and increased recycling (described in chapter five and [62]). We wanted to determine if this effect on EGFR stabilization would affect EGFR-dependent transformation. To investigate EGFR-dependent transformation, we utilized the WAP-TGF α mouse model of mammary gland carcinoma, which forms mammary gland tumors with complete incidence[94].

One of the characteristic features of WAP-TGF α mice is that they develop hyperplastic lesions after 3 pregnancies and these in turn serve as precursors to the development of

mammary tumors [94]. Therefore, we continuously bred the WAP-TGF α mice to induce tumor formation and collected the normal (N) mammary glands (that have gone through less than 3 pregnancies and hence are not yet hyperplastic), the hyperplastic mammary glands (H) and the tumors (T). We examined the interactions between EGFR and Muc1 in these various tissues, both by physical interaction (immunoprecipitation) and colocalization (immunofluorescence). Figure 4.1A demonstrates that although Muc1 and EGFR are expressed in normal, hyperplastic and tumor tissues from the WAP-TGF α animals, interactions between these proteins are not observed in the normal gland. To determine a potential mechanism for this difference, we examined localization of Muc1 and EGFR. We observed a significant difference in localization of these proteins which varied by the state of transformation. In the normal gland where apical/basolateral polarization is retained, Muc1 is found only at the apical surface, not interacting with basolateral EGFR (Figure 4.1B, Normal). As the gland transitions to hyperplasia, we begin to observe interactions between Muc1 and EGFR beneath the apical domain of the ductal epithelium (Figure 4.1B Hyper, arrows). In the tumor, upon loss of polarization, Muc1 and EGFR are found colocalizing throughout the cytoplasm of the transformed cells (Figure 4.1B Tumor, arrows).

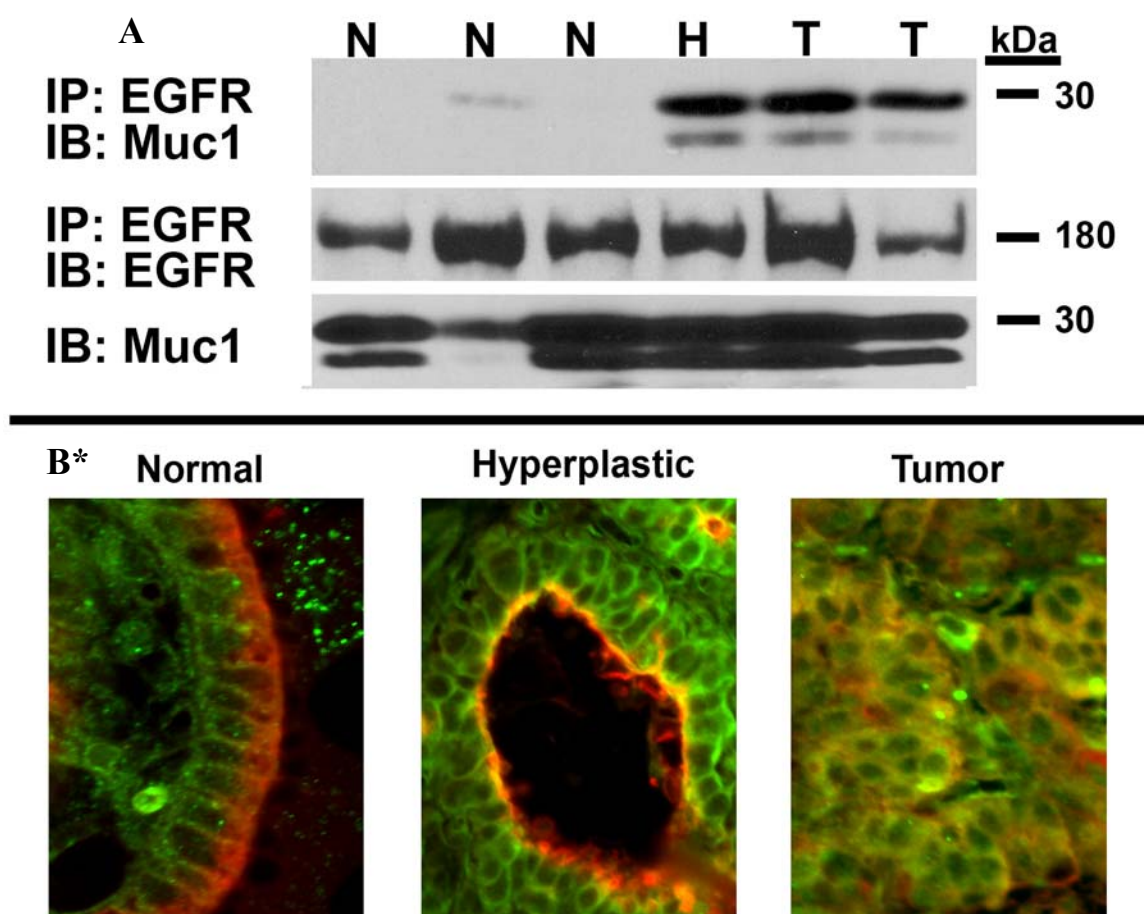


Figure 4.1: EGFR and Muc1 interactions occur in a transformed tissue dependent manner. (A) Tissue lysates from WAP-TGF α mice (normal (N), hyperplastic (H) and tumor (T)) were used to immunoprecipitate (IP) for EGFR and Muc1. These samples were then run on an SDS-PAGE gel and immunoblotted (IB) with anti-EGFR and anti-Muc1 antibodies. (B) Normal and hyperplastic mammary glands and tumors from WAP-TGF α mice were sectioned and used for immunofluorescence (green=anti-EGFR and red=anti-Muc1). (*The figure 4.1B was contributed by Benjamin Bitler)

Muc1 expression dramatically alters tumor incidence and latency in WAP- TGF α transgenic mice

To determine the effect of Muc1 expression and interaction on EGFR-dependent transformation, we generated WAP-TGF α transgenic mice on Muc1^{+/+}, Muc1^{+/-} and Muc1^{-/-} backgrounds and analyzed tumor progression. Previous studies by other groups using the WAP-TGF α model have been performed on both a mixed (C57Bl/6 X SJL) or inbred (FVB) background, but to perform our studies we crossed our animals to an inbred C57Bl/6 background (N>5). This allowed us to cross the WAP-TGF α to the Muc1^{-/-} (C57Bl/6) without introducing any modifier genes.

Animals were bred continuously to induce activation of the WAP promoter, and mammary glands were palpated once per week (every week after their third pregnancy) for approximately one year to evaluate tumor progression. Tumors were considered established upon palpation of a 0.5cm solid mass that remained after subsequent palpations. As previously reported, we observed 100% incidence of tumor formation in WAP-TGF α mice on Muc1^{+/+} background (29/29 animals developed tumors), with a mean onset of 6-9 months (Figure 4.2). This rate of tumor progression was dramatically impacted by the loss of Muc1 expression. The loss of Muc1 resulted in greater than 50% of WAP-TGF α /Muc1^{-/-} mice not developing any mammary tumors in the time span evaluated (11/30 animals developed tumors (Figure 4.2). Not only did the ablation of Muc1 expression affect tumor formation, but the age of onset of tumor formation (in

those animals that developed mammary tumors) was significantly delayed as well (p value = 0.0004). Evaluation of tumor histotype revealed no significant alteration in the presence or absence of Muc1 expression, with the majority of tumors being either fibroadenomas, myoepitheliomas and keratoacanthomas (Robert Cardiff, UC Davis, personal communication). While the WAP-TGF α /Muc1^{+/-} mice developed mammary tumors slightly later as compared to WAP-TGF α /Muc1^{+/+}, the latency period was not statistically significant (Figure 4.2). Evaluation of Muc1 expression from these WAP-TGF α /Muc1^{+/-} tumors revealed that there was not a significant difference in the expression levels of Muc1 protein.

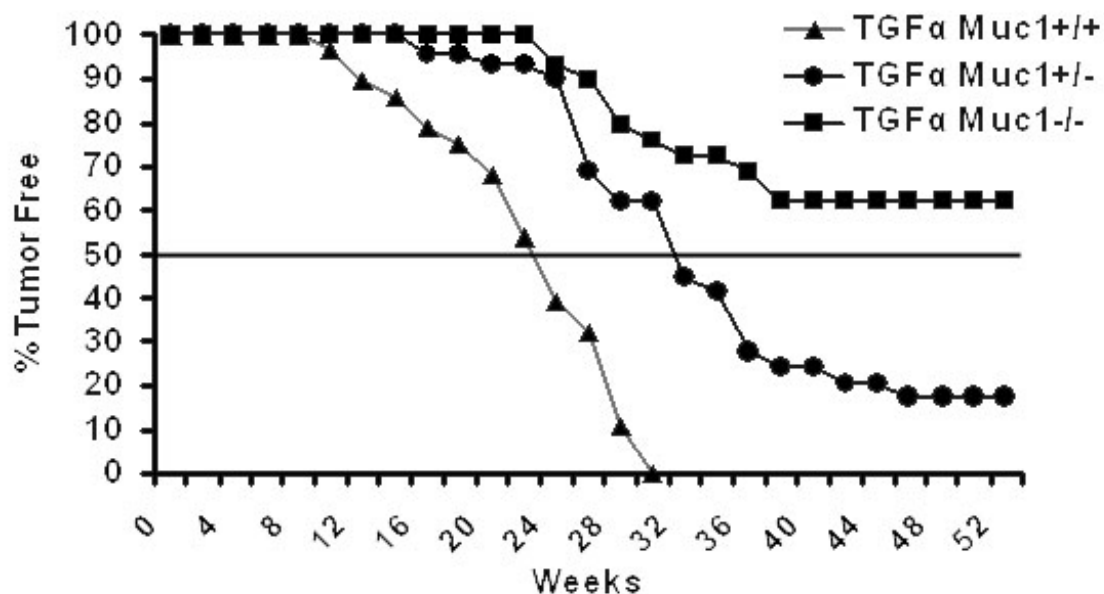


Figure 4.2: Loss of Muc1 expression increases the latency of mammary tumorigenesis in WAP-TGF α transgenic mice. WAP-TGF α transgenic animals (Muc1 $^{+/+}$ (\blacktriangle), Muc1 $^{+/-}$ (\bullet) or Muc1 $^{-/-}$ (\blacksquare) background) were continuously bred to activate transgene expression and palpated weekly to detect tumor formation. There is a significant difference in time to tumor onset between the Muc1 $^{+/+}$ and Muc1 $^{-/-}$ mice ($p=0.0004$). Kaplan-Meier curve denotes time from 1st parturition to time of palpable 0.5 cm diameter tumor (non-regressing). Dotted line indicates when 50% of animals were tumor free. WAP-TGF α /Muc1 $^{+/+}$, $n=28$, WAP-TGF α /Muc1 $^{+/-}$, $n=29$, and WAP-TGF α /Muc1 $^{-/-}$, $n=29$.

Overexpression of Cyclin D1 in WAP-TGF α correlates with expression of Muc1

Previous studies have demonstrated that cyclin D1 is upregulated both at mRNA and protein levels in WAP- TGF α transgenic mice [94]. In addition, it has been shown that transactivation of EGFR by Wnt1 and Wnt5a growth factors in the mammary epithelial cell lines results in the increased levels of cyclin D1 [96]. Therefore, we investigated the expression levels of cyclin D1 in hyperplastic glands and tumors derived from WAP-TGF α transgenic mice on Muc1^{-/-} and Muc1^{+/+} backgrounds. We found that cyclin D1 protein levels were not increased in hyperplasias respective to normal glands (data not shown) regardless of Muc1 background (Muc1^{-/-} versus Muc1^{+/+}). However, while cyclin D1 expression was significantly increased in tumors derived from WAP-TGF α /Muc1^{+/+} glands, there was little increase in cyclin D1 expression in WAP-TGF α /Muc1^{-/-} glands (Figure 4.3A, panel one). To determine potential mechanisms of loss of cyclin D1 expression in WAP-TGF α /Muc1^{-/-} versus WAP-TGF α /Muc1^{+/+} we evaluated the expression and activation of EGFR in the two backgrounds. We found that total levels of EGFR were relatively similar in both backgrounds, as shown by densitometry analysis (Figure 4.3B and 4.3D, respectively). Additionally, we evaluated the activation status of EGFR by analysis of phosphorylation levels of the Y992 residue. The densitometry analysis results further revealed that the activation of the receptor, although slightly diminished in the WAP-TGF α /Muc1^{-/-} tumors, was not statistically different (Figure 4.3B and 4.3 D).

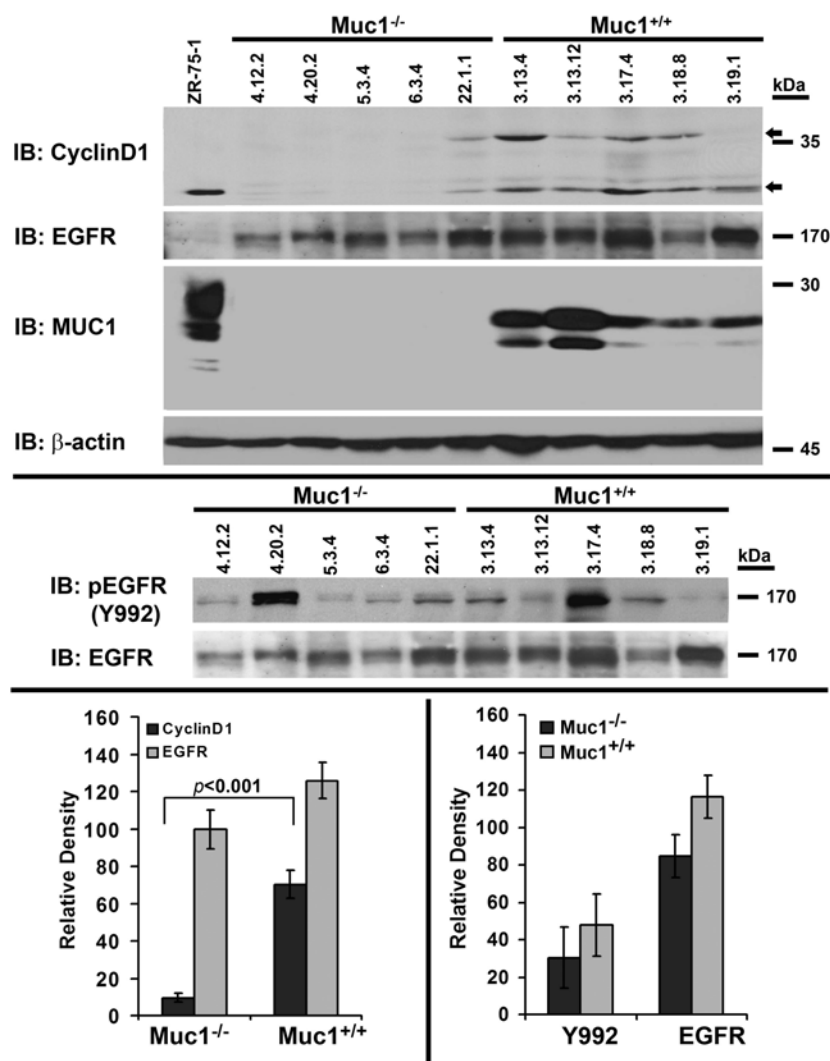


Figure 4.3. CyclinD1 over-expression in WAP-TGF α transgenic mice correlates with Muc1 expression. (A) Protein lysates from tumors derived from WAP-TGF α mice on Muc1^{-/-} and Muc1^{+/+} backgrounds were separated on SDS-PAGE and immunoblotted (IB) with anti-CyclinD1, EGFR, Muc1 and β -actin antibodies. ZR-75-1 human adenocarcinoma cell lysate was used as a positive control for CyclinD1 expression. Note the double bands (\blackleftarrow) detected in mouse lysates were confirmed to be CyclinD1 by using blocking peptide to CyclinD1 (data not shown). In addition, upon longer exposure, EGFR was detected in ZR-75-1. (B). Tumor lysates derived from WAP-TGF α mice on Muc1^{-/-} and Muc1^{+/+} backgrounds were separated on SDS-PAGE and immunoblotted with anti-phosphoEGFR (Y992) and EGFR(Ab-17) (C). Densitometry showed proteins levels of CyclinD1 were significantly elevated in Muc1 expressing tumors. (D). Levels of EGFR and phosphorylated-EGFR (Y992) were not significantly different between Muc1^{-/-} and Muc1^{+/+} tumor lysates. Error bars denote the standard error.

Muc1 expression promotes development of hyperplasias in the mammary gland

One of the unique characteristics of the WAP-TGF α model is the development of hyperplastic mammary glands prior to frank tumor development [94]. Previous studies demonstrate the development of greater than 100 hyperplastic foci per mammary gland in animals undergoing greater than 5 pregnancies. Since we determined that the removal of Muc1 expression delayed the onset of mammary tumors (Figure 4.2), we hypothesized that the progression of normal mammary glands to hyperplasias is modulated by the expression of Muc1.

First, we evaluated contralateral mammary glands from tumor bearing animals on the WAP-TGF α /Muc1^{-/-} versus WAP-TGF α /Muc1^{+/+} backgrounds, respectively. Whole mount analysis demonstrated a dramatic decrease in the amount of hyperplastic foci observed (Figure 4.4). Microscopic analysis of the lesions verified the presence of multiple hyperplastic foci (Robert Cardiff, UC Davis, personal communication).

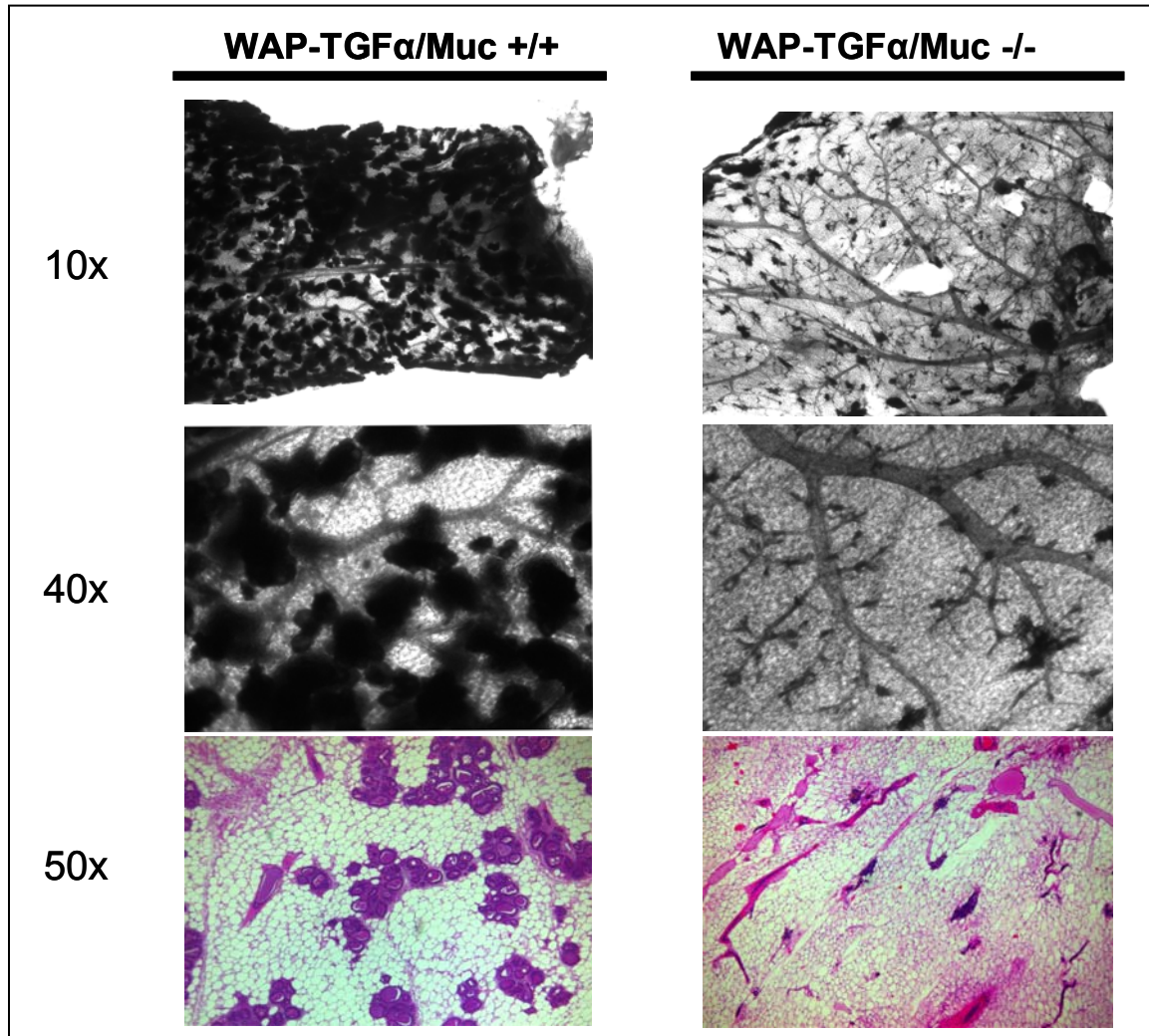


Figure 4.4: Removal of Muc1 expression inhibits the development of hyperplasias in the WAP-TGF α transgenic mice. The wholemounts of the hyperplastic mammary glands isolated from the tumor-bearing animals of WAP-TGF α on Muc1 +/+ and Muc1 -/- reveal that loss of Muc1 expression results in decreased number of hyperplasias (Two panels on the top). Shown in the bottom most panel is the H&E staining of the wholemounts showing the presence of numerous hyperplastic alveolar nodules in WAP-TGF α /Muc1 +/+ .

To evaluate the time at which Muc1 expression affects the progression to hyperplasia, we generated animals on the WAP-TGF α /Muc1^{-/-} versus WAP-TGF α /Muc1^{+/+} backgrounds, and allowed them to progress through 3-5 pregnancies each, harvesting the mammary glands prior to tumor formation. We next counted foci of approximately 1.0 mm in size after whole mount analysis of these glands, using a dissection microscope. This analysis showed that while WAP-TGF α /Muc1^{+/+} mice developed >100 foci by this time, WAP-TGF α /Muc1^{-/-} mammary glands developed only 10-20 foci (Figure 4.5). These data indicate that Muc1 expression affects early stages of EGFR-dependent breast cancer progression.

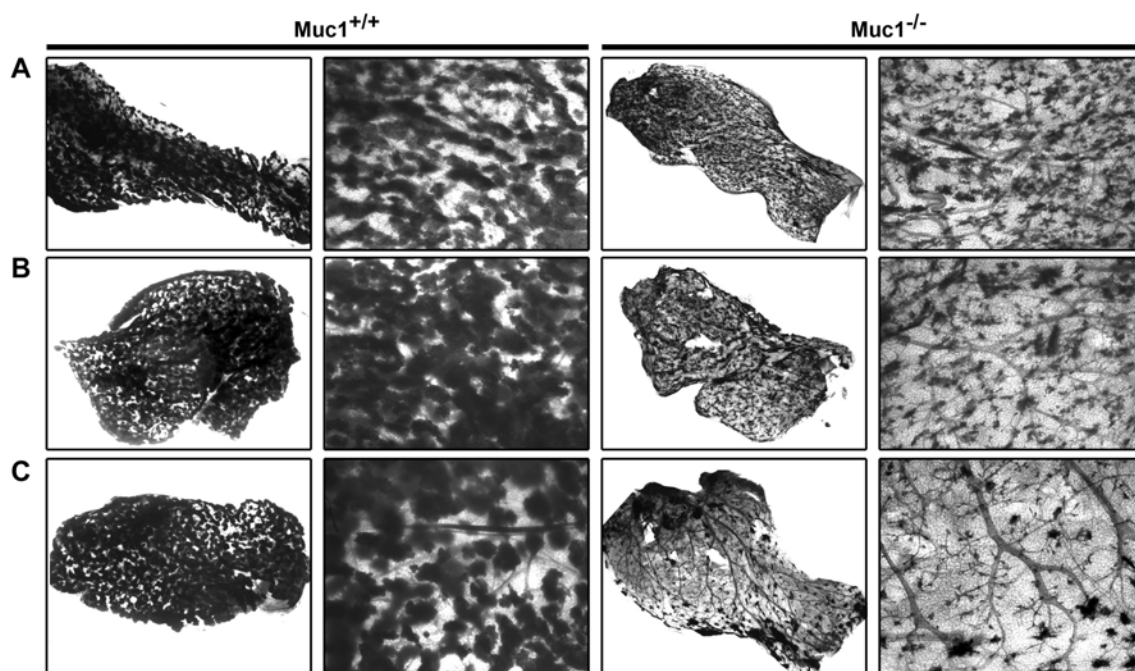


Figure 4.5: Removal of Muc1 expression inhibits the accumulation of hyperplasias in the WAP-TGF α transgenic mice. The wholemounts of the hyperplastic mammary glands were prepared from WAP-TGF α on Muc1 ^{+/+} and Muc1 ^{-/-} after three, four and five pregnancies (top, middle and bottom). Loss of Muc1 expression results in decreased number of hyperplasias (Three panels on the right).

Discussion

Our recent findings reported in the chapter three demonstrate that the expression of MUC1 increases EGFR stability as well as prolong the phosphorylation of EGFR in response to EGF by inhibiting the ubiquitination of EGFR. In addition, expression of MUC1 increases the Akt activation in response to EGF. Based on these observations, we sought to investigate the effects of Muc1 expression on mammary transformation using a mouse model in which EGFR is activated. Hence, we chose the WAP-TGF α mouse model in which the growth factor TGF α binds directly to EGFR and induces mammary transformation.

Analyses of the biochemical interactions between Muc1 and EGFR in the WAP- TGF α model revealed that Muc1 and EGFR interact in the transformed tissue-specific manner (Figure 4.1). We obtained similar results in the MMTV-Wnt1 mouse model as well (data not shown). Although the direct consequences of their interactions are not addressed in detail in this study, we do demonstrate that it possibly has an effect on the tumor progression.

Comparison of the tumor onset in WAP-TGF α transgenic mice revealed that knockdown of Muc1 expression dramatically alters mammary tumor development. Consistent with the previous studies [97], our studies indicate that the WAP-TGF α /Muc1^{+/+} mice develop tumors with 100% (29/29) incidence. However, in a striking contrast, we found that only

37% (11/30) of the WAP-TGF α /Muc1^{-/-} developed mammary tumors during the time analyzed (Figure 4.2). Most importantly, even in the WAP-TGF α /Muc1^{-/-} mice that developed mammary tumors, the age of onset of tumors was significantly delayed. While the mean age of onset of tumors (measured from the date of first parturition to the time at which the 0.5 cm is observed) in the WAP-TGF α /Muc1^{+/+} reported in the previous studies (on the C57/BL6XSJL background) was around 19 weeks [97], our current study indicated that the mean age of onset in the WAP-TGF α /Muc1^{+/+} mice is 24 weeks. This could possibly be accounted for the background differences.

As demonstrated by the studies reported in the chapter four, our studies revealed that the expression of MUC1 modulates the stability of EGF-activated EGFR. We established that MUC1 increases EGFR stability by preventing downregulation of EGFR. In our current studies, we found that there is no significant difference in the total levels of EGFR or its activation status in presence of Muc1 in response to TGF α (Figure 4.3 middle and bottom panels). The fate of EGFR stability and signaling is ligand specific and hence different ligands have different effects. It has been demonstrated previously that EGF and TGF α treatment results in alternate trafficking patterns of EGFR [95]. While EGF remains bound to EGFR as it transits from early to late endosomes and finally to lysosomes and hence target EGFR for degradation, TGF α dissociates from EGFR in the early endosomes and hence favors recycling to the cell surface instead of routing through degradative pathway. Therefore, the possibility of inhibition of EGFR degradation does not even arise

in response to TGF α treatment, and hence Muc1 expression might not play any role in stabilizing EGFR protein levels in that situation.

Consistent with the previous observations, we found that cyclinD1 is highly expressed in the WAP-TGF α mice. In addition, it has been reported previously in the mammary epithelial cell lines that EGFR activation results in the increased expression of cyclinD1. However, the key finding in our study is that even though EGFR is activated to a similar extent both in the presence and absence of Muc1, the expression of cyclin D1 is dependent on Muc1 expression. These results imply that the activation of EGFR alone does not lead to increased cyclin D1, but the expression of Muc1 has a huge impact.

One of the common features of WAP-TGF α and the MMTV-MUC1 transgenic mice is the delay in postlactational involution [49, 94]. It has been hypothesized that this delay allows for additional mutations that ultimately result in transformation. Hence, we surmised that if Muc1 and TGF α function in a synergistic pathway that delays involution, then ablation of Muc1 expression should inhibit the delay. To test this hypothesis, we compared the age-matched whole mounts of mammary glands after 3-5 pregnancies, and found that while the WAP-TGF α /Muc1^{+/+} developed numerous hyperplastic alveolar nodules after the third pregnancy that increased after successive pregnancy, the WAP-TGF α /Muc1^{-/-} accumulated very few. This indicates that the removal of Muc1 expression overcomes the delay in postlactational involution and hence does not accumulate the precursor mammary lesions. This result might explain the low

frequency of mammary tumors as well as the increase in latency in the WAP-TGF α /Muc1^{-/-} mice. The precise pathways that get deregulated and the mechanism of inhibition of delay in involution are currently under investigation.

V. MUC1 EXPRESSION PROMOTES INVASION OF BREAST CANCER CELLS AND ENHANCES PULMONARY METASTASES IN TRANSGENIC MICE

Introduction

Metastatic invasion is one of the six hallmarks of cancer that plays a pivotal role in the mortality of the cancer patients. This process is very complicated and involves many steps outlined in the Figure 5.1A and it requires concerted events mediated by multiple proteins. Metastatic invasion involves detachment of the tumor cell from its neighboring cells at the primary site, invasion through the basement membrane and other stromal components, intravasation and extravasation through the endothelial cells and then ultimately establishment of a colony at a distant/metastatic site. One of the major players identified that participates in many steps of this critical event is MUC1. The role of mucins in general during invasion is depicted in the Figure 5.1B.

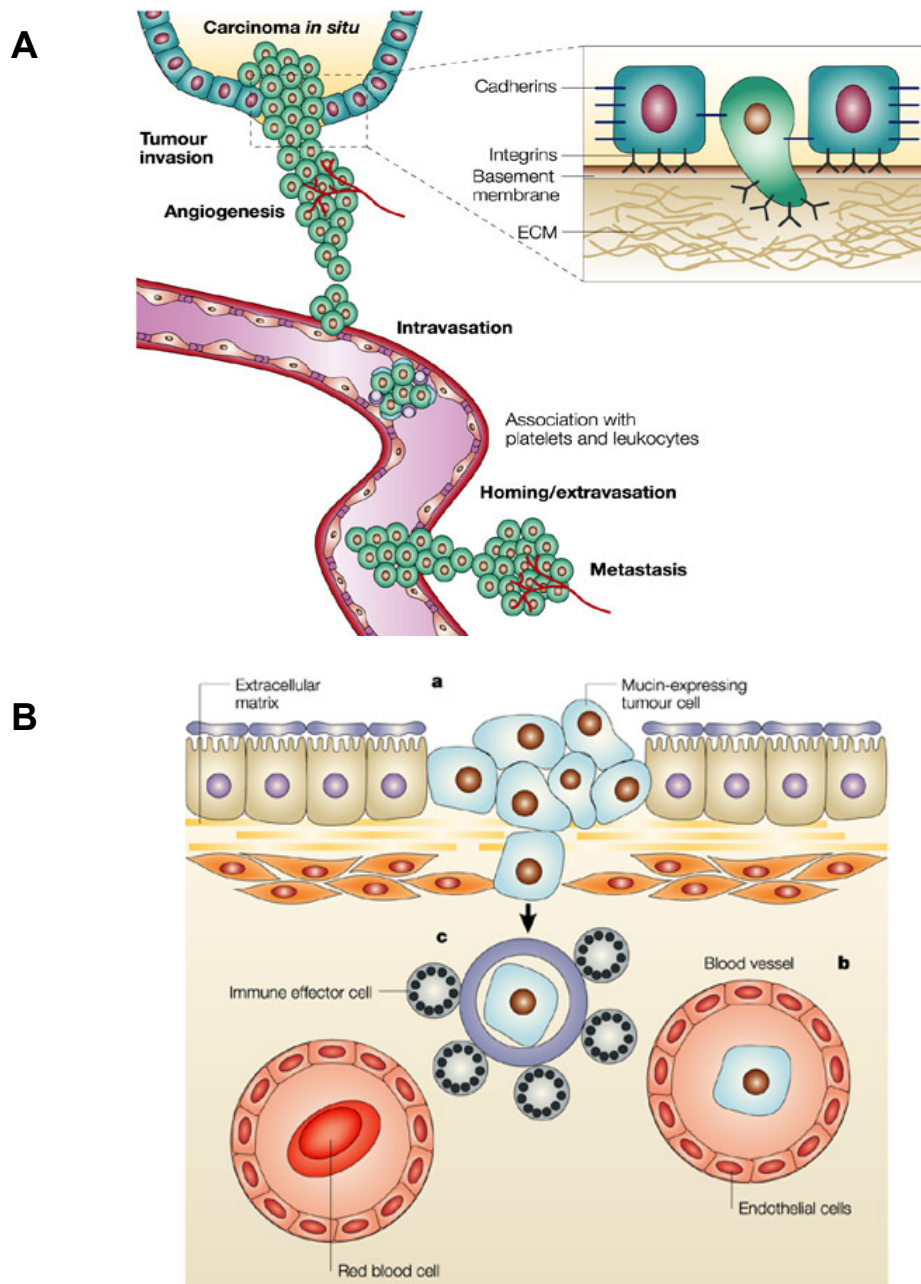


Figure 5.1: (A) Metastatic cascade: Depicts the various steps involved in the spread of a cancer cell from a primary site to a distant site [5] **(B) Tumors use mucins for invasion, metastases and protection.** Tumors use mucins to detach from the primary site and stroma and use adhesive effects of mucins to attach to endothelia and also to protect from immune surveillance [2]

The interesting feature of MUC1, which aids in invasion and metastases, is the presence of domains that regulate anti-adhesion as well as promote metastases. Evidence that MUC1 can contribute to this critical process comes from various studies, some of which are summarized as follows: a) Overexpression of MUC1 can contribute to decrease in aggregation of cells due to the steric hindrance and negative charge created by the extensive glycosylation [37] b) Decrease of MUC1 expression induces cell adhesion of breast cancer cell lines mediated by E-cadherin [98]. c) MUC1 overexpression can inhibit integrin mediated cell adhesion to the extracellular matrix [38]. d) The non-glycosylated form of MUC1 can affect the initial attachment of the breast cancer cells to the tissues at distant sites and facilitate establishment of metastatic foci [99]. e) MUC1 competes with E-cadherin binding to β -catenin and results in the disruption of cell adhesion complexes [100]. f) MUC1 and β -catenin associate at the sites of focal adhesion of breast cancer cells and enhance the invasive ability. Furthermore, MUC1 and β -catenin interactions are strongly upregulated in the lymph node metastases of the breast cancer patients [101]. g) Overexpression of MUC1 has been associated with the invasive and metastatic tumors of the breast, ovarian, colon, pancreas, gall bladder and oral epithelia (reviewed in [2]). Recent studies have shown that the knockdown of MUC1 using RNA interference resulted in decreased metastatic potential of pancreatic adenocarcinomas [102].

The role of EGFR family members, including the receptors and the ligands that activate them, in invasion and metastases of multiple cancers has been well documented. In view of this fact, the constitutive activity of EGFR and ErbB2 confers highly aggressive

properties on tumors and promotes metastases (reviewed in [56]). In addition, studies have shown that the co-expression of EGFR along with its ligand TGF α correlates with worse prognosis in the breast cancer patients [91]. Furthermore, the ligands that activate ErbB receptors have been shown to play a role in the production of proteases that degrade the basement membrane and allow the tumor cells to invade (reviewed in [56]).

Despite several studies implicating the roles of MUC1 and ErbB receptors in invasion and metastases, there is very little evidence of the effect of their synergistic interactions. Hence, the primary objective of the study described in this chapter is to analyze the effects of MUC1 expression on EGFR dependent invasion and metastases.

Results

MUC1 expression results in EGFR-dependent activation of p38 MAPKinase

Recent studies in our lab have demonstrated that MUC1 expression enhances EGFR stability and signaling in the highly invasive breast cancer cell line MDA-MB-231[62]. Hence, we screened for various EGFR- dependent, invasion promoting signaling pathways that are modulated by the expression levels of MUC1. As shown in the Figure 5.2, p38 MAPkinase is activated 5 minutes after EGF treatment only in the cells expressing MUC1. Furthermore, the activation persisted for 45 minutes after EGF treatment, while in the cells that have decreased MUC1 expression did not exhibit the activation at any of these time points tested.

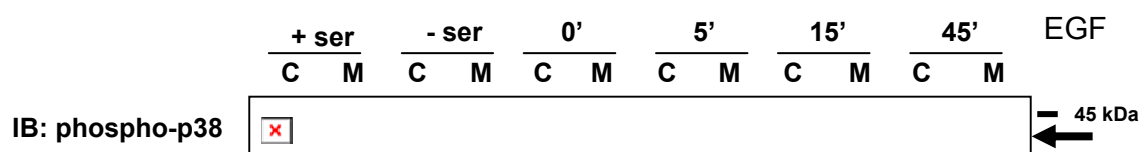


Figure 5.2: MUC1 expression induces the activation of p38 MAPKinase in the breast cancer cells. MDA-MB-231 cells were stably transfected with the CMV(C) or CMV-MUC1(M) and subjected to the endocytosis assays for the timepoints mentioned as described in the chapter three. The lysates were run on a SDS-PAGE gel and immunoblotted (IB) with the anti-phospho p38 antibody

MUC1 mediated increase in the breast cancer cell invasion is dependent on the ligand as well EGFR kinase activity

We generated the stable transfectants of full length MUC1 in the MDA-MB-231 cell line that expresses low levels of MUC1. Since our results showed an increase in the activity of p38 MAPKinase involved in invasion, we sought to determine if MUC1 expression alters the invasive ability of this cell line. We subjected the MDA-MB-231 cell lines with the control transfectant (CMV) and MUC1 (CMV-MUC1) to the *in vitro* transwell collagen assay and the results are shown in the Figure 5.3. The breast cancer cells that overexpressed MUC1 invaded more than the control at both 12 and 24 hours. We then addressed the question of whether the MUC1 mediated increase in invasion is dependent on EGFR. We first treated the 231-CMV-MUC1 cells with EGF and measured the invasion 24 hours after EGF treatment. As shown in the Figure 5.3, invasion of MDA-MB-231 cells overexpressing MUC1 is enhanced in the presence of EGF, implicating the role of EGFR. We then confirmed this observation by using an EGFR-kinase specific inhibitor, Tyrphostin AG1478. As shown in the Figure 5.3, treatment of cells with tyrphostin decreased the invasive ability, demonstrating the importance of EGFR Kinase activity. Therefore, MUC1 expression increases the invasive ability of breast cancer cell lines and it is dependent on EGFR activation.

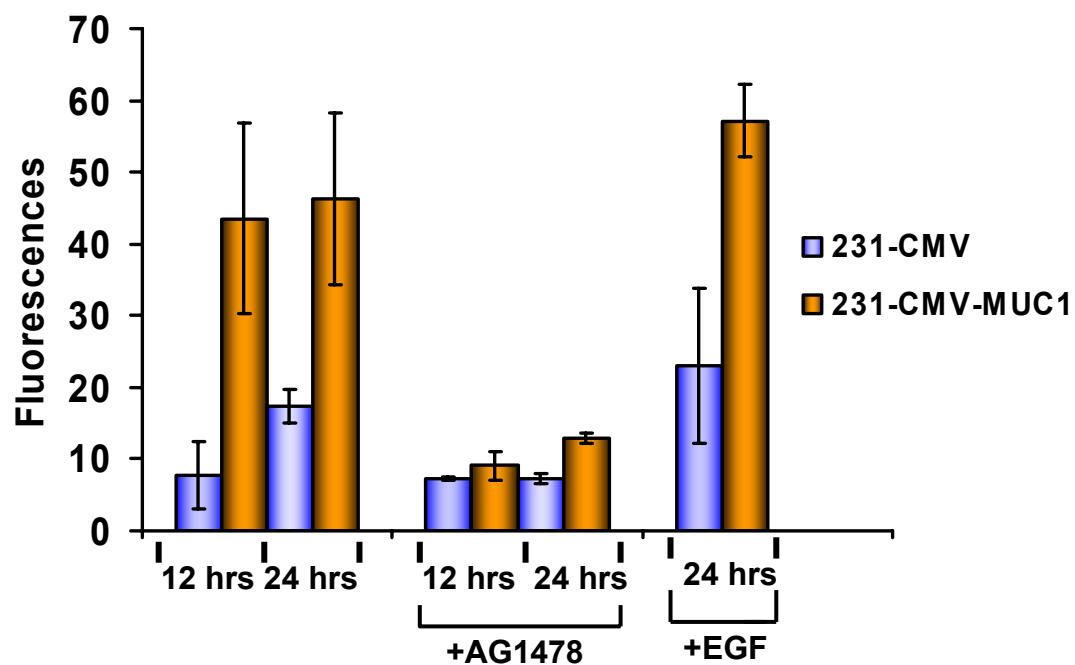


Figure 5.3: MUC1 mediated increase in MDA-MB-231 cells is dependent on EGFR kinase activity as well as EGF. MDA-MB-231 cells with the CMV (control) and CMV-MUC1 (MUC) were allowed to invade through type I collagen for 12 and 24 hours. MDA-MB-231 cells were either pretreated with 20 μ M EGFR kinase inhibitor (AG1478) or EGF to determine the effect of EGFR kinase activity.

Effects of Muc1 expression on pulmonary metastases in WAP-TGF α transgenic mice

MUC1 overexpression has been shown to correlate to metastatic progression in a number of studies of breast cancer [101]. Therefore, we sought to determine if Muc1 expression induced/altered pulmonary metastases in the WAP-TGF α mouse model. While WAP-TGF α mice on a mixed SJL X C57Bl/6 background show no lung pathology, the WAP-TGF α mice we generated on the inbred C57Bl/6 background displayed cellular foci in 28/29 lungs. Microscopic examination revealed perivascular lymphocytic infiltrate and perivascular lymphomas (P) of unknown origin in 28/28 of the lungs, and a metastatic adenocarcinoma (A) in 1/28 lungs (Robert Cardiff, personal communication). Interestingly, there was no lung pathology observed in any of the WAP-TGF α /Muc1^{-/-} animals (Figure 5.4). Note that neither the thymus nor spleen was preserved from these animals, leaving the origin of the lymphomas a subject for future evaluation.

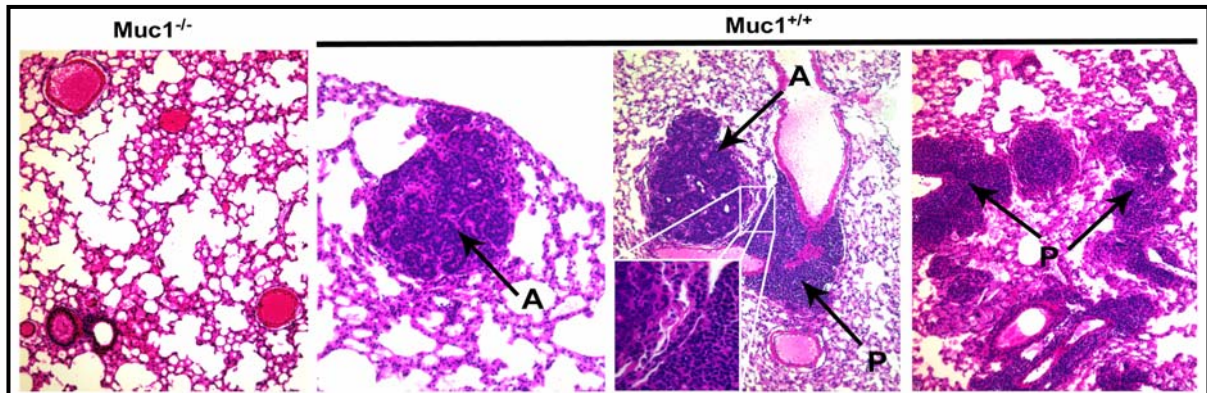


Figure 5.4: Metastatic Adenocarcinoma (A) and perivascular lymphomas (P) are induced in the lungs of WAP-TGF α /Muc1^{+/+} but not in WAP-TGF α /Muc1^{-/-}. H&E staining of the lung sections reveal an accumulation of perivascular lymphomas of unknown origin in WAP-TGF α /Muc1^{+/+} (Analysis by Dr. Robert Cardiff, UC Davis). There was a very rare occurrence of adenocarcinoma (A) in the lungs of WAP-TGF α /Muc1^{+/+} as well. However, note that these were not detected in WAP-TGF α /Muc1^{-/-}.

MUC1 expression enhances the rate of pulmonary metastases in MMTV-Wnt1 transgenic mice

As lungs from WAP-TGF α transgenic mice developed perivascular lymphomas, we analyzed the effects of Muc1 expression on an additional mouse model of breast cancer, the MMTV-Wnt-1.

Rationale for choosing MMTV-Wnt1 mouse model

Previous studies reported that removal of Muc1 expression delays tumor onset in the MMTV-Wnt1 transgenic mice, implying the significance of Muc1 expression in mammary transformation in this mouse model [101]. In addition, the Wnt family of growth factors have been demonstrated to transactivate EGFR by mediating the maturation and release of the growth factors that bind EGFR [56] [96]. Hence, this mouse model offers the potential to evaluate the importance of Muc1 expression as well as EGFR activation in mammary transformation. Furthermore, as the MMTV-Wnt1 mice do not develop metastatic mammary tumors, we crossed them to MMTV-MUC1 transgenic mice that overexpress the full length human MUC1. These bitransgenic mice (MMFW) provided us the tools to evaluate the pulmonary metastases as they did not have any endogenous lung pathology. Furthermore, we crossed the MMTV-Wnt1 transgenic mice to Muc1^{-/-} to use a negative control for Muc1 expression.

We first verified that Muc1 and EGFR formed a biochemical complex in the tumors and then investigated the effect on pulmonary metastases. Analysis of tumors and normal mammary gland from each compound mouse mutant demonstrated a tumor-specific interaction between MUC1 and EGFR (Figure 5.5B). Analysis of the bitransgenic mice revealed a striking induction of pulmonary metastases. We observed that 60 % (6/10) of the MMTV-Wnt-1/MMTV-MUC1 bitransgenics developed metastases while none were detected in the MMTV-Wnt-1/Muc1^{-/-} animals (Figure 5.5A). Investigation of localization of MUC1 and EGFR demonstrated that EGFR and MUC1 colocalize in the these metastases (Figure 5.5C)

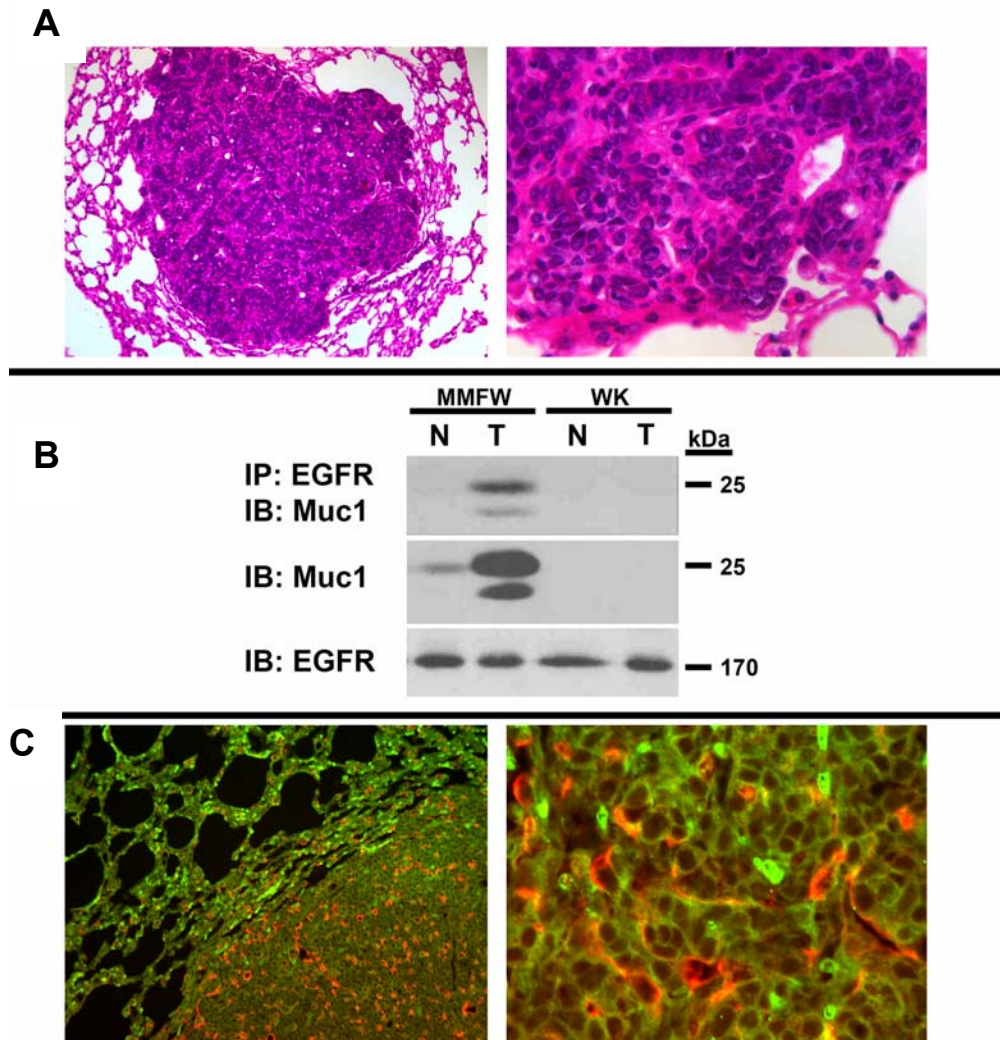


Figure 5.5: MUC1 expression increases the rate of pulmonary metastases in the MMTV-Wnt1/MMTV-MUC1 (MMFW) bitransgenic mice. (A). H&E staining of the lung sections from the MMFW mice reveal the metastases **(B).** MUC1 and EGFR form biochemical complexes in the tumors (T) but not in the contralateral normal mammary gland (N). Normal and Tumor lysates from the MMTV-Wnt1/Muc1^{-/-} are used as negative control. **(C)** MUC1 (red) and EGFR (green) colocalize in the metastases in the MMFW lungs

Discussion

The objective of the study described in this chapter is to investigate the effects of MUC1 expression on EGFR-dependent invasion and metastases. To evaluate the role of MUC1 expression on invasion, we stably transfected the metastatic breast cancer cell line MDA-MB-231 with CMV alone (control) and CMV-MUC1. We treated both the stable transfectants with EGF and performed endocytosis assay as described in the chapter three.

Analysis of the various invasion promoting signaling molecules revealed that the p38 MAPKinase is activated only in the presence of MUC1 five minutes after EGF treatment and the activation persisted 45 minutes after EGF treatment. Although many other pathways might contribute to the invasive potential in this cell line, we have identified that p38 MAPK as one of them. In fact recent studies have suggested that p38MAPK activation results in increased invasion of the breast cancer cell lines [103].

In addition, our experiments using the transwell collagen in vitro invasion assay revealed that MUC1 expression increases the invasion of MDA-MB-231 cells both at 12 and 24 hours. Furthermore, our results demonstrate that the increase in invasion is dependent on EGF as well as the kinase activity of EGFR.

The observation of perivascular lymphomas in the WAP-TGF α model is a novel development, and appears to be a result of expression of the transgene in an inbred C57Bl/6 background. While the development of the lymphomas is of unknown cause, it is still interesting to observe that a loss of Muc1 expression completely removes the occurrence of these lymphomas in the lung. It has been previously established that Muc1 is expressed in B and T cell lymphocytes and MUC1 was also detected in patients with T cell lymphoma. In a T cell lymphoma cell line (Jurkat), MUC1 was shown to play a critical role in activation and proliferation. It is therefore tempting to speculate that Muc1 loss is also affecting the spontaneous development of lymphomas in this model.

Investigation of effects of transgenic expression of MUC1 in the MMTV-Wnt1 revealed that MUC1 expression increases the rate of pulmonary metastases significantly. Taken together, with the exception of WAP-TGF α mouse model, we found that MUC1 expression enhances invasion and metastases.

VI. *WAVED-2* MUTATION IN EGFR RESULTS IN EMBRYONIC LETHALITY OF MICE ON FVB GENETIC BACKGROUND

Introduction

The epidermal growth factor family of receptors and the ligands that activate them are expressed in multiple organs and at various stages of development. These proteins play critical roles in the early stages of embryo development as well as in adult organs and the physiological effects include cell proliferation, differentiation, apoptosis, angiogenesis, etc. Given that there are several other molecules that contribute to similar processes, delineation of the specific roles of EGFR can possibly be achieved by the analysis of the EGFR knock out mice. Knock out studies of EGFR have been performed by two independent groups in parallel in 1995 using mice of various genetic backgrounds and the results varied depending on the mouse strain used [104].

Briefly, the studies revealed that the fetuses lacking EGFR were retarded in growth and died at mid-gestation in a 129/Sv genetic background, some survive until birth in 129/Sv X C57BL6 cross, and some even survive up to postnatal day 20 in a 129/Sv X C57BL6 X MF1 background [104]. The analyses of the reasons for death revealed that the deficiency of EGFR disrupted the proliferation and survival of the spongiotrophoblast cells of the placenta. The EGFR knockout studies performed by Threadgill *et al* also revealed similar results in CF-1 and CD-1 backgrounds[105]. The death of offspring occurred during peri-implantation in CF-1 strain while the mutant EGFR mice lived up

to 3 weeks post-gestation in CD-1 strain and displayed abnormalities in skin, kidney, brain, liver and gastrointestinal tract. Hence, the lethality in EGFR null mice poses a limitation to studying the specific role of EGFR activation in transgenic mouse models. However, the spontaneous mutation, (referred to as *waved-2*) in EGFR that results in reduction of autophosphorylation as well as drastically decrease kinase activity by as much as 90%, has provided an invaluable surrogate to study the importance of EGFR activation [81].

The genetic alteration involved in *Egfr*^{wa2/wa2} phenotype is the spontaneous mutation in a single base pair that results in a Valine to Glycine substitution at residue 743 (residue 741 in the human EGFR) located within the tyrosine kinase domain of EGFR [106]. *In vitro* experiments utilizing the liver membranes derived from the *waved-2* mice revealed that the autophosphorylation ability of EGFR was reduced by 5 to 10 fold as compared to the normal EGFR. In addition, the kinase activity which is measured by ability to phosphorylate an exogenous substrate was reduced as much as by 90%[106]. Since EGFR activation results in the recruitment of multiple effector molecules that bind to phosphorylated tyrosine residues, which in turn are the substrates for EGFR Kinase activity, the reduction in EGFR kinase activity can have a significant impact on a plethora of signaling pathways. Therefore, in lieu of EGFR knock out mice, the *Egfr*^{wa2/wa2} mice offer the potential to explore the significance of EGFR activation in transgenic mice in the development of mammary tumors.

The relationship between MUC1 and EGFR in the transgenic mice comes from the studies in MMTV-MUC1 transgenics. Studies in these mice showed that MUC1 and the erbB family of receptor tyrosine kinases biochemically interact in the lactating mammary gland [60]. In addition, experiments in the MMTV-MUC1 transgenic model have shown that this interaction results in the potentiation of EGF-dependent signaling pathways. Examination of the Ras/MAP Kinase pathway in these transgenic mice demonstrated that the overexpression of MUC1 vastly increases EGF-dependent p42/44 ERK activation during lactation [60]. Taken together, these results imply that MUC1 and EGFR biochemical interactions occur in the transgenic mice that overexpress full length human MUC1. Studies in the same mice showed that overexpression of MUC1 results in the development of mammary tumors [49], thus demonstrating the function of MUC1 as an oncogene. However, the importance of EGFR activation in mammary transformation induced by MUC1 is not known to date.

Hence, we intended to delineate the significance of EGFR activation in mammary tumor progression in the MMTV-MUC1 mouse model by crossing them to *Egfr*^{wa2/wa2} mice that express mutant EGFR. We report here that the *Egfr*^{wa2/wa2} mutation in the FVB background results in embryonic lethality and hence the study of crossing them to MMTV-MUC1 could not be accomplished. Furthermore, analysis of the cause for embryonic lethality revealed defects in the development of aortic valve in the *Egfr*^{wa2/wa2} embryonic hearts.

Materials and Methods

The details of the reagents and methods that are common to other studies can be found in chapter two. Following are the methods pertinent to this study.

Waved-2 mice: One pair of mice heterozygous for *Waved-2* mutation (hereafter referred to as *Egfr*^{wa2/+}) on the FVB genetic background were provided by Dr. Noreen Luetke from the University of South Florida, Tampa.

PCR genotyping: DNA was isolated from tails of the mice using an ethanol precipitation method. PCR analysis was utilized to verify genotype of animals. *Egfr*^{Wa2/Wa2} mice were confirmed using the following primers: Wa2 Forward 5'-CCCAGAAAGGGATATGCG-3' and Wa2 Reverse 5'-GCAACCGTAGGGCATGAG-3'. This reaction was amplified for 45 cycles of 30sec at 94°C, 30sec at 55°C, and 30sec at 72°C. The PCR product was then digested with *FokI* at 37°C for 4hrs followed by 65°C for 20mins. This digest reaction produced two fragments 170bp uncut (*Egfr*^{wt}) or a 95 and 75bp pieces (*Egfr*^{wa2}). The digested PCR product was run on a 2% agarose/ethidium bromide gel to confirm the sizes.

Results

Egfr^{wa2/wa2} mutation is embryonic lethal on FVB genetic background

In order to explore the importance of EGFR signaling in the MMTV-MUC1 mouse model, we attempted to cross them to *Egfr*^{wa2/wa2} mice. Since the MMTV-MUC1 mice were on a FVB background, we chose to use the *Egfr*^{wa2/wa2} on FVB background as well. The study began with one pair of *Egfr*^{wa2/+} mice and hence initially required generation of homozygous *Egfr*^{wa2/wa2}. The crossing of *Egfr*^{wa2/+} mice surprisingly did not result in the expected number of *Egfr*^{wa2/wa2} mice. We then pursued the crosses for many generations to exclude any random errors and the results are depicted in the table 6.1. From a *Egfr*^{wa2/+} to a *Egfr*^{wa2/+} cross, 25% of the offspring are expected to be *Egfr*^{wa2/wa2}. However, out of a total of 161 offspring generated, we only obtained 3 (1.86%) *Egfr*^{wa2/wa2} pups as compared to the expected 40.25 (25%).

Litter Name	Total no. of offspring	No. of $Egfr^{wa2/wa2}$ Expected (25%)	No. of $Egfr^{wa2/wa2}$ Observed
2.1	8	2	1
2.2	5	1.25	0
2.3	9	2.25	0
2.4	3	0.75	0
2.5	8	2	0
2.6	8	2	0
2.7	8	2	0
2.8	5	1.25	0
2.9	9	2.25	0
2.10	9	2.25	0
2.11	2	0.5	0
2.12	8	2	0
2.13	12	3	1
2.14	6	1.5	1
2.15	6	1.5	0
2.16	14	3.5	0
2.17	6	1.5	0
2.18	12	3	0
2.19	5	1.25	0
2.20	18	4.5	0
Total	161	40.25	3

Table 6.1: Summary of expected and observed $Egfr^{wa2/wa2}$ offspring from a $Egfr^{wa2/+}$ vs. $Egfr^{wa2/+}$ cross.

Note that this cross generated only 3/161 $Egfr^{wa2/wa2}$ instead of the expected 40/161

Egfr^{wa2/wa2} mutation is embryonic lethal on FVB genetic background

We then crossed the *Egfr*^{wa2/wa2} male to a *Egfr*^{wa2/+} female to check if it will increase the number of *Egfr*^{wa2/wa2} mice we needed for the study. The results from these crosses are summarized in the table 6.2. Again, we noticed a striking percentage of lethality since out of total 111 pups generated, while the expected number of *Egfr*^{wa2/wa2} is 55.5 (50%) we only obtained 13(11.8%). It should be noted that an attempt to cross the *Egfr*^{wa2/wa2} male to *Egfr*^{wa2/wa2} female was also undertaken, and that cross did not generate any pups.

Litter Name	Total no. of offspring	No. $Egfr^{wa2/wa2}$ Expected (50%)	No. $Egfr^{wa2/wa2}$ Observed (50%)
3.1	10	5	2
3.2	16	8	2
3.3	9	4.5	2
3.4	8	4	2
3.5	14	7	2
3.6	9	4.5	2
3.7	5	2.5	0
3.8	7	3.5	0
3.9	7	3.5	5
3.10	4	2	0
3.11	8	4	0
3.12	4	2	0
3.13	10	5	1
Total	111	55.5	18

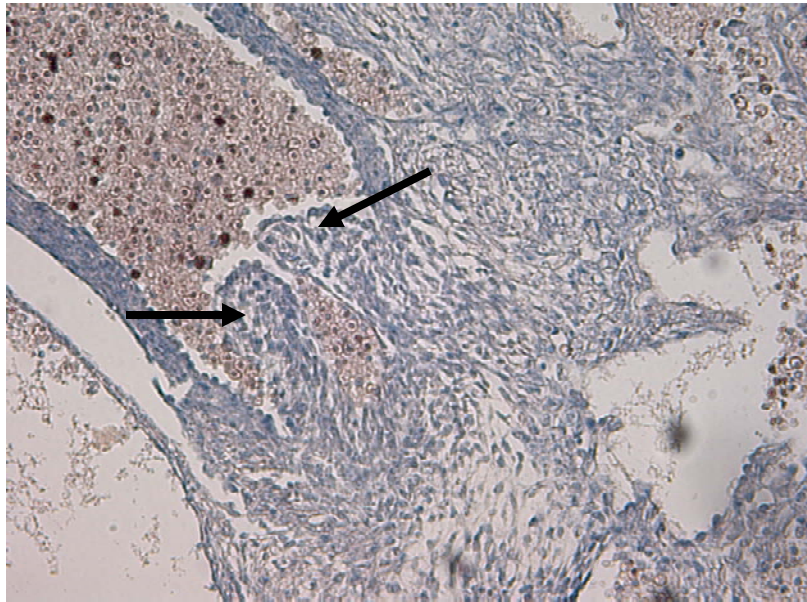
Table 6.2: Summary of expected and observed $Egfr^{wa2/wa2}$ offspring from a $Egfr^{wa2/wa2}$ vs. $Egfr^{wa2/+}$ cross.

Note that this cross generated only 18/111 $Egfr^{wa2/wa2}$ instead of the expected 55.5/111

Hearts from *Egfr*^{wa2/wa2} embryos reveal abnormalities in the aortic valves

It has been previously reported that the *Egfr*^{wa2/wa2} mice on the BLE1C3H background had enlarged semilunar valves in the hearts [107]. Hence, we speculated that the possible cause of embryonic lethality in the *Egfr*^{wa2/wa2} mice was due to a defect in the heart development. Therefore, we isolated the embryonic hearts at various time points and compared those from *Egfr*^{wa2/wa2} to *Egfr*^{wa2/+}. The sections revealed that the hearts derived from the *Egfr*^{wa2/wa2} embryos had abnormal aortic valves (Figure 6.3, bottom panel, arrows).

***Egfr*^{wa2/+}**



***Egfr*^{wa2/wa2}**

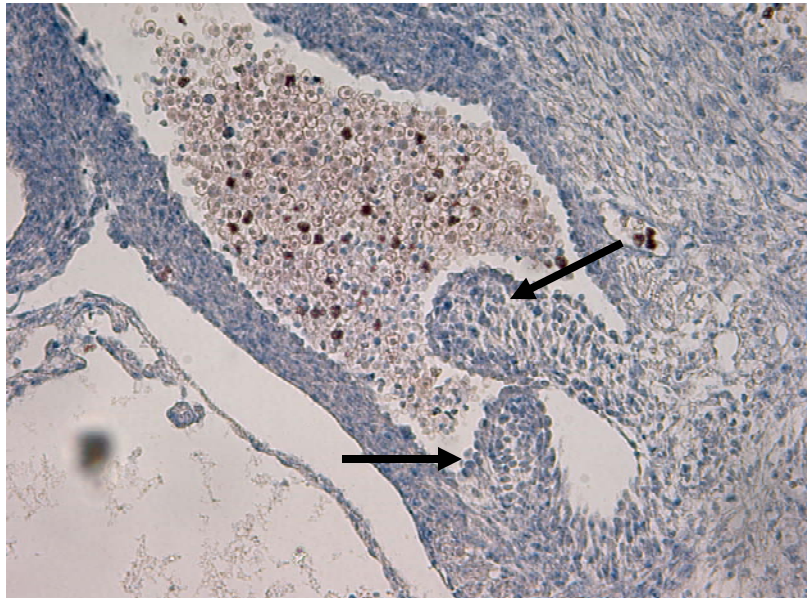


Figure 6.3: Hearts derived at E 14.5 reveal abnormal aortic valves in *Egfr*^{wa2/wa2}

Discussion

EGFR signaling has been demonstrated to play a critical role in various aspects of development as well in neoplastic progression. Recent studies in the MMTV-MUC1 revealed that EGFR and MUC1 form biochemical complexes in the lactating mammary gland and that MUC1 expression increases EGF dependent ERK1,2 MAP Kinase activation [60]. In addition, the MMTV-MUC1 mice develop mammary tumors [49]. Moreover, the significance of EGFR activation in the induction of mammary carcinoma in MMTV-MUC1 mice is unknown. Hence, the ideal way of determining the role of EGFR in MMTV-MUC1 mice would be to cross them to EGFR knockout mice and analyze effects on tumor onset and development. However, the critical limitation to perform such a study is the availability of EGFR knockout models, as it results in embryonic lethality. However, *Egfr*^{wa2/wa2}, with a point mutation in the EGFR kinase domain with highly diminished EGFR kinase activity offer invaluable tools to study the importance of EGFR activation.

This study attempted to generate MMTV-Wnt1 mice on the *Egfr*^{wa2/wa2} background. However, the efforts were unsuccessful as it resulted in the embryonic lethality and very few numbers of *Egfr*^{wa2/wa2} mice were obtained (Tables 6.1 and 6.2). Analysis of the possible cause (s) of death revealed that the hearts isolated at embryonic day 14.5 from *Egfr*^{wa2/wa2} embryos exhibit enlarged valves as compared to the hearts from the wild type (Figure 6.3). Although enlarged valves alone might not be the direct cause of death,

further complications could eventually result in the non-viability of the offspring before birth.

Therefore, the current studies demonstrated that the *wa-2* mutation results in the embryonic lethality in *FVB* genetic strain. It has been previously shown that the EGFR knock out mice die at different stages depending on the genetic background used [104] [105]. Strain-specific genetic modifiers have been suggested as the reasons for the difference. Currently, we are backcrossing the *Egfr*^{*wa2/wa2*} mice to BL6 background which could provide valuable tools to delineate the significance of EGFR kinase activity in MMTV-MUC1 transgenic mice.

VII. CONCLUDING STATEMENTS

Cancer encompasses a disarray of a myriad signal transduction processes all of which ultimately result in a huge imbalance in the cell proliferation and death. The EGFR family of receptor tyrosine kinases together with the ligands that activate them are frequently deregulated in many cancers, plausibly contribute to all the six hallmarks inherent to all cancers and hence provide a strong rationale for therapeutic interventions. Consequently, the discovery of the first ligand EGF in 1962 by Dr. Stanley Cohen and further cloning and characterization of EGFR in 1984, followed by the identification of three other receptors and multiple ligands in this family, have grabbed the attention of many researchers all over the world.

The contributions of various researchers on the role of the ErbB receptors have led to the elucidation of the critical importance of these receptors in the normal mammary gland development as well as in breast cancer progression. In fact, Herceptin is the first drug designed towards an oncogenic cell surface receptor Erbb2 and represents a paradigm shift in the treatment of breast cancer. The fact that this drug is effective only in a fraction of breast cancer patients that overexpress Erbb2, demands identification of other molecules that serve as prognostic and diagnostic indicators. In addition, it has been found that cancers that display expression of Erbb2 together with EGFR correlate with worse prognosis in some cancers[108, 109]. Since no ligand has been identified so far that directly binds to Erbb2, the activation of Erbb2 in most instances depends on the

heterodimer formation. Hence, it is important to elucidate the mechanisms of regulation of activation of other receptors that either cooperate with ErbB2 or even function alone.

Recent studies in the human breast carcinoma cells as well as in the transgenic mouse models have led to the identification of the protein MUC1 as one of the substrates for the activated EGFR. In addition, the formation of MUC1 and EGFR complex potentiates the activation of ERK1,2 MAP Kinase in the lactating mammary gland. Furthermore, phosphorylation of MUC1 on the YEKV motif by EGFR leads to binding of C-Src and β -catenin to MUC1. The overall effect of these interactions is to increase the cell proliferation, cell migration and invasion, etc all of which are the hallmarks of cancer. Therefore, given that both MUC1 and EGFR are oncogenes that are expressed in a high percentage of breast cancers and also the studies demonstrating the potentiation of EGFR signal transduction pathways by MUC1, urged us to hypothesize that the biochemical interactions between MUC1 and EGFR play a pivotal role in the breast cancer progression.

Ligand binding results in the dimerization and activation of the EGF receptors at the cell surface which then ultimately induce a plethora of signaling pathways that culminate in various important biological processes in the cell. Since the two key features that determine the function of EGFR are the levels of protein as well as the duration of activation, we first tested if MUC1 expression has an effect on one or both of these two aspects. In order to adequately address the effect of MUC1 expression on EGFR protein

levels and activation, we used two human breast cancer cell lines: BT-20(expresses high levels MUC1) and MDA-MB-231(expresses very low levels of MUC1). In addition, we used an immortalized normal mammary epithelial cell line MCF10A (expresses high levels of MUC1) to determine if the effects of MUC1 on EGFR are unique to breast cancer cells alone, or if it occurs in the normal mammary epithelial cells as well.

We demonstrate through RNAi-mediated loss of MUC1 expression that MUC1 inhibits the degradation of phosphorylated EGFR after ligand binding, in both BT20 breast cancer cells and MCF10A breast epithelial cells. Overexpression of MUC1 in MDA-MB-231 cells recapitulates this effect by stabilizing ligand-activated erbB receptor. While we were pursuing the studies of effects of MUC1 expression on EGFR protein levels, another group reported that the knockdown of MUC1 expression using siRNA in the KB epidermoid carcinoma cells, AGS human gastric adenocarcinoma cells as well as in 293 human embryonic kidney cells, results in significant reduction of EGFR mRNA and protein levels [86]. This group, however, speculated that MUC1 expression could be regulating the expression of EGFR at the transcriptional level, although did not provide any evidence to substantiate the same. In contrast to their study, we focused on the EGFR expression at the posttranslational level and did not detect any significant changes in the total steady state levels of EGFR (in the cells growing in serum with no exogenous EGF) with or without MUC1.

In light of the recent findings that the endocytic and signaling machineries do not exist independent of each other, but signaling continues to occur even after the EGFR is internalized, we investigated if MUC1 expression had an effect on the activation of different signaling pathways. Our studies indicate that both ERK1,2 MAPKinase and Akt activation continue to occur even after stimulation of EGFR endocytosis . Most importantly, our results indicate that activation of ERK1,2 occurs independent of MUC1 expression, whereas the activation of Akt is detected only in the presence of MUC1 at later time points after EGF treatment. This indicates that the ultimate effect of MUC1 on EGFR dependent signaling pathways is pathway specific.

It is important to note that the MUC1 dependent alteration of intracellular trafficking might not be unique to EGFR. In deed, a recent study has shown that expression of MUC1 in Chinese Hamster Ovary (CHO) cells results in upregulation of Fas receptor at the surface upon stimulation with the Fas ligand[110]. However, the mechanism by which the modulation of intracellular trafficking can be achieved was not addressed in this study Hence, we investigated the plausible mechanisms by which MUC1 alters EGFR trafficking. Since it has been well established that the prerequisite for EGFR downregulation is the EGF-induced ubiquitination, we speculated that MUC1 expression might be altering the ubiquitination of EGFR upon ligand binding. Our results demonstrate that MUC1 expression indeed inhibits the ubiquitination of EGFR and thereafter favors recycling of EGFR to the membrane instead of trafficking to late endosomes/lysosomes for degradation. We believe that our current studies do not rule out

other possibilities of EGFR trafficking and hence the current studies are investigating the same.

Since our studies demonstrated that MUC1 expression enhances EGFR stability and signaling and hence possibly alter the pathways leading to cellular transformation, we hypothesized that MUC1 expression plays a pivotal role in the EGFR-dependent mammary tumor progression. To test this, we utilized the WAP-TGF α transgenic mice and compared the tumor onset and development with and without Muc1. Our results illustrate that the removal of Muc1 expression dramatically delays mammary tumor development, in that only ~37% of the WAP-TGF α mice on the Muc1 null background develop mammary tumors, in contrast to 100% of WAP-TGF α that express Muc1. In addition, the time to develop mammary tumors is delayed significantly in the absence of Muc1 expression. Furthermore, we did not detect any significant difference in the EGFR levels with and without Muc1 in this model, activated by the ligand TGF α . Though this observation is in contrast to what we observed with the ligand EGF in our studies, it is consistent with the studies reported previously revealing that different ligands result in alternate EGFR trafficking [111]. while EGF in general, promotes degradation, TGF α favors recycling of the EGFR to the cell surface.

Delineation of the possible signaling pathways that resulted in altered latency revealed that the expression of the cell cycle protein cyclin D1 is significantly reduced in the absence of Muc1 expression. It must be noted that cyclin D1 upregulation in the WAP-

TGF α mice was reported in the previous studies [94]. In a separate study, it has been shown that MUC1 induces Cyclin D1 expression by binding to the transcription factor β -catenin [112]. Our studies demonstrate that EGFR activation alone is not sufficient to induce high levels of cyclin D1, but the expression of MUC1 together with EGFR activation contributes to upregulation of cyclin D1.

Our studies on effect of MUC1 expression on invasion of breast cancer cell lines substantiated the earlier reports that showed that MUC1 increases the invasion of cell lines *in vitro*. Importantly, we determined that the MUC1-dependent increase in invasion of breast cancer cell lines is dependent on the ligand EGF and also the EGFR kinase activity. In line with this, we investigated the effect of MUC1 expression on metastases in an *in vivo* mouse model, and found that transgenic expression of MUC1 increases the rate of pulmonary metastases in the MMTV-Wnt1.

In essence, our studies have demonstrated that MUC1 is a novel regulator of EGFR trafficking and signaling in breast cancer. In addition, ablation of Muc1 expression dramatically alters EGFR-activation induced mammary tumor development. Furthermore, MUC1 increases the rate of pulmonary metastases and hence contributes to many features of the hallmarks of cancer. Based on the current findings, we propose a model for how MUC1 might be enhancing EGFR signaling and is depicted in Figure C1. However, we do believe that MUC1 might be contributing to cancer progression in many ways and not merely by modulating the trafficking of EGFR upon EGF treatment. .

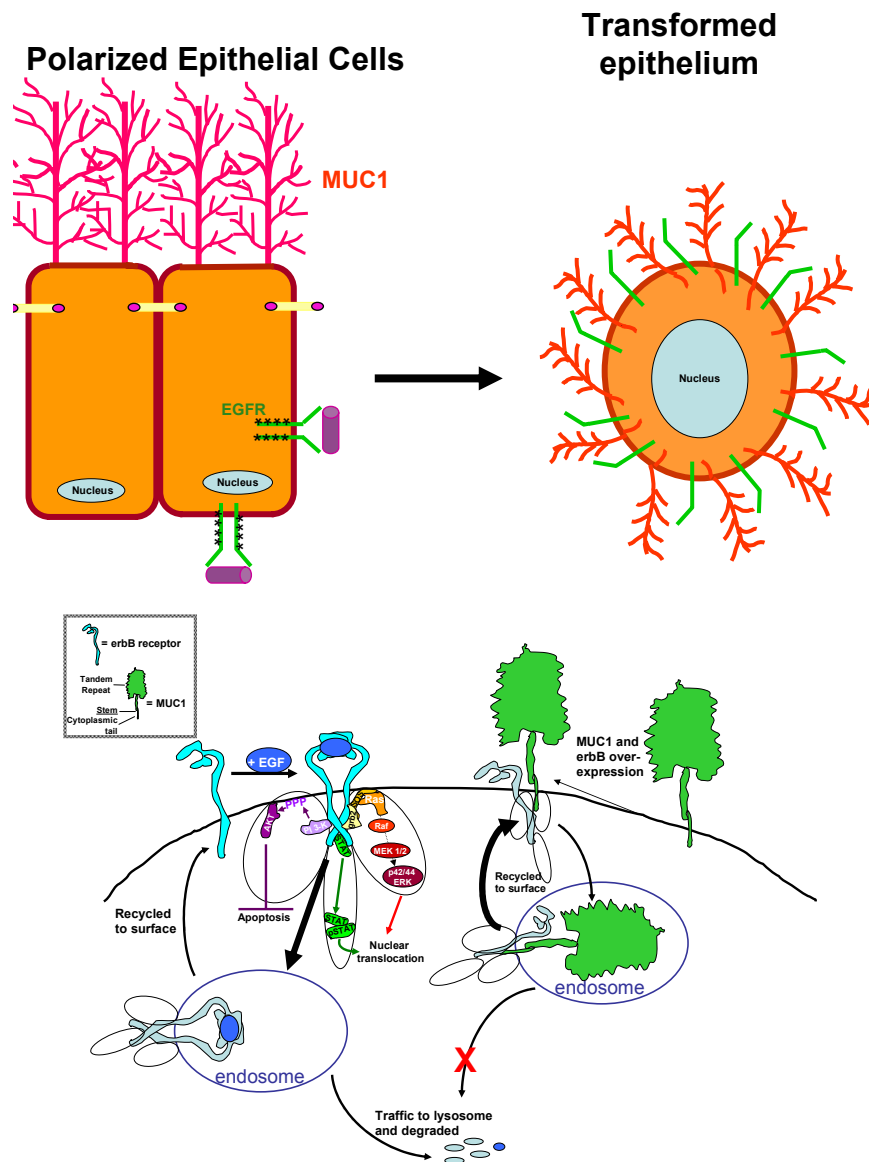


Figure C1: Model for the proposed mechanism of MUC1-mediated modulation of EGFR stability. In a normal polarized epithelial cell, MUC1 is apically localized, whereas EGFR is basolateral. Hence, they do not biochemically interact and therefore activation of EGFR by EGF ultimately results in degradation and attenuation of signaling. However, in the transformed cells in which polarity is lost, MUC1 and EGFR interact and MUC1 prevents ubiquitination of EGFR and also favors the recycling pathway due to its inherent constant recycling property

Therapeutic implications

The cloning and characterization of MUC1 in 1984 has attracted the attention of many researchers in various fields of biology, including immunology, glycobiology, biochemistry, cellular biology and oncology. The research efforts from all these disciplines have contributed to a great understanding of the role of MUC1 in cancers in general and breast cancer in particular. Indeed, MUC1 is a very attractive therapeutic target for various reasons including:

1. MUC1 is overexpressed in a very high percentage of different cancer types.
2. Knock out of Muc1, at least in mice, reveal no obvious phenotypes and hence disruption of its expression in normal cells might not be fatal in humans.
3. MUC1 interacts with various signaling molecules implicated in various cancers including, EGFR, C-Src, β -catenin, p53, Estrogen receptor α , Grb2, etc. The interactions with these molecules have been demonstrated to increase cell proliferation, migration, invasion, decrease in aggregation of cells, decrease in apoptosis, transcription of cyclin D1, etc all of which contribute to majority of the hallmarks of cancer.

Therefore, based on the above reasons, the MUC1 molecule offers a great potential for therapeutic interventions. Since, the cytoplasmic tail of MUC1 which is necessary for mammary transformation, is relatively very short consisting of only 72 amino acids, one can envision a drug or toxin that can “chew up” or degrade the entire cytoplasmic region,

preferably only in the cancer cells and inhibit the interactions with other molecules and hence inhibit the cancer progression. Ideally, such a drug holds a lot of promise, but has not been designed so far. However, we have developed a mimetic peptide fused to a viral “TAT” sequence that mimics the cytoplasmic domain of MUC1 that binds to EGFR and β -catenin to test if it can act in a dominant negative manner and halt tumor progression. The rationale for choosing this region was based on the findings that this domain impinges on the EGFR, C-Src and Wnt signaling pathways. In addition, the established significance of EGFR in breast carcinoma, together with our recent findings reported in this study demonstrating that binding of MUC1 to EGFR has a huge impact on EGFR stability and signaling formed the basis for the design of this peptide.

We have first demonstrated in the breast cancer cells that this peptide can actually be delivered into the cell (Figure C.2). The consequent studies involving administration of this peptide into *in vivo* mouse models is already showing us some promising results (data not shown).

Therefore, in summary, while we are aware that EGFR signaling is an extremely complicated pathway and involves diverse stimuli and multiple biological outcomes, we believe that the modulation of EGFR by MUC1 will have a significant impact on breast cancer therapy.

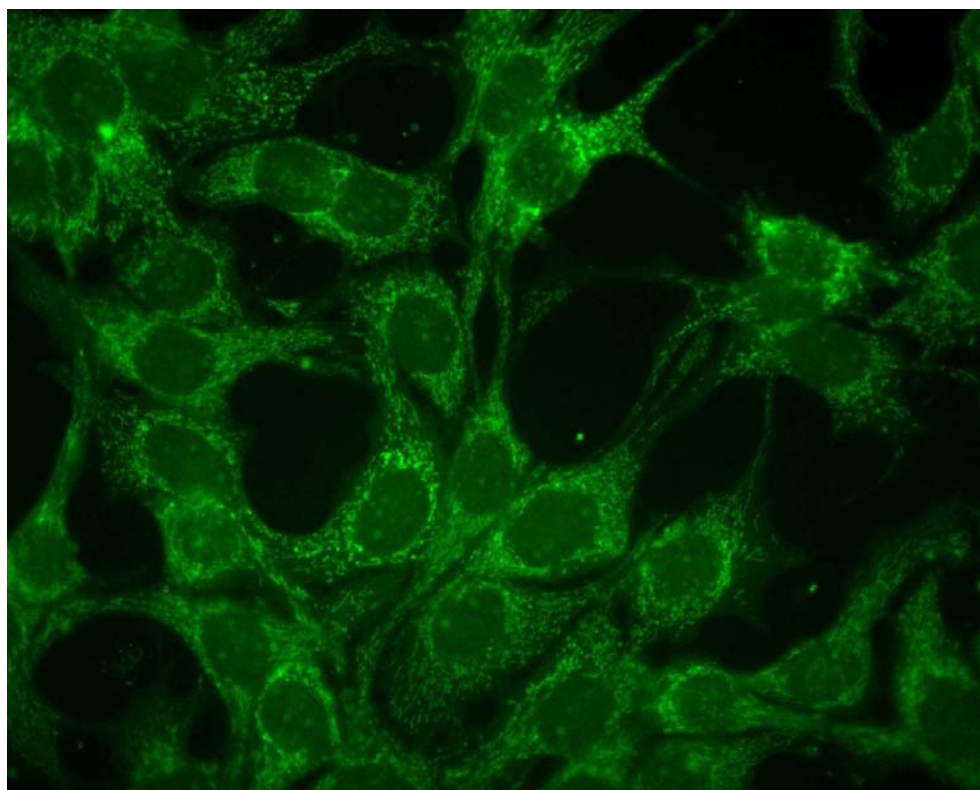


Figure C2: TAT-fused mimetic peptide can be delivered into the breast cancer cells with very high efficiency

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