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Muc4 Modulation of Ligand-Independent ErbB2 Signaling

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MUC4 MODULATION OF LIGAND-INDEPENDENT ERBB2 SIGNALING

By
Goldi A. Kozloski

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
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MUC4 MODULATION OF LIGAND-INDEPENDENT ERBB2 SIGNALING

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The membrane mucin Muc4 is a heterodimer, bi-functional glycoprotein complex that is normally expressed in epithelial tissue. Functional studies on the extracellular mucin subunit of Muc4 have shown that it acts to promote anti-adhesion properties by sterically interfering with cell-cell and cell-matrix interactions and that the extent of this effect is directly associated with the number of tandem repeats on this subunit.

Functional studies on the transmembrane subunit of Muc4 have shown that this subunit participates in intracellular signaling through interaction with the receptor tyrosine kinase ErbB2. This role of Muc4 was shown to be mediated by stabilizing the heregulin ligand-induced ErbB2-ErbB3 heterodimer through interference with the internalization process of these receptors, thus potentiating the PI3K, a survival-signaling pathway that is mediated by this heterodimer. However, Muc4 was also shown to potentiate ErbB2 phosphorylation in the absence of heregulin by an unknown mechanism. The aim of this work was to examine the role of Muc4 in intracellular signaling by evaluating the ligand-independent Muc4-ErbB2 interaction. Biochemical analyses of A375 human melanoma cells expressing Muc4 under different cell treatments, and probed with phospho-specific antibodies, were used to understand the mechanism. An antibody microarray screen was
used to decipher the intracellular activated signaling pathways. The results of the mechanistic analysis indicated that Muc4 potentiates ErbB2 signaling significantly by interacting with ErbB2 and ErbB3 and by stabilizing the kinase active ErbB2 receptor, thus increasing its phosphorylation signal half-life and resulting in sustained ErbB2 signaling. The signaling pathway analysis suggests that through Muc4 direct interaction with ErbB2, signaling pathways that promote loss of cell polarity are activated. Loss of cell-cell adhesion is mediated by interference with the cadherin-catenin complex stability, and loss of cell-matrix adhesion is mediated by facilitating focal adhesion turnover. Together, these results suggest that Muc4 is a potent oncogenic factor, and further enhance our understanding of the role that Muc4 plays in ligand-independent intracellular signaling.
To my husband

Walter H. Kozloski
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Finally, I am extremely grateful for the guidance and support of my husband, Walter H. Kozloski, and for the continued inspiration of my children, Adam and Ilana.
PREFACE

Molecular components that are embedded in the plasma membrane of cells play important roles in communicating signals from the “outside-in.” In motile cells these components provide information about the extracellular matrix conditions and help guide cell movement. In sessile cells within a tissue environment, these components provide information about the conditions of neighboring cells as well as the extracellular matrix condition. These signals are important for normal cell function because they guide spatial and temporal cellular decisions at the molecular level that can affect cell fate. Failure to properly integrate these signals often results in aberrant cellular functions that are associated with a disease state.

Communication of signals from “outside-in” are dependent on structural features that are common to all signaling membrane components; an extracellular domain that interacts with the outside environment, a transmembrane domain that allows their positioning within the membrane, and a cytoplasmic domain through which the outside information is communicated into the cell. Each of these domains is important in successfully communicating signals, and a vast array of molecular mechanisms for each of these domains have been explored.

The work presented here focuses on two membrane components, Muc4 and ErbB2 and on their joint role in communicating “outside-in” signaling that is associated with a disease state. In the first chapter of this work, Muc4 and ErbB2 are introduced. The second chapter is an analysis of their signaling potential at the cell surface. In the
third chapter, their joint signaling effects on intracellular signaling is evaluated. Chapter four includes the major conclusions of this study.
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CHAPTER ONE

INTRODUCTION

Muc4, a member of the Mucin family

Mucins are characterized as glycoproteins that contain a polymorphic central domain composed of a variable number of tandem repeats (VNTRs) rich in serine, threonine and proline residues. The serine and threonine residues serve as O-linked glycosylation sites and are important factors in the increased mass of mucins, resulting in 50-90% increased O-linked oligosaccharides mass. The subsequent carbohydrate moieties however vary depending on the mucin type, the site of mucin expression, and the physiological or pathological conditions (Lamblin et al., 2001). Mucins are actively studied due to the finding that their expression and glycosylation is altered with the differentiation state of cells and with neoplastic transformation (Gendler and Spicer, 1995). Their major function is to protect and lubricate the ducts and the lumens within the human body (Hollingsworth and Swanson, 2004), but they are also involved in the differentiation and renewal of the epithelium, and modulation of cell adhesion, immune response, and cell signaling (Chaturvedi et al., 2008a). Mucins are classified based on their structural properties and include the gel- forming/secreted, the small soluble, and the membrane bound. Membrane mucins are anchored to the plasma membrane via their single transmembrane hydrophobic segment, and this localization domain increases their participation in signaling and allows them to serve as sensors of the external environment (Carraway et al., 2000). This occurs via extracellular domain mediated ligand binding or as a consequence of altered conformation from changes in external biochemical
conditions such as pH, ionic composition, or physical interactions (Singh and Hollingsworth, 2006). Their extracellular domain provides a steric barrier that can limit the direct access of other cells, or even large molecules to the associated cell surface (Carraway et al., 2003b). Seven membrane mucin genes have been identified in the human genome; \textit{MUC1, MUC3, MUC4, MUC12, MUC13, MUC15, MUC16}, and \textit{MUC20}. This work focuses on the membrane mucin Muc4. Please note that human mucins are noted in capital letters (MUC), and all other species with lower case letters, (Muc). Here, Muc4 is used to refer to the rat protein, also known as the sialomucin complex.

\textbf{Muc4, the heterodimeric SialoMucin Complex (SMC)}

Muc4 was originally discovered as a highly over expressed glycoprotein complex on the surface of rat ascites 13762 in mammary adenocarcinoma cells (Sherblom et al., 1980). Studies showed that the SMC is transcribed from a single gene as a 9-kilobase pair transcript and translated into a single large polypeptide (Sheng et al., 1992). Early in its biosynthesis, Muc4 is proteolytically cleaved into two subunits, a large mucin subunit, ASGP1, and a smaller transmembrane subunit, ASGP2 (Sheng et al., 1990). Despite the cleavage, the two subunits remain tightly, but non-covalently attached, and display a large cell surface complex at the plasma membrane. Figure 1-1 is a cartoon model of the SMC at the plasma membrane. One important feature to note is that the intracellular portion of SMC-ASGP2 is short, only 23 amino acids long (UniProtKB/Swiss-Prot: Q63661), thus most of this complex is extracellular. The ASGP1 subunit contains 5 putative N-linked glycosylation sites and a NIDO domain whose function is not clear.
The ASGP2 subunit contains a von Willebrand factor type D domain (VWD) that is required for multimerization, and two epidermal growth factor-like (EGF-like) domains (SMART domain database).

![SMC Model](image)

Figure 1-1. **SMC Model.** Branched structures represent attached oligosaccharides. The parallel lines represent the lipid bilayer of the plasma membrane. Adapted from Carraway *et al.*, 2005.

Muc4 serves as a protective agent for the epithelia by providing steric protection at cell surfaces through potent anti-adhesive properties that lead to loss of cell-cell and cell-matrix interactions. This function of Muc4 is mediated by glycosylation modifications on the tandem repeat domain, and is dependent on the number of tandem repeats in the ASGP1 subunit (Komatsu *et al.*, 2000).

Muc4 is normally expressed in many epithelial tissues both during development and in the adult. It is expressed in the airway and in bodily fluids like saliva, tear-film, ear fluid, and breast milk. In addition, Muc4 is also expressed in the blood vessels of luminal surfaces and in endothelial cells (Zhang *et al.*, 2005).
**Muc4 and MUC4 are orthologs**

The homology between the rat Muc4 and the human MUC4 was difficult to establish because of differences in the sequence homology and organization of the ASGP1 subunit compared with the corresponding human subunit MUC4α. As shown in Figure 1-2, the size and number of the tandem repeat (TR) domains vary between the rat and the human protein. In Muc4 there are 12 TR of 117-124 amino acids, and in MUC4 there are 3 long TR (126 amino acids) and 146-400 short TR (16 amino acids). Within the ASGP2 and corresponding MUC4β subunits however, similar domain organization, size, and sequence homology is shared, providing strong evidence that Muc4 and MUC4 are homologous proteins. (Escande et al., 2002; Moniaux et al., 1999). Later studies also established that these proteins have similar functions and can be studied using common antibodies. The homology was also important to establish for functional studies involving exogenous introduction of the protein by expression vectors because the gene processing features exhibited by the human protein is more complex. The gene encoding MUC4 is present in cells in a diverse array of transcripts that are generated by several mechanisms including alternative use of cassette exons, exon-skipping, or use of cryptic splice donor/acceptor sites, giving rise to about two dozen different transcript variants (Escande et al., 2002), and making such studies more complex to carry out. An investigation of alternative products for the Muc4 gene indicated that no splice variants are generated in the rat, thus establishing Muc4 as a more attractive substrate for these types of studies.
Figure 1-2. Domain organization and sequence homology comparison between the rat (Muc4) and human (MUC4) proteins. Adapted from Carraway et al. 2000.

**Muc4 roles in tumor progression**

Neoplastic transformation is promoted by modified cellular behavior at different levels including proliferation, cell-matrix adhesion, cell-cell adhesion, movement, apoptosis control, transcription, and membrane transport. The Muc4 functions outlined above suggest that Muc4 could potentially facilitate tumor development and progression through multiple mechanisms including anti-adhesion at the cell-cell and cell-matrix levels, and in cell motility. These hypothesized roles are actively studied using Muc4 and MUC4, and data from these studies support these hypothesized roles (Chaturvedi et al., 2007; Komatsu et al., 1997a; Ponnusamy et al., 2008). In addition, aberrant expression of MUC4 has been reported in a variety of carcinomas where it is also associated with poor prognosis, thereby establishing its importance as a molecular marker for advanced metastasis tumor state (Singh et al., 2007). The Muc4 overexpression effect on primary tumor growth, and on tumor progression by xenotransplantation studies in mice revealed
that Muc4 can promote tumor growth of primary tumors (Masanobu Komatsu et al., 2000), and promote metastasis (Komatsu et al., 2001) by repressing apoptosis. These findings highlight Muc4 as an important target in studies of tumor metastasis, and in signal transduction mechanisms that promote metastasis.

**Muc4 roles in signal transduction**

Signal transduction from “outside-in” is promoted by changes at the cell surface that are successfully transmitted into the cytoplasm. One possible mechanism by which Muc4 can promote such signaling is through its C-terminal tail (CTT). A similar CTT dependent signal transduction role has been established in MUC1, the best characterized transmembrane mucin. MUC1 participates in a wide range of intracellular signaling through the ability of its CTT to act as a target for several kinases, and as a docking site for components of cytoplasmic signaling pathways (Hattrup and Gendler, 2008). MUC1 CTT is 72 amino acids long and contains six conserved tyrosine residues which are potential sites for phosphorylation and signaling (Carraway et al., 2005). Muc4 CTT is shorter, only 23 amino acids, but it displays a ligand for PDZ domain, which mediates assembly of large multi-protein complexes through binding of PDZ domain-containing proteins. No other features, such as post translation modification or cleavage sites, were identified in Muc4 CTT sequence to provide clues for other potential roles in signal transduction.

Additional mechanisms of signal transduction potential for Muc4 could be mediated through extracellular interactions with other membrane components that display potent cellular signaling potential. Such a target was identified for Muc4 as the receptor
tyrosine kinase, ErbB2. Complex associations between Muc4 and ErbB2 have been demonstrated in co-immunoprecipitation experiments from detergent lysates in a number of systems including the 13762 ascites cell membranes (Carraway et al., 1999), Muc4-transfected A375, MCF-7 and Cos-7 cells (Jepson et al., 2002), lactating rat mammary tissue (Price-Schiavi et al., 2005) and others. Similarly, MUC4-ErbB2 association was demonstrated in human tumors (Chaturvedi et al., 2008b; Miyahara et al., 2008; Ponnusamy et al., 2008). More significantly, this association was shown to result in changes in the CTT of ErbB2, displaying induction and potentiation of tyrosine phosphorylation (Jepson et al., 2002; Ramsauer et al., 2006a). The CTT of ErbB2 contains several sequence dependent and post translation modification dependent domains that facilitate the recruitment of intracellular signaling proteins (Table 3-2), thus providing a link for Muc4 role in signal transduction through its interaction with ErbB2.

The ErbB receptor family

The subclass I of the receptor tyrosine kinase (RTK) superfamily consists of four members, ErbB1-ErbB4, and 13 polypeptide extracellular ligands, which contain a conserved epidermal growth factor (EGF) domain. The basic functional unit in ErbB signaling is a receptor dimer to which each partner contributes unique features. ErbB receptors share significant sequence and structure homology and contain an extracellular ligand-binding region that is composed of four domains (L1, S1, L2, S2), a single transmembrane spanning helix, and a cytoplasmic portion with a tyrosine kinase domain and a C-terminal tail. However, despite structure similarity, only ErbB1 and ErbB4 are fully functional in terms of ligand-binding and kinase activity, ErbB3 kinase activity is
impaired and ErbB2 lacks the capacity to bind EGF ligand (Citri and Yarden, 2006a; Warren and Landgraf, 2006). Despite this lack of function, both ErbB2 and ErbB3 form heterodimeric complexes with other ErbB receptors that are capable of generating potent cellular signals (Franklin et al., 2004; Sliwkowski et al., 1994; Warren and Landgraf, 2006). Figure 1-3 is a cartoon depicting the general structure features of ErbB2 and ErbB3 monomers, and the structure of a functional signaling unit that results from their interaction (Citri et al., 2003). Note that the structure of the receptor ErbB2 in the absence of a ligand is similar to the ligand bound ErbB3 receptor, suggesting that ErbB2 is ready to dimerize and that its activation is only limited by stable dimerization with other ligand-bound ErbBs (Garrett et al., 2003).

Figure 1-3. **Heregulin-ligand induced ErbB3 forms a potent heterodimer with ErbB2.** Cysteine rich domains (S1 and S2), ligand-binding domains (L1 and L2), ErbB3 (blue), ErbB2 (orange), heregulin (NRG in red), P inside circles represent phosphorylated tyrosine residues. Adapted from Citri et al. 2003.
The ErbB receptor signaling network involves a wide array of signaling pathways through combinatorial control that include 1) the spatial and temporal expression of their ligands, (Sweeney and Carraway, 2000), 2) formation of different homo and hetero-dimer sets (Pinkas-Kramarski et al., 1996; Riese and Stern, 1998), and 3) activation of the intrinsic kinase domain resulting in specific phosphorylated tyrosine residues on the intracellular tails of the receptors (Schulze et al., 2005). The cell surface combinatorial control generates in turn signaling diversity which is defined by 1) the recruitment specificity of signaling molecules that bind their tyrosine phosphorylated CTT, determined by the amino acid sequence surrounding the phosphorylated residues (Csiszar, 2006), and 2) by the duration of the phosphorylation signal they display (Holbro and Hynes, 2004; Hynes and Lane, 2005; Jorissen et al., 2003; Pinkas-Kramarski et al., 1996; Warren and Landgraf, 2006). The specific intracellular phosphorylation patterns on the CTT have significant implications to intracellular signaling because they provide the “signature” through which specific cellular signaling components are recruited to initiate a biological output. The binding of effector/adaptor proteins containing phosphotyrosine binding domains, PTB or SH2, provide the first-line of signaling specificity (Klapper et al., 1999; Schulze et al., 2005; Songyang et al., 1995).

Combinatorial control of ErbB receptors allows for the activation of a vast signaling network involving different signaling pathways. These are mediated via the downstream effector molecules Ras, Raf, mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), Akt, phospholipase-C, Rho, and signal transducer and activator of transcription (STAT) (Morandell et al., 2008; Prenzel et al., 2001), and result in various cellular functions as diverse as proliferation, differentiation, apoptosis,
migration, and adhesion (van der Geer et al., 1994; Yarden and Sliwkowski, 2001).
Abnormal activation of these receptors has been implicated in various forms of human
cancers and is often the result of amplification, overexpression, and constitutive
activation of mutant receptors or autocrine growth factor loops (Hynes and Lane, 2005).

The Muc4-ErbB2 complex

As noted above, the interaction between Muc4 and ErbB2, along with the Muc4
role in potentiating ErbB2 phosphorylation provide the link through which Muc4 can
participate in intracellular signaling. This role of Muc4 has been examined in several
studies that were carried out in different systems and resulted in two different proposed
models depicted in figure 1-4; 1) the ligand-independent model, which suggests that
Muc4 induces ErbB2 phosphorylation without the addition of the ErbB3 ligand, but
through interaction with ErbB2 alone (Jepson et al., 2002; Ramsauer et al., 2006a), and 2)
the ligand-dependent model, which suggests that Muc4 can potentiates phosphorylation
of ErbB2 but requires the interaction with ErbB3 and the addition of the ErbB3 ligand
heregulin, also noted as the “quad-complex” (Muc4-ErbB2-ErbB3- HRG1β), (Carraway
et al., 1999; Funes et al., 2006; Jepson et al., 2002). In these models, Muc4 was referred
to as an intramembrane ligand for ErbB2, a term describing a special case of autocrine
receptor-ligand relationship in which in addition to being produced by the same cells, the
ligand and the receptor are also associated in the same membrane (Carraway and
Carraway, 2007). The basis for the usage of this term was established through studies
showing that the Muc4-ErbB2 complex formation requires co-expression of Muc4 and
ErbB2 in the same cell, that they form a stable complex, and that the EGF1 domain of
Muc4 is critical for this interaction (Carraway et al., 1999). In addition, this specific interaction resulted in potentiated ErbB2 phosphorylation on tyrosine 1139 and 1248 in the absence of soluble ligand (Jepson et al., 2002; Ramsauer et al., 2006a).

Figure 1-4. **Models of Muc4-ErbB2 complexes.**
A. Muc4 (green)-ErbB2 (red) ligand-independent model. B. Muc4-ErbB2-ErbB3 (purple)-NRG (orange) ligand-dependent “quad-complex.”
Adapted from Carraway *et al.* 2005

The mechanism for the ligand-dependent model was shown to result from the ability of Muc4 to maintain stable quad-complexes at the cell surface and interfere with the normal internalization of stimulated receptors (Funes et al., 2006). Based on our understanding of ErbB2-ErbB3 receptor activation mechanisms, the ligand-dependent model was readily accepted because it contained all the components necessary for receptor activation, *i.e.* an ErbB dimer and a soluble ligand. However, the ligand-independent model presented a challenge because Muc4 is not an ErbB member or a soluble EGF ligand; therefore it lacked two necessary components that are required for ErbB2 activation. To better understand the mechanistic role that Muc4 plays in the ligand-independent model, I analyzed the complex components and the cell surface
associated signaling potential of ErbB2 in the A375 human melanoma cells expressing
Muc4. This analysis is presented in chapter 2 of this work.

**Muc4-ErbB2 signaling**

Muc4 dependent changes on the CTT of ErbB2 predict that ErbB2 signaling
pathways will become activated, and that through identification of these pathways, the
role of Muc4 in “outside-in” signaling will be better understood. Both models (Figure 1-4)
were examined for changes in ErbB2 signaling in the A375 human melanoma cell line.
The results indicated that in the presence of heregulin, Muc4 enhances the recruitment of
PI3K to ErbB3, and augments ErbB2-ErbB3 signaling through the PI3K pathway (Funes
et al., 2006; Jepson et al., 2002), a regulator of cellular proliferation and survival
signaling. In the absence of heregulin, studies showed that Muc4 alone fails to induce
downstream phosphorylation of components in the PI3K pathway (Funes et al., 2006;
Jepson et al., 2002). One observation under this condition showed that Muc4 expression
enhances the expression levels of p27kip (Jepson et al., 2002), a cell cycle regulator shown
to modulate apoptosis (Sgambato et al., 2000), suggesting that through this molecule
Muc4 may participate in differentiation signaling. Thus the role of Muc4 in signaling in
the absence of heregulin was not clear. To better understand the role that Muc4 plays in
“outside-in” signaling, I used an antibody microarray screen to identify major signaling
molecules that are differentially regulated under Muc4 expression in the A375 cells. This
analysis is presented in chapter 3 of this work.
Hypotheses and aims

Previous findings suggested that Muc4 participates in signal transduction through its extracellular association with ErbB2. The purpose of this work was to analyze changes in ligand-independent ErbB2 signaling activity in response to Muc4 expression in order to better understand the role that Muc4 plays in “outside-in” signaling. Signaling analyses focused on ErbB2 signaling potentiation and on ErbB2 intracellular signaling pathways.

Two main questions were addressed:
1: What is the mechanism by which Muc4 can alter the phosphorylation state of ErbB2 without ligand treatment?
2: What are the signaling pathways that are activated in Muc4 expressing cells?

My hypothesis for the first question was that ErbB2 phosphorylation is induced without ligand through increased ErbB2 conformation stability at the membrane in response to Muc4 association, and through consequent increased ErbB2 signal duration. Regarding the downstream signaling pathways, my hypothesis was that Muc4 expression would result in sustained ErbB2 signaling pathways.
CHAPTER 2

MUC4-ERBB2 CELL SURFACE SIGNALING POTENTIAL ANALYSIS

Overview

The membrane mucin MUC4 is aberrantly expressed in numerous epithelial carcinomas and is currently used as a cancer diagnostic and prognostic tool. MUC4 can also modulate the receptor ErbB2 phosphorylation and potentiate its cellular signaling. To better understand the mechanism by which MUC4 modulates ErbB2 phosphorylation in the absence of a soluble ligand, I performed analyses of receptor complexes and signaling using a transient transfection of Muc4 construct into the A375 human melanoma and BT-474 breast cancer cell lines. Quantitative and comparative signaling modulations were evaluated by immunoblots using phospho-specific antibodies and densitometry analysis. Signaling complex components were identified by chemical cross-linking, fractionation by gel filtration, and immunoblots. The mechanistic analysis indicates that the Muc4 modulation phenotype is dependent on a direct interaction with the heterodimer ErbB2-ErbB3. This interaction results in a significant \( p=0.0002 \) increase in ErbB2 phosphorylation without changing the total receptor levels. This ErbB2 phosphorylation does not increase ErbB3 phosphorylation levels. Addition of the ErbB3 ligand heregulin does induce ErbB3 phosphorylation. The model suggests that in the absence of a soluble ligand, Muc4 acts as a stabilizing membrane component for the
ErbB2-ErbB3 complex. This work highlights Muc4 as a potent oncogenic-promoting factor, and further enhances our understanding of ligand-independent ErbB2 activation.

**Background**

Rat Muc4 is a heterodimeric glycoprotein that is synthesized from a single polypeptide precursor and cleaved early after synthesis, resulting in a tightly but non-covalently associated complex of two subunits, ASGP-1 and ASGP-2 (Rossi et al., 1996; Wu et al., 1994). Muc4 is classified as a cell surface mucin and is normally expressed in epithelial tissues where it protects epithelia by lubricating the surfaces and protecting them from infections and injuries (Hattrup and Gendler, 2008). Aberrant expression of Muc4 has been reported in a variety of human carcinomas (Yonezawa et al., 2008) where Muc4 is implicated in affecting cellular phenotypes that promote cancer development, including cell adhesion (Komatsu et al., 1997b), cell polarity (Ramsauer et al., 2003), promotion of oncogenesis (Karg et al., 2006; Moniaux et al., 2007; Ponnusamy et al., 2008), and cell signaling through modulation of the receptor ErbB2 phosphorylation (Funes et al., 2006; Jepson et al., 2002; Ramsauer et al., 2003; Ramsauer et al., 2006b).

The epidermal growth factor receptor ErbB2 belongs to the subclass I of the receptor tyrosine kinase super family. Other members of this subclass are ErbB1 (EGFR), ErbB3, and ErbB4. ErbB receptor signaling regulates large numbers of important cellular functions, including proliferation, differentiation, survival, adhesion and migration (Citri and Yarden, 2006b; Warren and Landgraf, 2006). These functions are mediated via an array of signaling pathways involving the downstream effector molecules Ras, Raf,
mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), Akt, phospholipase-C, Rho, and signal transducer and activator of transcription (STAT) (Morandell et al., 2008). In cancer, these receptors become constitutively activated as a result of autocrine ligand production, receptor overexpression, and/or mutations leading to aberrant cellular behaviors that promote neoplastic transformation and oncogenesis (Hynes and Lane, 2005).

Signaling through these receptors is complex. In general, ErbB receptor activation mechanisms are said to be affected by the spatial and temporal expression of ligand, formation of different homo and heterodimers, and activation of their kinase domains that result in specific tyrosine phosphorylation on their intracellular tails (Burgess, 2008; Linggi and Carpenter, 2006). However, unique structural features in ErbB2 and ErbB3, the closed ligand binding domain of ErbB2 (Garrett et al., 2003), and the inactive kinase domain of ErbB3 (Plowman et al., 1993), define ErbB2 and ErbB3 receptors as obligate heterodimers with a unique signaling relationship due to their inability to signal as homodimers. ErbB2 exhibits a dimerization competent conformation (untethered) in the absence of a soluble ligand, but cannot form active ErbB2 homodimers (Garrett et al., 2003), and ErbB3 assumes its dimerization competent conformation upon soluble ligand binding, but its enzymatically inactive kinase domain interferes with the formation of ErbB3 active homodimers. Thus ErbB2 and ErbB3 rely on complementary signaling where ligand-bound ErbB3 provides ErbB2 with a heterodimer partner, and ErbB2 provides the kinase activity.
The effect of Muc4 aberrant expression in human carcinomas on ErbB2 signaling modulation has been examined in several studies (Carraway et al., 1999; Funes et al., 2006; Jepson et al., 2002; Ramsauer et al., 2003; Ramsauer et al., 2006b), and has resulted in two proposed models (Figure 1-4). In the first model, Muc4 increases ErbB2 phosphorylation without the addition of the ErbB3 ligand in a mechanism that depends on Muc4-ErbB2 association, the soluble ligand-independent model. In the second model, Muc4 potentiates phosphorylation of ErbB2 but requires the addition of the ErbB3 ligand and formation of a stable quad complex, (Muc4-ErbB2-ErbB3- heregulin) (Jepson et al., 2002), the ligand-dependent model. The mechanism for the ligand-dependent model was shown to be the result of the ability of Muc4 to maintain stable quad-complexes at the cell surface and interfere with the normal internalization of stimulated receptors (Funes et al., 2006). Based on our understanding of ErbB2-ErbB3 receptor activation mechanisms, the ligand-dependent model was readily accepted because it contained all the components necessary for receptor activation, *i.e.* an ErbB dimer and a soluble ligand. However the ligand-independent model presented a challenge because Muc4 is not an ErbB member or a soluble EGF ligand; therefore it lacked two necessary components that are required for ErbB2 activation. To better understand the mechanistic role that Muc4 plays in the ligand-independent model, I analyzed receptor signaling and complex components of the two models. The mechanistic analyses show that the key to the Muc4 modulation phenotype is its direct association with ErbB2 and ErbB3, but the essential difference between these is that in the ligand-independent model, ErbB2 kinase activity results in significant ErbB2 phosphorylation without affecting ErbB3 phosphorylation. These results indicate that in the ligand-independent model, Muc4 serves as a stabilizing
membrane component for ErbB2 and ErbB3. This work suggests that Muc4 is a potent oncogenic-promoting factor and further enhances our understanding of ligand-independent ErbB2 signaling.

**Materials and Methods**

**Materials** - Reagents were obtained as follows: Human recombinant heregulin (EGF domain), and anti B-actin (clone AC-15) mouse monoclonal antibody from Sigma; Chemical cross linking reagents BS3 (bis [sulfo succinimidyl] suberate) and DTSSP (3,3’-Dithiobis[sulfosuccinimidylpropionate]) from Termo Scientific; Anti-erbB-2/HER-2 rabbit polyclonal IgG, anti-phospho-erbB-2/HER-2 (Tyr1248) rabbit polyclonal IgG, and anti-EGFR rabbit polyclonal IgG from Upstate; c-erbB-2/HER-2/neu AB-17 (clone e2-4001 +3B5) mouse monoclonal antibody from Lab Vision Neomarkers; Anti-c-ErbB2/c-Neu (Ab-5) mouse monoclonal antibody and Anti-c-ErbB2/c-Neu (Ab-3) Mouse monoclonal antibody (3B5) from Calbiochem; Phospho-HER3/ErbB3 (Tyr1289) (21D3) rabbit monoclonal antibody from Cell Signaling Technology; ErbB-3 (C-17) polyclonal antibody, and ErbB-4 (C-7) polyclonal antibody from Santa Cruz Biothechnology; Anti-human c-erbB-2 oncoprotein polyclonal rabbit from Dako; Anti-rabbit IgG and Anti-mouse IgG coupled to peroxidase secondary antibodies from Promega; Alexa Fluor fluorescent secondary antibodies from Molecular probes; FuGENE HD transfection reagents from Roche Applied Science; BT-474 (# HTB-20TM) breast cancer cell line from American Type Culture Collection; Ready-gels 4-15% Tris-HCl from Bio-Rad; Muc4 rCpep polyclonal rabbit antibody raised against the cytoplasmic tail of Muc4 and Muc4 monoclonal 4F12 antibody raised against Muc4β (ASGP-2) subunit have been previously
described (Rossi et al., 1996); Kinase inhibitor Lapatinib (GW572016) was a gift from GlaxoSmithKline.

**Cell Culture and Muc4 Induction/Transfection**- Construction of A375 stable clone expressing Muc4-Rep3 under tetracycline regulation (*tet-off*) has been previously described (Komatsu et al., 1997b). The Muc4-Rep3 clone contains the ASGP-2 (transmembrane) subunit of Muc4 and 3 tandem repeats from the ASGP-1 (mucin) subunit of Muc4. The stable clone was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% v/v penicillin/streptomycin, 0.4 mg/ml G418, 0.15 mg/ml hygromycin, and 2 µg/ml tetracyclin. To induce Muc4 expression, cells were grown to 60% confluence, rinsed three times with PBS, and then cultured for 48 h. in the absence of 2 µg/ml tetracycline. The BT-474 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% v/v penicillin/streptomycin. Transient transfections of Muc4-Rep1 construct (Komatsu et al., 1997b) were carried out for 48 h. using FuGene HD reagent as recommended by the manufacturer. Cells were serum-starved overnight (0.1% fetal calf serum) prior to growth factor treatment. All cells were incubated at 37°C with 5% CO2.

**Analysis of Receptor Phosphorylation**- The Muc4 effect on receptor phosphorylation was carried out in serum starved cells (24 h.) and under different conditions including 50 nM heregulin (saturating concentration) treatment for various times at 37°C, 50 µM (sodium orthovanadate) phosphatase inhibitor treatment for 2 h., and 0.2 µM kinase inhibitor (Lapatinib-GW572016) treatment for 6 h. Cells grown in
100 mm dishes, were rinsed twice with ice-cold PBS saline after the different treatments, and lysed in 250 µl lysis buffer, pH 7.2 (20 mM MOPS, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM B-glycerophosphate pH 7.2, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyfluoride, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin, and 1 mM dithiothreitol). Cells were scraped, collected, sonicated (3 Amplitude Microns on ice for 5 seconds, 2 cycles), and lysates cleared by centrifugation at 14,000 rpm for 30 min. at 4° C and then added to SDS-PAGE sample buffer. Lysates were resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Membranes were blocked in 5% non-fat dry milk in TBST overnight, incubated with primary antibody for 2-4 h., washed three times in TBST, incubated in secondary antibody in 5% non-fat dry milk in TBST for 1 h., and washed three times in TBST. Phospho-specific signaling proteins were immunoblotted (IB) as recommended by the respective manufacturers. Blotted proteins were visualized by western lighting chemiluminescence reagent (PerkinElmer) and visualized on Blue Lite Autorad Film (BioExpress).

Quantitative and Statistical Analysis- To evaluate quantitative changes in signaling in response to Muc4 expression, at least three samples were analyzed on the same gel in order to reduce background error. Using densitometry software (ImageJ NIH) signals were quantified along with control bands that provided the baseline for signal normalization. Data were expressed as the mean ± standard deviation for a series of at least 3 experiments. Student’s t test was used to compare mean values as
appropriate. \( P \) values < 0.05 were considered to represent significant differences (ImageJ NIH).

**Immunofluorescence**- To examine the relevant receptor expression and localization in the model system, human melanoma A375 induced cells (70% confluence) on 25-mm round cover slips were fixed in 4% paraformaldehyde for 20 min. and permeabilized for 10 min. in PBS plus 0.15% Saponin. Antibodies were diluted in PBS with 1% BSA and incubated for 1 h. Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse were used as secondary antibodies. Slides were mounted in ProLong Gold antifade solution (Invitrogen) and visualized on a Leica DMRB microscope.

**Chemical Cross Linking and Analysis**- To examine the role of Muc4 in forming direct stable complexes with the ErbB receptors, and to determine the components that make the complexes in each of the models, cellular cross-linking studies were carried out using a non-cleavable cross linker following IB analysis, and a cleavable chemical cross linker following size exclusion purification of complexes, cleavage, and IB analysis. A375 cells (60-70% confluence) were rinsed three times with PBS and then incubated with 1 mM BS3 dissolved in 20 mM sodium phosphate, 0.15 M NaCl buffer, pH 7.5 for 2 h. on ice. The reaction was quenched by adding 1 M Tris-HCl, pH 7.5 to a final concentration of 20-50 mM Tris, and incubated at room temperature for 15 min. For cleavable chemical cross linking, 1 mM DTSSP dissolved in water was added to cells for 2 h. on ice, and the reaction was stopped by adding 1 M Tris, pH 7.5 to a final
concentration of 10-20 mM and incubated at room temperature for 15 min. Cross-linked cells were lysed in 250 µl lysis buffer without phosphatase inhibitors and without dithiothreitol in the DTSSP treated samples. Cells were scraped, collected, sonicated, and the lysate was cleared by centrifugation at 14,000 rpm for 30 min. at 4° C. The supernatant was directly loaded by FPLC (1 mL/min) onto a high resolution Superose 6 analytical gel filtration column equilibrated at 4° C in lysis buffer without Triton X-100 or inhibitors. The column run was programmed for flow consistency and uniform elution profile. Different proteins in the lysate elute based on their size and were collected as 1 ml fractions. Protein fractions were then TCA precipitated, loaded with 5% β-mercaptoethanol containing SDS-PAGE sample buffer and run on 4-15% gradient gels (Bio-Rad) for IB analysis.

Results

Model System Characterization- In order to better understand the mechanistic role that Muc4 plays in the ligand-independent model, Muc4 and the ErbB receptor levels had to be carefully considered in the model system. The A375 human melanoma adherent cell line does not express Muc4, therefore allowing the examination of changes in ErbB signaling in response to regulated Muc4 expression. The receptors of interest, ErbB2 and ErbB3, are endogenously expressed, and the receptor ErbB4 is conveniently absent, allowing for an unobstructed comparative analysis of the heregulin ligand treatment to which both ErbB3 and ErbB4 receptors can bind, albeit to ErbB4 with lower affinity (Jones et al., 1999). The laboratory has generated several A375 stable clones expressing Muc4 under the tetracycline off regulation system (Komatsu et al., 1997b). All of these
clones express the transmembrane subunit of Muc4 (ASGP-2), but differ in the number of tandem repeats they contain from the mucin subunit (ASGP-1), *i.e.* Rep3, Rep5, and Rep8. Although all clones robustly express Muc4 upon induction, the Rep3 clone was used primarily in this study because it was utilized in the previous studies that established the ligand-independent model, and it did not exhibit leakiness. Figure 2-1 A shows by immunoblotting that Muc4 induction in this clone is robust and that ErbB2 and ErbB3 are endogenously expressed. Figure 2-1 B shows immunofluorescence analyses that further establish receptor expression and co-localization as indicated by the overlay panel. The other Muc4 free cell line that was used was the BT-474 breast cancer cell line. The Muc4-Rep1 construct was introduced to this line using transient transfection in the kinase inhibitor experiments.

**Figure 2-1. The adherent, A375 melanoma cell line model system.** A. A375 Muc4-Rep3 cells turned off and on for 48 h. and serum starved (0.1% FBS) for 24 h. Cell lysates were blotted with antibodies to ErbB2, Muc4, and ErbB3. B. A375 cells were fixed and incubated with antibodies to the indicated receptors and visualized by Immunofluorescence microscopy on a Leica DMRB. Scale bar 50 µm.
Muc4 activation of ErbB2 Phosphorylation in the Absence of heregulin treatment:

Muc4 Expression Significantly Augments Phospho-ErbB2 Signal - It was previously shown that Muc4 expression in the absence of soluble ligand treatment can result in an elevated ErbB2 phosphorylation signal in different systems (Jepson et al., 2002; Ponnusamy et al., 2008; Ramsauer et al., 2006b). However, the extent of observed changes in phospho-ErbB2 varied, thus I carried out a quantitative analysis of the Muc4 effect on phosphorylation of ErbB2 in order to assess whether the Muc4 effect is significant. Immunoblots and quantitative analyses of phospho-ErbB2 (Y1248) and total ErbB2 band intensity levels in response to Muc4 induction were carried out by densitometry analysis in triplicate samples. Figure 2-2 A shows the immunoblot results for the Muc4-Rep3 clone, indicating that the phospho-ErbB2 band intensity changed markedly upon Muc4 induction. Note that the total ErbB2 receptor levels did not change upon Muc4 induction across all 6 lanes ($\sigma=36.55 \pm 1.10$). In addition, Muc4 induction resulted in near phospho-ErbB2 signal saturation, as reflected by the ratio of phospho-ErbB2 (On)/total ErbB2 (0.72), evaluated with antibodies that are raised to the same region on ErbB2. Figure 2-2 B quantitative densitometry shows that Muc4 treatment resulted in a 2-fold increase of the phospho-ErbB2 signal. The Student’s t test of significance indicated that this change was extremely significant ($p=0.0002$). Muc4-Rep1 transient expression in the BT-474 cell line shown in Figure 2-6, also resulted in a robust increase in the phospho-ErbB2 signal. These results indicated that Muc4 expression enhances phospho-ErbB2 significantly.
A. Muc4 expression augment ErbB2 phosphorylation signal significantly ($p=0.0002$). A375 Muc4-Rep3 cells turned off and on for 48 h. and starved (0.1% FBS) for 24 h. A. Triplicate samples of cell lysates under each condition were blotted with antibodies to Muc4, pY-ErbB2 (Y1248), ErbB2, and actin. B. Quantitative analysis of pY-ErbB2 signal (Y1248). Using densitometry software (ImageJ NIH), phosphotyrosine signals were normalized with actin signal and then expressed as the mean ± standard deviation for a series of at least 3 experiments. Student’s $t$ tests were used to compare mean values as appropriate. $P$ values < 0.05 were considered to represent significant differences (ImageJ NIH).
Muc4 Alone Has No Effect on ErbB3 Phosphorylation - The elevated ErbB2 phosphorylation signal is believed to occur in response to formation of active ErbB2 heterodimers with ligand bound ErbB members, resulting in functional units that display phosphorylation on the intracellular tails of both receptors (Holbro et al., 2003). In the model, the most likely active ErbB2 heterodimer unit is ErbB2-ErbB3. Because I observed elevated ErbB2 phosphorylation in the absence of soluble ligand treatment and without changes in total ErbB2 receptor levels, I considered the possibility that Muc4 expression induced ErbB2 in response to autocrine expression of ErbB3 ligand, resulting in active ErbB2-ErbB3 heterodimer. To test this possibility, I examined the ErbB3 phosphorylation, anticipating that autocrine ligand expression in the system will result in ligand-bound ErbB3 receptor which can then bind ErbB2 and result in the elevated ErbB2 phosphorylation I observed. The immunoblot in Figure 2-3 shows that Muc4 expression has no effect on ErbB3 phosphorylation, while the positive control lanes of samples treated with the ErbB3 soluble ligand heregulin do generate phospho-ErbB3 signal upon stimulation for 3 min. These results suggested that Muc4 augments ErbB2 phosphorylation by a unique mechanism that is independent of ErbB3 phosphorylation or ErbB2-ErbB3 active dimer formation, and that the Muc4 effect was not a result of autocrine ligand expression.
Figure 2-3. **Muc4 expression does not lead to ErbB2 phosphorylation of ErbB3 in the absence of ErbB3 ligand.** A375 Muc4-Rep3 cells turned off and on for 48 h. and serum starved (0.1% FBS) for 24 h. Cells were treated with heregulin ligand at [50 nM] for 3 min. Triplicate samples of cell lysates under each condition were blotted with antibodies to phospho-ErbB3 (Y1289), ErbB3, and actin.

**Muc4 Expression Results in Large Muc4-ErbB2-ErbB3 Containing Complexes—**

The lack of changes in phospho-ErbB3 phosphorylation in response to Muc4 expression was surprising because it suggested that Muc4 results in elevated ErbB2 phosphorylation by a unique mechanism. To verify that ErbB2 and ErbB3 form a complex, and to further understand the mechanism by which Muc4 potentiates phospho-ErbB2 without affecting phospho-ErbB3, I carried out cross-linking analyses. I considered both a direct and an indirect Muc4 effect. In a direct effect, Muc4 expression may result in the formation of stable complexes with ErbB2 and ErbB3, and in an indirect effect, Muc4 expression may change the association dynamics between the receptors ErbB2 and ErbB3 by possibly driving ErbB2 homodimer formation by two-dimensional molecular crowding and/or by sterically interfering with ErbB3 co-localization with ErbB2. I started the analysis to distinguish between these possible Muc4 mechanisms by cross-linking cells expressing
Muc4 with BS3, a water-soluble, non-cleavable, membrane-impermeable chemical cross-linker with a spacer arm length of 11.4 Å. I predicted that the cross-linked Muc4-expressing samples would result in changes in the immunoblot pattern of Muc4, ErbB2, and ErbB3, possibly through generation of additional, unique slower mobility bands, which will help in differentiating between these scenarios. In Figure 2-4 the arrows point to the additional slower mobility unique bands that were observed in Muc4, ErbB2 and ErbB3 immunoblots from the cross-linked samples. These slowest-migrating bands suggested that Muc4 expression resulted in Muc4-ErbB2-ErbB3 complex formation since cross-linked products of slow mobility at the same position on the gels were observed in all immunoblots (Muc4, ErbB2, and ErbB3).

Figure 2-4. Muc4 expression facilitates formation of unique ErbB2, ErbB3 and Muc4 cross-linked products. A375 Muc4-Rep3 cells turned off and on for 48 h. and serum starved (0.1% FBS) for 24 h. were crossed linked with the chemical cross linking agent BS3 [1 mM] for 2 h. at 4°C. Cell lysates were blotted with antibodies to Muc4, ErbB2, ErbB3, and actin. Yellow arrows indicate cross-linked products.
To further establish the direct Muc4 mechanism effect, I proceeded to characterize these large complexes by identifying their composition. I repeated the cross-linking experiment with a reversible chemical cross linker analogue, DTSSP, and included a fractionation step by gel filtration chromatography on a Superose6 column prior to cleavage and immunoblot analysis of samples, in order to examine the composition of the relevant complexes. Evidence of Muc4 in unique fractions, *i.e.* without ErbB2 and ErbB3, would support an indirect Muc4 mechanism, while evidence of Muc4 in the same fractions as ErbB2 and ErbB3 would support a direct Muc4 mechanism. The overall gel filtration fractionation profiles of cross-linked Muc4-Off vs. Muc4-On samples were similar; however, a higher protein concentration was observed in the early fractions of Muc4-On samples within the void volume of the column and extending through fraction 16 (Figure 2-5 A). This observation showed that the Muc4-On samples contained more large complexes since the different proteins in the lysates elute based on their size. The eluted fractions were TCA-precipitated, and the disulfide cross-links cleaved prior to immunoblot analysis. The results in Figure 2-5 B show that in the Muc4-Off and Muc4-On samples, ErbB2 and ErbB3 were eluted together (fractions 9-10 ml), suggesting that ErbB2-ErbB3 dimers form and that Muc4 is not interfering with the receptor co-localization. In the Muc4-On sample, Muc4 was eluted in the same fractions (8-10 ml.) that were observed for the receptors, indicating that Muc4 expression results in Muc4-ErbB2-ErbB3 complexes, and further supporting a direct role for Muc4 in the mechanism by which it potentiates phospho-ErbB2 signaling.
A.

**Figure 2-5. Muc4 expression results in large Muc4-ErbB2-ErbB3 complexes.** A375 Muc4-Rep3 cells turned off and on for 48 h. and serum starved (0.1% FBS) for 24 h. were crossed linked with the reversible chemical cross linking agent DTSSP [1 mM] for 2 h. Cell lysates were fractionated using gel filtration chromatography on Superose 6 column, collected fractions were TCA precipitated and cleaved, and samples were blotted with the indicated antibodies. A. Superose 6 gel filtration fractionation profile of A375 Muc4-Rep3 cells turned off and on. B. Processed fractions (as described above) from Muc4-On and Muc4-Off were blotted with antibodies to Muc4, ErbB2, and ErbB3.
Muc4 Potentiates the Activity of the ErbB2 Kinase- The preceding results suggested that Muc4 potentiates phospho-ErbB2 significantly without effecting phospho-ErbB3 signaling by a direct mechanism that involves Muc4-ErbB2-ErbB3 complex formation. Next, I wanted to investigate the mechanistic role of Muc4 in affecting phospho-ErbB2 kinase activity. Two possibilities were considered. 1) Muc4 association with ErbB2 facilitates ErbB2 autocatalytic activity leading to increased phospho-ErbB2. 2) Muc4 facilitates an increase in phospho-ErbB2 by trapping ErbB2 at the membrane and allowing other intracellular kinases to phosphorylate the ErbB2 cytoplasmic tail. The essential difference between these two mechanisms is that the first requires ErbB2 kinase activity, while the second does not. With regard to the Muc4 mechanism, the kinase-dependent effect implies that Muc4 stabilizes the active ErbB2 receptor, whereas the kinase independent mechanism implies a membrane-trapping role for Muc4. To distinguish between these mechanisms, I examined the effect of ErbB2 kinase inhibition on the ability of Muc4 to promote ErbB2 phosphorylation. I used the kinase inhibitor Lapatinib, a reversible dual inhibitor of EGFR and ErbB2 autophosphorylation and activation, in the breast cancer cell line BT-474. The BT-474 line was used in this experiment because it was established as a Lapatinib sensitive cancer cell line at relatively low Lapatinib concentrations (Hegde et al., 2007). In addition, BT-474 has no detected endogenous Muc4 expression. The Muc4 plasmid is readily transfected into this cell line, resulting in ErbB2 phosphorylation similar in response to Muc4 (Figure 2-6) as was observed in A375-Rep3 (Figure 2-2). The results in Figure 2-6 indicate that kinase inhibition represses the increase in phospho-ErbB2 induced by Muc4. These data suggest that Muc4 acts through potentiation of ErbB2 autocatalytic activity.
Muc4 increase in ErbB2 phosphorylation signal is ErbB2 kinase activity dependent. BT-474 cells transiently transfected with Muc4-Rep1 plasmid for 48 h. and serum starved (0.1% FBS) for 24 h. were treated with the kinase inhibitor Lapatinib [0.2 µM] for 6 h. Cell lysates were blotted with antibodies to Muc4, phospho-ErbB2 (Y1248), ErbB2 and actin.

Muc4 modulation of ErbB Phosphorylation in the Presence of heregulin

Muc4 Stabilizes Phospho-ErbB2 Signal in Cells Treated with Heregulin – The preceding results suggested that Muc4 expression resulted in a significant change in ErbB2 signal intensity (Figure 2-2) in a mechanism that depended on stable interaction of Muc4 with the active ErbB2 receptor (Figure 2-6). These data raised the possibility that Muc4 might also change ErbB2 signal duration, an important factor in cell surface signaling output (Holbro and Hynes, 2004; Jorissen et al., 2003; Warren and Landgraf, 2006). Ideally, this could be tested by comparing ErbB2 signal duration in response to Muc4 with another ErbB2 stimulating ligand. The best choice of stimulating ErbB2 ligand in the system is heregulin because treatment with heregulin leads to the formation of active ErbB2-ErbB3 heterodimers resulting in phosphorylation on both receptors.
However, such a signal duration comparison is not valid because Muc4 expression requires 24-48 hours of processing time by transient transfection or induction before changes in ErbB2 phosphorylation can be observed, whereas heregulin stimulation is rapid, resulting in changes in ErbB2 phosphorylation within minutes of addition to cells. I therefore chose to study changes on ErbB2 signal duration by comparing ErbB2 signals in cells treated with heregulin ligand (human recombinant EGF domain) and cells expressing Muc4 and treated with heregulin. The immunoblot analyses in Figures 2-3 and 2-7 A (circles) indicate that the model system indeed resulted in the expected active ErbB2-ErbB3 heterodimers upon ligand treatment even after 1 min. incubation. A comparison of the generated ErbB2 phosphorylation signal however indicates that the ErbB2 signal duration was different. In the Muc4-Off cells treated with heregulin at saturating concentration, a short-lived signal was generated, peaking at the 5 min. time point and decreasing to almost background levels by 11 min. (Figure 2-7 circles). In the Muc4-On cells treated with heregulin, a stable ErbB2 phosphorylation signal was generated with increasing heregulin incubation time (Figure 2-7 squares). This result indicated that both ErbB2 phosphorylation signal intensity and signal duration factors were affected by Muc4 expression, increasing the duration of the phospho-ErbB2. Because alterations in receptor signaling duration are implicated in aberrant cellular proliferation and oncogenic phenotypes, these results indicate that Muc4 can act as a potent cell-surface oncogenic signaling modulator for the receptor ErbB2.
Figure 2-7. **Muc4 stabilizes ErbB2 phospho-tyrosine signal in cells treated with heregulin ligand.** A375 Muc4-Rep3 cells (off or on) were starved (0.1% FBS) and treated with heregulin [50 nM] for the indicated times. Quantitative signal density analysis of triplicate phospho-ErbB2 (Y1248) IB signal is shown. Using densitometry software (ImageJ NIH), phospho-tyrosine signal was normalized with actin signal and then expressed as the mean ± standard deviation for a series of at least three experiments. Circles represent Muc4-Off cells treated with ligand; squares represent Muc4-On cells treated with ligand, and triangles, Muc4-Off cells treated first with Na$_3$VO$_4$ [50 µM] for 2 h. and then with ligand.

**Muc4-generated Phospho-ErbB2 Signal Pattern is Unique**- The stable ErbB2 phosphorylation signal pattern that was observed in Muc4 expressing cells treated with heregulin is similar to a pattern that is observed in systems where down regulation mechanism is lost. A common mechanism for regulating receptor tyrosine kinase signaling involves dephosphorylation. To address the possibility that Muc4 blocks
phosphatase activity, I sought to generate a similar stabilized phospho-ErbB2 signal by using phosphatase inhibition of Muc4-Off cells treated with heregulin. Although the phosphatase inhibition increased phospho-ErbB2 at an early time point, it did not increase the duration of the phosphorylated receptor (Figure 2-7 triangles). These results suggested that the phospho-ErbB2 was not stabilized by Muc4 interference with phosphatase activity, but due to another Muc4 mechanism, consistent with the earlier kinase inhibition analysis indicating that Muc4 stabilizes the active ErbB2 receptor.

**Discussion**

ErbB receptor signaling is diverse and complex, producing a wide range of biological outputs by a mechanism that involves specific receptors and ligand combinations (Prenzel et al., 2001; Warren and Landgraf, 2006). Constitutive activation of these receptors leads to aberrant cellular behaviors that promote neoplastic transformation and oncogenesis. Aberrant Muc4 expression and ErbB receptor activation have been linked by studies showing that Muc4 can potentiate ErbB2 and ErbB3 phosphorylation in systems treated with the ligand heregulin (Carraway et al., 1999; Funes et al., 2006). In the present study, I examined the role of Muc4 in potentiating ErbB2 phosphorylation in a ligand-independent manner.

Previous observations in support of the ligand-independent model include an increase in phospho-ErbB2 signal in several cell lines co expressing Muc4 (Jepson et al., 2002; Ramsauer et al., 2003; Ramsauer et al., 2006b), co-immunoprecipiation of ErbB2 with Muc4, and identification of the critical region within Muc4 that is required for this association (Carraway et al., 1999). I started the test of this model by first establishing
that the ErbB2 effect of Muc4 is significant. I showed that Muc4 can promote significant ErbB2 phosphorylation without the involvement of soluble ligand and without changing ErbB2 receptor levels.

Next, I characterized the ErbB components that are involved in this signaling. I showed that Muc4 forms a complex with both ErbB2 and ErbB3. This finding improved the previous ligand-independent model (Kermit L. Carraway, 2007; Ramsauer et al., 2006b) by providing added details regarding the complex components (i.e. ErbB3) and by providing information about the signaling potential of ErbB2 and the phosphorylation state of ErbB3. Lack of ErbB3 phosphorylation in this complex was surprising because ErbB2-ErbB3 association predicts that ErbB2 phosphorylation will also result in ErbB3 phosphorylation, and because ErbB3 is readily phosphorylated when the ligand heregulin is added (Tao and Maruyama, 2008). These results suggest that in this model, Muc4 and ErbB3 provide the conformation stability that is required for ErbB2 phosphorylation.

The comparative analyses of the phospho-ErbB2 signal under different treatments indicate that Muc4 also effects ErbB2 signal duration. I showed that Muc4 stabilizes the phospho-ErbB2 signal with time, in agreement with the suggested Muc4 mechanism in the ligand-dependent model indicating that Muc4 interferes with the normal internalization process of stimulated receptors by retention of receptors at the cell surface (Funes et al., 2006). I also showed that phospho-ErbB2 signal stability is not mediated via a mechanism that involves blocking phosphatase action, but via the ability of Muc4 to potentiate stable kinase activity of ErbB2. Together these findings indicate that Muc4 is not merely a membrane-trapping component but a potent potentiation factor of stable ligand-independent ErbB2 activity.
The model in Figure 2-8 depicts Muc4 effect on ErbB signaling in the presence and absence of heregulin, portraying that the essential difference between these is that in the presence of ligand, ErbB2 and ErbB3 phosphorylation is potentiated, and in the absence of ligand, only ErbB2 phosphorylation is affected. Thus the model indicates that Muc4 potentiates ErbB2 phosphorylation in a ligand-independent manner.

**Figure 2-8. Structure-based model of ERBB2 and ERBB3 receptors association and signaling without Muc4 (top) and with Muc4 (bottom).** A. The structure of untethered ERBB2 monomer in green (1), tethered ERBB3 monomer in orange (2), receptors co-localization in the absence of ligand (3), and receptors active dimer in the presence of a ligand (4). B. The structure of Muc4 transmembrane subunit with truncated mucin subunit (1), Muc4 association with the receptors in the absence of a ligand (2), and Muc4 association with the receptors in the presence of a ligand (3).
The mechanistic analyses suggest that the Muc4 effect is mediated through its ability to stabilize the heterodimer ErbB2-ErbB3. The exact membrane interaction of Muc4 with the heterodimer is not clear at this time, but studies of ligand-independent activity of isolated receptor domains treated with reagents that induce receptors oligomerization suggest that several possibilities exist (R. Bose, 2009). One, is that this effect is mediated via extracellular domain interaction, also in agreement with earlier Muc4-ErbB2 association studies indicating that the N-terminal EGF-1 domain of Muc4 extracellular domain is critical for co-association with ErbB2 (Carraway et al., 1999).

Another possibility, is that the Muc4 effect is mediated by stable transmembrane (TM) complex association. The presence of GXXXG motifs within the TM domains of ErbB2, ErbB3 and Muc4 which are implicated in facilitating ligand-independent ErbB activity and stability (Mendrola et al., 2002; Samna Soumana et al., 2007; Sharpe et al., 2000; Tanner and Kyte, 1999), suggests that this motif might also contribute to a stable signaling complex. Finally, other not well-described associations, or a combination of the above noted possibilities, might also contribute to this Muc4 effect. An integrated, detailed view of all of the receptors’ parts through studies of the intact ErbB structure will help increase our understanding of the regulation of this allosteric receptor family (Lemmon, 2008), and help provide a more detailed Muc4 role in ligand-independent ErbB signaling.
CHAPTER 3
ANALYSIS OF MUC4-ERBB2 DEPENDENT INTRACELLULAR SIGNALING PATHWAYS

Background

The rat membrane mucin Muc4 is a heterodimeric glycoprotein complex that is normally expressed in epithelial tissue. The heterodimer complex is a product of a precursor protein that undergoes proteolytic cleavage early after synthesis, resulting in a non-covalent but tightly associated complex of two subunits ASGP1 and ASGP2. The ASGP1 subunit is exclusively extracellular. The ASGP2 subunit is a single pass type-I membrane protein that is mostly extracellular, and displays a short cytoplasmic tail of 23 amino acid residues. Both subunits carry numerous glycosylation sites, which significantly alter the mass of the complex with glycosylation, resulting in a large membrane bound extracellular complex. The human orthologue MUC4, exhibits significant structure and domain organization homology, and is similarly positioned in the membrane with its subunits, MUC4α and MUC4β.

Muc4 expression has been reported in a variety of human cancers, and this has been associated with cellular processes that promote epithelial tissue differentiation and cancer development including survival, anti adhesion and anti-apoptotic properties. Moreover, Muc4 potentiates ErbB2 phosphorylation and also interferes with the treatment of herceptin antibody binding to ErbB2 (Nagy et al., 2005). ErbB2 is an epidermal growth factor receptor tyrosine kinase that contributes to the malignancy of
breast, ovarian and other tumor types (Moasser, 2007). ErbB2 and other members of its subclass, ErbB1 (EGFR), ErbB3 and ErbB4, have been implicated in signaling that regulate several important cellular functions including proliferation, differentiation, survival, adhesion, and migration via the well established MAPK, PI3K, PCK, Rho, and STAT signaling pathways (Morandell et al., 2008). All members of this subclass are type I single pass receptors with an Extracellular ligand binding domain and a cytoplasmic portion containing a kinase domain and a C-terminal tail with multiple tyrosine residues and other binding motifs that serve as the binding domain for cytoplasmic molecules that participate in its downstream signaling pathways.

Several studies have focused on the role of Muc4 in ErbB2 signaling, and this has resulted in two different models representing different tumor types. One model indicates that Muc4 potentiates ErbB2 and ErbB3 phosphorylation in response to the ligand heregulin by forming a quad complex, Muc4-ErbB2-ErbB3-heregulin (Carraway et al., 1999; Funes et al., 2006). This model represents systems in which autocrine expression of heregulin play a role in the activation state of ErbB2 and ErbB3 and in turn in potentiating survival signaling via the PI3K pathway. The other model indicates that Muc4 potentiates ErbB2 phosphorylation without heregulin by forming a stable direct Muc4-ErbB2-ErbB3 complex, as established in chapter 2 of this work. This model represents a system in which Muc4 expression can act to increase and stabilize the phosphorylation of ErbB2, independent of heregulin autocrine production or other ligands that can result in ErbB3 phosphorylation. The essential difference between these models regarding intracellular signaling is evident on the cytoplasmic display that is
produced in each model. The analysis of tyrosine phosphorylation of the complexes generated by both models suggests that in the heregulin-dependent complex several phosphorylated tyrosine residues on both ErbB2 and ErbB3 are present, and in the heregulin-independent complex fewer phosphorylated tyrosine residues on ErbB2 alone are observed. The primary function of the phosphorylated tyrosine residues on the cytoplasmic tail of the receptors is to recruit effector/adaptor proteins and initiate signaling pathways from the cytoplasmic surface of the plasma membrane into the cell (Csiszar, 2006). Thus, differences in tyrosine phosphorylation on the cytoplasmic tails of these complexes predict the recruitment of a different set of cytoplasmic components, possibly initiating different cellular signaling pathways.

In agreement with this prediction, Muc4-ErbB2 signaling studies in the A375 human melanoma cell line indicate that Muc4 expression stimulates different ErbB2 signaling pathways in the presence and absence of heregulin. In the presence of heregulin, Muc4 acts to enhance phosphorylation of signaling components in the protein kinase B/Akt in the PI3K pathways and the MAPK pathways, as evident by the analysis of changes in the phosphorylation levels of the major components in these pathways (Funes et al., 2006; Jepson et al., 2002). In the absence of heregulin, studies showed that Muc4 alone fails to induce phosphorylation of the MAPK pathways or of proteins in the PI3K pathway (Funes et al., 2006; Jepson et al., 2002). Thus the results of Muc4 signaling in the absence of heregulin presented a challenge, and the Muc4 effect on pY1248-ErbB2 downstream signaling was not clear. One clue came from a study which showed that Muc4 expression enhances the expression of p27kip (Jepson et al., 2002), a cell cycle regulator shown to modulate apoptosis (Sgambato et al., 2000), suggesting that
through this molecule Muc4 may participate in differentiation signaling. In an effort to better understand the Muc4 associated signaling pathways downstream of pY1248-ErbB2 in the absence of heregulin, I used an antibody microarray analysis. The aim was to identify the major signaling molecules that are differentially regulated under this treatment and to classify these into signaling pathways in order to learn more about the role that Muc4 plays in oncogenesis. Such analysis is also beneficial in identifying targets for drug treatments. The analysis surveyed over 650 signaling components from the major established signaling pathways, and probed both phosphorylated and total levels of proteins. Using the A375 human melanoma cell line as a model system, I compared signaling hits between cells expressing Muc4 and cells not expressing Muc4. The screen generated 18 hits that were at least two fold different between the Muc4 expressing cells and the control cells. Using established signaling pathway maps along with experimental data on ErbB2 tyrosine phosphorylation under this condition, i.e. pY1139, and pY1248 (Ramsauer et al., 2006a), I analyzed the signaling pathways that are activated downstream of this complex. The analysis indicates that Muc4 acts to negatively regulate signaling pathways that promote cell-cell adhesion and cell-extracellular matrix adhesion via the adherens junction pathway and the focal adhesion pathway respectively. Implication for Muc4 involvement with the activation of Rho-GTPases signaling is also suggested via the non-canonical planer cell polarity Wnt signaling pathway.
Materials and Methods

Cell Culture and Muc4 Induction- Construction of A375 stable clone expressing Muc4-Rep3 under tetracycline regulation (tet-off) has been previously described (Komatsu et al., 1997b). The Muc4-Rep3 clone contains the ASGP-2 (transmembrane) subunit of Muc4 and 3 tandem repeats from the ASGP-1 (mucin) subunit of Muc4. The stable clone was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% v/v penicillin/streptomycin, 0.4 mg/ml G418, 0.15 mg/ml hygromycin, and 2 µg/ml tetracycline. To induce Muc4 expression, cells were grown to 60% confluence, rinsed three times with PBS, and then cultured for 48 h. in the absence of 2 µg/ml tetracycline. Cells were incubated at 37°C with 5% CO2.

Antibody microarray analysis with Kinexus™ Bioinformatics Corporation

1) Antibody microarray analysis standard screen (KAM-1.1)- This screen surveys 650 signaling proteins in duplicate in two samples on the same microarray slide and provides an output that includes qualitative and semi-quantitative analyses of the expression and phosphorylation states of protein kinases and cell signaling proteins. The qualitative analyses includes TIFF Files of immunoblots optimized to reveal band shifts in signaling proteins, and the quantitative analyses show the strength of an enhanced chemiluminescence signal for each target protein and is provided in a Microsoft Excel spreadsheet. This screen is recommended by the Kinexus™ Company as the initial step in signaling analysis.

Sample preparation: Culture media was removed from A375 cells (1x10^6 to 2x10^6) expressing Muc4 for 48 h. and from control samples. Cells were rinsed twice with ice-
cold PBS, and lysed in 200 µl lysis buffer, pH 7.2 (20 mM MOPS, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM B-glycerophosphate, pH 7.2, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyfluoride, 3 mM benzamidine, 5 µM pepstatin A, 10 µM leupeptin, and 1 mM dithiothreitol). Cells were scraped in lysis buffer, collected, and transferred into a 1.5 ml micro centrifuge tube. Cells were sonicated four times for 10 seconds each time on ice and then spun at 90,000 g for 30 min at 4°C in an ultracentrifuge. The resulting supernatant fractions were transferred into a 1.5 ml micro centrifuge tube, and the protein concentration was assayed using standard protocol for Bradford. The samples concentration was adjusted to 2 mg/ml and shipped on dry ice.

2) Antibody microarray custom (multi-Antibody) screen (KCPS 1.0)-This screen tests 18 antibodies of choice from over 650 antibodies, and provides a follow up validation study for hits that were identified in the standard initial screen.

Sample preparation: Samples for this analysis were generated at the same time as the samples for the preceding screen and stored in a -80°C freezer. Before shipment, samples were thawed on ice and boiled with SDS-PAGE sample buffer. The samples’ final concentration was 1 mg/ml, and samples were shipped at room temperature in a 1.5 ml Eppendort screw cap vials.

Database analysis tools- To analyze signaling pathways, the KEGG pathway site was used. This site provides a collection of manually drawn pathway maps representing our current knowledge of molecular and reaction networks for several cellular processes and is located at: http://www.genome.jp/kegg/pathway.html.

To analyze the binding motifs on the C-terminal tails of ErbB2, ErbB3 and Muc4 I used
the proteomic tool ELM site, a eukaryotic linear motif resource for predicting functional sites, located at http://elm.eu.org.

To analyze putative phosphorylation sites on the C-terminal of ErbB2, ErbB3, and Muc4 C-terminal tails I used the NetPhos server, a prediction of Ser., Thr. and Tyr. phosphorylation sites in eukaryotic proteins located at http://www.cbs.dtu.dk/services/NetPhos.

To examine kinase specific phosphorylation sites on a specific protein substrate in a pathway, I used the NetPhosK 1.0 Server located at http://www.cbs.dtu.dk/services/NetPhosK/

Results

The results of the standard screen included several signaling molecules from a variety of signaling pathways. The data was filtered based on the criteria established by the service provider, Kinexus\textsuperscript{TM} and included several factors involving the quality of the spot such as the morphology, background, intensity, and a minimum of 1.5 fold increased/decreased threshold. These filtering criteria narrowed our list of hits to 44. I further narrowed it down imposing stricter criteria by raising the fold increased/decreased level to 2.0. The 18 selected hits that are listed in table 3-1 provided the platform for the signaling analyses. As signaling pathways were identified however, participating molecular components that were not within this range (2 fold) were also examined in the data set.

Next, I clustered these hits into established signaling pathways. Here I used the KEGG server as well as a survey of the literature. In addition, I used the binding motifs on the cytoplasmic surfaces of Muc4, Erb2 and ErbB3 to guide the signaling pathway initiation
analyses. Binding motifs on the C terminal tail of membrane proteins are dictated by their sequences and by post translation modifications. To this end, I generated a list of all of the relevant cytoplasmic binding motifs on ErbB2, ErbB3 and Muc4 using the ELM server. A summary of this list is provided in Table 3-2. It should be noted that post translation binding motifs were only considered for the phosphorylated tyrosine ErbB2 on positions 1248 and 1139, for which experimental data was available (in chapter 2 of this work, and in Ramsauer et al. 2006 respectively). ErbB3 modifications were excluded based on the analysis described in chapter 2 of this work, and Muc4 modifications were excluded based on the NetPhos 2.0 Server indicating no phosphorylation predictions.

**Table 3-1.** Kinexus™ microarray hits

<table>
<thead>
<tr>
<th>Target Protein Name</th>
<th>Phospho Site (Human)</th>
<th>Full Target Protein Name</th>
<th>Swiss-prot Link</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSK1/2</td>
<td>S363/S369</td>
<td>Ribosomal S6 protein-serine kinase 1/2</td>
<td>Q15418</td>
<td>4.41</td>
</tr>
<tr>
<td>FAK</td>
<td>S722</td>
<td>Focal adhesion protein-tyrosine kinase</td>
<td>Q05397</td>
<td>3.83</td>
</tr>
<tr>
<td>PKCq</td>
<td>S676</td>
<td>Protein-serine kinase C theta</td>
<td>Q04759</td>
<td>3.27</td>
</tr>
<tr>
<td>Csk</td>
<td>Pan-specific</td>
<td>C-terminus of Src tyrosine kinase</td>
<td>P41240</td>
<td>2.90</td>
</tr>
<tr>
<td>SOX9</td>
<td>S181</td>
<td>SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)</td>
<td>P48436</td>
<td>2.58</td>
</tr>
<tr>
<td>FAK</td>
<td>S843</td>
<td>Focal adhesion protein-tyrosine kinase</td>
<td>Q05397</td>
<td>2.41</td>
</tr>
<tr>
<td>PP4/A’2</td>
<td>Pan-specific</td>
<td>Protein-serine phosphatase 4 - regulatory subunit</td>
<td>Q8TF05</td>
<td>2.39</td>
</tr>
<tr>
<td>PDK1</td>
<td>Pan-specific</td>
<td>3-phosphoinositide-dependent protein-serine kinase 1</td>
<td>O15530</td>
<td>2.34</td>
</tr>
<tr>
<td>Rb</td>
<td>T821</td>
<td>Retinoblastoma-associated protein 1</td>
<td>P06400</td>
<td>2.24</td>
</tr>
<tr>
<td>Cofilin 1</td>
<td>S3</td>
<td>Cofilin 1</td>
<td>P23528</td>
<td>2.21</td>
</tr>
<tr>
<td>STAT1</td>
<td>Y701</td>
<td>Signal transducer and activator of transcription 1</td>
<td>P42224</td>
<td>2.19</td>
</tr>
<tr>
<td>Tau</td>
<td>S720</td>
<td>Microtubule-associated protein tau</td>
<td>P10636</td>
<td>2.03</td>
</tr>
<tr>
<td>Paxillin 1</td>
<td>Y118</td>
<td>Paxillin 1</td>
<td>P49023</td>
<td>0.39</td>
</tr>
<tr>
<td>Catenin b</td>
<td>S45</td>
<td>Catenin (cadherin-associated protein) beta 1</td>
<td>P35222</td>
<td>0.46</td>
</tr>
<tr>
<td>CDK10</td>
<td>Pan-specific</td>
<td>Cyclin-dependent protein-serine kinase 10 PISSLRE</td>
<td>Q15131</td>
<td>0.46</td>
</tr>
<tr>
<td>CaMKK (CaMKK2)</td>
<td>Pan-specific</td>
<td>Calcium/calmodulin-dependent protein-serine kinase</td>
<td>Q8NS9</td>
<td>0.47</td>
</tr>
<tr>
<td>Histone H2B</td>
<td>S14</td>
<td>Histone H2B</td>
<td>P33778</td>
<td>0.47</td>
</tr>
<tr>
<td>PI3KR4</td>
<td>Pan-specific</td>
<td>Phosphoinositide-3-kinase, regulatory subunit 4</td>
<td>Q99570</td>
<td>0.47</td>
</tr>
</tbody>
</table>
One potential difference, however, between the Muc4 rat and the MUC4 human proteins regarding the signaling analysis, is that in the human protein two serine phosphorylations (score 0.549 in GARFSYFLN and 0.829 in YFLNSAEAL) are predicted, possibly expanding the signaling initiation potential to include serine kinases docking proteins under the same conditions. Signaling pathway clustering analysis pointed to three major signaling pathways; the adherens junction pathway, the Wnt signaling pathway, and the focal-adhesion pathway. The list of potential binding motifs suggested that the intracellular portion of the Muc4-ErbB2-ErbB3 complex presents several important binding motifs for the recruitment of a variety of intracellular signaling proteins with affinity to PTB, SH2, SH3, PDZ, WW and others. As noted at the bottom of Table 3-2, Muc4 displays a single binding motif, a PDZ ligand. This finding suggested that Muc4 might not participate directly in intracellular signaling, but indirectly through the C terminal tails of ErbB2 and ErbB3.
<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Motif Sequence in ErbB2</th>
<th>Motif Sequence in ErbB3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin recognition site</td>
<td>Site interacts with cyclin</td>
<td>RSLL, KTLSP</td>
<td>RYLVI</td>
</tr>
<tr>
<td>EVH1 ligand, Prolin-rich sequence</td>
<td>Binds the signal transduction modules EVH. Re-organization of the actin cytoskeleton, and modulation of actin dynamics and actin-based motility</td>
<td>PPPAF, PPSTF</td>
<td></td>
</tr>
<tr>
<td>PDZ ligand</td>
<td>Mediate interactions for the assembly of large multi protein complexes</td>
<td>DVPV, NEDL, GDLV, EYL, PEY, PEY</td>
<td>LEEV, EDNL, QEKV, EDEY, LEEL</td>
</tr>
<tr>
<td>PTB_1 domain</td>
<td>Phosphotyrosine binding motif at cytosol, side of plasma membrane</td>
<td>NPEY-1196, 1248</td>
<td></td>
</tr>
<tr>
<td>PTB_2 domain</td>
<td>NPYX PTB domain binding motif whose tyrosine is non-phosphorylated</td>
<td>NPEYL, NPEYL</td>
<td>NPDYW</td>
</tr>
<tr>
<td>SH2_Grb2</td>
<td>GRB2-like Src Homology 2 (SH2) domains binding motif. Recognize small motifs containing a phosphorylated Tyrosine residue</td>
<td>YVNQ-1139</td>
<td></td>
</tr>
<tr>
<td>SH2_STAT3</td>
<td>YXXQ motif found in the cytoplasmic region of cytokine receptors that bind STAT3 SH2 domain</td>
<td>YVNQ-1139</td>
<td></td>
</tr>
<tr>
<td>SH3_1</td>
<td>This motif is recognized by class I SH3 domains (focal adhesion)</td>
<td>RPQPPSP</td>
<td>RHSPPHP</td>
</tr>
<tr>
<td>SH3_2 binding motif</td>
<td>This is the motif recognized by class II SH3 domains (focal adhesion)</td>
<td>PDPY</td>
<td>PGDDY</td>
</tr>
<tr>
<td>SH3_3 binding motif</td>
<td>This is the motif recognized by those SH3 domains with a non-canonical class I recognition specificity (focal adhesion)</td>
<td>DLGPASP, EEAPRSP</td>
<td>LLTPVT, PVTLSP</td>
</tr>
<tr>
<td>SH3_5 binding domain</td>
<td>PXDDY motif recognized by some SH3 domains</td>
<td>DPEQDY</td>
<td>RHSPHP</td>
</tr>
<tr>
<td>PKB_1 motif</td>
<td>PKB Phosphorylation site</td>
<td>RRRSQSSTRS</td>
<td>PRRGDSAYH, RRRHSPPH</td>
</tr>
<tr>
<td>Plk site</td>
<td>Site recognised and phosphorylated by the Polo-like-Kinase</td>
<td>EDPTVPPL, LDSTFYR</td>
<td>REGTLLSS, SDLASL</td>
</tr>
<tr>
<td>MAPK Site</td>
<td>Proline-Directed Kinase</td>
<td>GPASPLQ, APRSPLA</td>
<td>SLLSPPS, RSPPSP</td>
</tr>
<tr>
<td>Y-based sorting signal</td>
<td>Tyrosine-based sorting signal responsible for the interaction with mu subunit of AP (Adaptor Protein) complex</td>
<td>YRSL, YLGL</td>
<td>YLVI, YMPM, YEYM</td>
</tr>
<tr>
<td>Endosome-Lysosome-Basolateral sorting signals</td>
<td>Sorting and internalisation signal found in the cytoplasmic juxta-membrane region of type I transmembrane proteins</td>
<td>DMGDLV</td>
<td>YEYM, YMDV, YEYM</td>
</tr>
<tr>
<td>CK1/CK2 sites</td>
<td>CK1/CK2 Phosphorylation sites</td>
<td>SSSTRSG, STRSSGG</td>
<td>SALSPLV, SLLSPSS</td>
</tr>
</tbody>
</table>

* PDZ ligand binding motif was the only motif identified in the C-terminal tail of Muc4, with the sequence DSEL (AEAL in MUC4).
The adherens junction (AJ) pathway - AJs are the most common type of intercellular adhesions, and are important in maintaining tissue architecture and cell polarity. At AJs, E-cadherins serve as essential cell adhesion molecules. The cytoplasmic tail of E-cadherins binds beta-catenin, which in turn binds alpha-catenin. Alpha-catenin is associated with F-actin bundles thereby linking the E-cadherin to the actin cytoskeleton (Jamora and Fuchs, 2002). The integrity of the cadherin-catenin complex is regulated by beta-catenin phosphorylation. Positive regulation is achieved through beta-catenin serine phosphorylation by the Ser/Thr casein kinase II (CKII) which stabilizes the complex, and negative regulation is achieved through tyrosine phosphorylation by receptor tyrosine kinases (RTKs) and cytoplasmic tyrosine kinases (Fer, Fyn, Yes, and Src), which disrupts the cadherin-catenin association (Piedra et al., 2003; Roura et al., 1999) and weakens the cadherin-catenin complex. Alternatively, dephosphorylation activity by tyrosine and serine phosphatases can also antagonize these effects. Thus in general, tyrosine kinase activity on beta catenin in this pathway results in loss of cadherin mediated cell-cell adhesion. Figure 3-1 is an excellent overview of some of the structural and functional regulation of the cadherin-catenin complex stability by the balance of tyrosine kinase and phosphatase activities.
The screen results indicated that in Muc4 expressing cells, beta catenin serine phosphorylation (S45 -2.18) and the kinase activity levels of KCII [as indicated by the signal for its catalytic subunit (-1.35)] are decreased. In addition, the activity of the serine phosphatase PP1 was increased (2.09 fold), suggesting that the stability of AJs is compromised under Muc4 overexpression. This is further supported by the increased ErbB2 Y1248 activity (2 fold) I observed. Thus, the analysis of the state of molecular components in this pathway that regulates the cadherin-catenin complex integrity indicates that the Muc4-ErbB2-ErbB3 complex formation may act to interfere with the cadherin-catenin complex, disrupt the cytoskeleton organization, and weaken AJs. Formation of the Muc4-ErbB2-ErbB3 complex is then predicted to facilitate cell-cell
dissociation. Further detail on how the Muc4-ErbB2-ErbB3 complex mediates weaker cell-cell adhesion is provided through examination of Src activity in Muc4 expressing cells. In figure 3-1, the role of cellular tyrosine kinases in promoting anti-adhesion is emphasized, noting specifically the action of Src. A filtered value for Src activity was not provided in the initial screen; however, Csk levels, a tyrosine kinase that inactivates Src on its C-terminus, were increased (2.41), suggesting that Src is down regulated. Src activity was re-examined in the custom screen, specifically probing with Src phospho-Y529 antibody representing its inhibitory status, and the data indicates that Src activity is indeed down regulated (1.8 fold). Together the results from both screens suggest that in Muc4 expressing cells beta catenin post translation modifications lead to negative regulation of the cadherin-catenin complex integrity. This effect, however, seems to be mediated independent of the action of Src. The signaling analysis summary of the adherens junction pathway is presented in Figure 3-2.

The role of Muc4 in this pathway can be further established by examining beta catenin tyrosine phosphorylation levels and the cellular localization of E-cadherins, which requires the chaperon activity of beta catenin for successful translocation to the cell membrane (Chen et al., 1999). Increased beta catenin tyrosine phosphorylation, and retention of newly synthesized E-cadherin in the ER compared with cell surface expression of E-cadherin, will further establish the negative regulation effect of Muc4 on this signaling pathway.
Figure 3-2. **Muc4 expression weakens cadherin-catenin interaction independent of Src.** Kinexux™ generated values are presented for molecules that participate in the adherens junction signaling pathway. PP4/A’2 is a serine phosphatase.

**The Wnt signaling pathway**- Beta catenin is a major component of the Wnt/Wingless signaling pathway in which regulation of beta catenin levels is critical (Cadigan and Nusse, 1997). Changes in beta catenin levels lead to changes in steady state and cell surface levels of cadherins, and to cadherin-mediated cell-cell adhesion (Yanagawa, 1997). In addition to its stabilizing role in AJs, in the Wnt pathway beta catenin acts as a transcription co-factor with T cell factor/lymphoid enhancer factors TCF/LEF (Cadigan and Nusse, 1997). In this pathway, beta catenin signaling is regulated by its accumulation in the cytoplasm. The Wnt pathway acts as a positive regulator of beta-catenin by inhibiting beta-catenin degradation and leading to its
cytoplasmic accumulation. Beta-catenin is then free to enter the nucleus and activate Wnt-regulated genes, which regulate several cellular processes that involve cell-cell adhesion. Thus, changing levels of beta-catenin is a reflection of the Wnt signaling pathway activity. The initial screen indicated that beta catenin levels were decreased in Muc4 expressing cells (-1.53 fold). This result was puzzling since the preceding implications of its role in destabilizing the cadherin-catenin complex predicted that beta catenin levels would increase, unless the Wnt pathway was not active and beta catenin was quickly degraded in Muc4 expressing cells. To distinguish between these two scenarios, the levels of beta catenin were re-probed in the follow-up validation study.

The results indicated that beta catenin levels were indeed increasing in Muc4 expressing cells (1.62 fold). False positive reports in the initial screen are expected in a large-scale data set such as a microarray screen, and this reflects the importance of including a follow up validation of the results. Overall, however, the data set of the initial screen was in agreement with the data set obtained in the custom validation screen and also with previous experimental results. Further analysis of the role of Muc4 in promoting the Wnt signaling pathway can be evaluated by examination of cytoplasmic and nuclear levels of beta catenin and also by evaluating the expression levels of some of its unique transcription targets.

The Wnt pathway may not directly involve transcriptional end points via beta-catenin, but may signal toward the cytoskeleton and cell polarity in tissues. This is mediated by the non-canonical planer cell polarity (PCP) Wnt pathway which is best understood in *Drosophila*, but is also implicated in epithelial tissue polarity in vertebrates (Veeman et al., 2003). This pathway differs from the canonical pathway in requiring
Dishelvelled to be localized to the cell membrane via its DEP domain. A main branch downstream of Dishelvelled involves the small GTPases of the Rho family, RhoA (Ras homologue gene-family member A) and Rac. Dishelvelled activates the GTPases which in turn activates the stress kinase JNK (Jun N-terminal kinase) and ROCK (Rho-associated coiled-coil-containing protein kinase 1), resulting in remodeling of the cytoskeleton and changes in cell adhesion and motility (Veeman et al., 2003). A simplified version of this pathway is presented in Figure 3-3. Please note that the values for some of the components in this pathway from the initial screen are included in this figure, and overall support the role of this pathway in Muc4 expressing cells. Specifically, the small GTPase Rac1 is up regulated (1.61). The role of Rac1 in cytoskeletal reorganization is to promote actin polymerization at the leading edge of migrating cells (Rottner et al., 1999). No other components in the screen were beneficial in understanding the link between Muc4 and Rac1 action; however, the requirement for membrane localization with Dishelvelled may be helpful in better understanding the Muc4-GTPases link. To further establish this pathway action in Muc4 expressing cells, components of this pathway were re-probed in the follow-up validation analysis. One interesting observation was that the level of the stress kinase ROCK was increased 36 fold, suggesting that in Muc4 expressing cells the PCP-Wnt pathway may act through the action of RhoA. At this time, however, a validated value for RhoA is not available. Thus, the role of this small GTPase in Muc4 expressing cells is yet to be elucidated. The results of the validated screen are summarized in Table 3-3.
Figure 3-3. The planar cell polarity Wnt signaling pathway. Values in parenthesis reflect fold increase in the signal intensity of marked proteins (initial screen) in Muc4 expressing cells compared with the control sample.

Table 3-3. KCPS-1.0 Kinetworks™ validation screen results

<table>
<thead>
<tr>
<th>Full Name of Protein</th>
<th>Abbrev</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paxillin 1 [Y118]</td>
<td>Paxillin 1</td>
<td>208</td>
</tr>
<tr>
<td>RhoA protein-serine kinase beta</td>
<td>ROKb (ROCK1)</td>
<td>36</td>
</tr>
<tr>
<td>Focal adhesion protein-tyrosine kinase [Y397]</td>
<td>FAK</td>
<td>3.2</td>
</tr>
<tr>
<td>Integrin beta 1 [S785]</td>
<td>Integrin b1</td>
<td>2.6</td>
</tr>
<tr>
<td>Src proto-oncogene-encoded protein-tyrosine kinase [Y529]</td>
<td>Src</td>
<td>1.8</td>
</tr>
<tr>
<td>Catenin (cadherin-associated protein) beta 1</td>
<td>Catenin b</td>
<td>1.62</td>
</tr>
<tr>
<td>Src proto-oncogene-encoded protein-tyrosine kinase [Y529]</td>
<td>Src</td>
<td>1.6</td>
</tr>
<tr>
<td>Protein-serine kinase C theta [T538]</td>
<td>PKCt</td>
<td>1.28</td>
</tr>
<tr>
<td>Ribosomal S6 protein-serine kinase 1/2 [S363/S369]</td>
<td>RSK1/2</td>
<td>1.26</td>
</tr>
<tr>
<td>Protein-tyrosine phosphatase 1D (SHP2, SHPTP2, Syp, PTP2C)</td>
<td>PTP1D</td>
<td>1.16</td>
</tr>
<tr>
<td>Ribosomal S6 protein-serine kinase 1/2 [S363/S369]</td>
<td>RSK1/2</td>
<td>0.74</td>
</tr>
<tr>
<td>Cyclin-dependent protein-serine kinase 1/2 [T14+Y15]</td>
<td>CDK1/2</td>
<td>0.7</td>
</tr>
<tr>
<td>Glycogen synthase-serine kinase 3 beta [S9]</td>
<td>GSK3b</td>
<td>0.7</td>
</tr>
<tr>
<td>MAPK/ERK protein-serine kinase 1 (MKK1) [T291]</td>
<td>MEK1 (MAP2K1)</td>
<td>0.64</td>
</tr>
<tr>
<td>Glycogen synthase-serine kinase 3 alpha [S21]</td>
<td>GSK3a</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Fold change was calculated from normalized C.P.M. values obtained in A375 Muc4 expressing cells and in control A375 cells.
**The focal adhesion signaling pathway**- Focal adhesions are contact points of specialized structure where integrin receptors mediate extracellular matrix adhesion links to the actin cytoskeleton and inform the cell about the condition of the extracellular matrix. These adhesion complex sites are composed of more than 50 different proteins including structural, signaling and adaptor molecules, and are involved in considerable signaling functional diversity (Wozniak et al., 2004). In addition to their role in cell adhesion, they act as signaling hubs and mediate signaling that are involved in essential biological processes including cell motility, cell proliferation, cell differentiation, regulation of gene expression and cell survival. In sessile cells, focal adhesions are quite stable under normal conditions, but in moving cells focal adhesions are constantly being assembled and disassembled as the cell establishes new contacts at the leading edge, and breaks old contacts at the trailing edge of the cell. Two of the major kinases found in focal adhesions are the focal adhesion kinase (FAK) and Src, which bind to different partners to regulate adhesion dynamics and cell behavior. Other tyrosine kinases such as Abl, Csk and PYK2 and Ser/Thr kinases such as ILK, PAK and PKC are also found in focal adhesions (Zamir and Geiger, 2001). The results of the microarray screen indicated that several components that participate in focal adhesion were differentially regulated in Muc4 expressing cells. I therefore examined the data to evaluate the role of Muc4 in focal adhesion signaling.

The preceding noted implications in Muc4 weakening AJs, suggested that Muc4 expression might also weaken cell-matrix interaction by destabilizing the focal adhesion complexes. To determine the stability state of focal adhesion complexes in sessile cells expressing Muc4, signaling molecules that can mediate focal adhesion complex stability
or instability had to be identified. This task was proven difficult because focal adhesion components are dynamically changing in living cells. Focal adhesions initially form focal complexes (FC), small focal adhesions at the periphery of spreading or migrating cells, which precede the larger and more mature focal adhesions (FA) that are associated with matrix adhesion (Wozniak et al., 2004). Thus, a cell must be able to continuously remodel FC into FA and vice versa to signal through focal adhesion, but cells that are prone to migration show a higher proportion of FC as compared to mature FA. Figure 3-4 presents a view of focal adhesion type and composition.

**Figure 3-4. Focal adhesion types and composition.** Cartoon depicting the three predominant types of adhesion typically found in an adherent cell plated on extracellular matrix; focal complex (FC), focal adhesion (FA) and fibrillar adhesion (FB) are shown. Cartoon schematics of the typical protein composition defining each adhesion type are also shown. Scale bar 5 µm. Adapted from Worth & Parsons et al. 2008

One way to meaningfully analyze the data and examine the role of Muc4 expression in cytoskeleton reorganization and in regulating extracellular matrix adhesion was to evaluate the proportion of FC and FA by distinguishing between the state of
molecular components that are uniquely associated with each adhesion complex type.

Some of the major components that regulate FC vs. FA are listed in table 3-4.

**Table 3-4.** Molecular components in focal complexes and focal adhesion

<table>
<thead>
<tr>
<th>Focal Complexes (FC)</th>
<th>Focal Adhesion (FA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAK S-phosphorylation</td>
<td>FAK Y-phosphorylation</td>
</tr>
<tr>
<td>FAK Y-auto phosphorylation</td>
<td>Src phosphorylation</td>
</tr>
<tr>
<td>Src phosphorylation</td>
<td>Rho/ROCK</td>
</tr>
<tr>
<td>Rac/Cdc42</td>
<td>Ras</td>
</tr>
<tr>
<td>P130Cas</td>
<td>Paxillin Y-phosphorylation</td>
</tr>
<tr>
<td></td>
<td>Vinculin Y-phosphorylation</td>
</tr>
<tr>
<td></td>
<td>Talin</td>
</tr>
</tbody>
</table>

FAK is a key player in FA formation and turnover both as an enzyme and as an adaptor molecule. FAK is activated upon integrin engagement with its ligand via auto-phosphorylation on Y397 (Schaller et al., 1994). This phosphorylation in turn provides a binding site for Src. The formation of Src-FAK complex is a prerequisite for Src activation. Active Src then phosphorylates FAK on other tyrosine residues, creating docking sites for other SH2 domain-bearing molecules such as Grb, linking FAK to activation of Ras and the MAPK pathway (Schaller et al., 1994). Src also phosphorylates other FAK associated proteins such as paxillin and tensin (Parsons and Parsons, 1997). FAK is also phosphorylated on serine residues, and these have been shown to be involved in mitosis and in detachment from the extracellular matrix (Ma et al., 2001), suggesting a role in FA turnover. The data from the screen indicated that in Muc4 expressing cells, FAK tyrosine phosphorylation (Y576) is increased (1.82 fold), but serine phosphorylation (S843, S722) is also increased (2.41 and 3.83 fold respectively), suggesting that in Muc4 expressing cells, FAs are dynamically remodeled.
FAK Y397, a key auto-phosphorylation site that is activated upon integrin engagement with the extracellular matrix, was important to evaluate in order to better understand the role of Muc4 in the focal adhesion complex assembly. This site is specifically associated with FC since its signal represents the first step in the assembly of focal adhesion complexes. This site was probed in the validation analysis and showed a signal increase (3.2 fold), suggesting that FAK does engage with integrins, and that FAK displays the necessary binding site for Src. In addition, a value for integrin beta1 S785 site was also provided in the validation analysis, showing a 2.6 fold increase in this signal. This phosphorylation site also represents integrin engagement with the matrix and provides GRB2 and SHC binding sites for intracellular signaling molecules. However, consequent FAK tyrosine phosphorylations by Src, which is required for the continued maturation of FA complexes, seem to be compromised in Muc4 expressing cells. Because Src phosphorylation of FAK is necessary for creating binding sites for other intracellular signaling proteins, it is possible that through the down regulation effect of Src activity in Muc4 cells, FCs are more common than mature FA complexes. At the plasma membrane domain level, the formation of large Muc4-ErbB2-ErbB3 complexes may generate signaling microdomains that act to antagonize newly forming focal adhesion complexes by promoting anti-adhesive signaling, and acting to interfere with sustained adhesion. This antagonizing effect could then interfere with the maturation of FC into FA and promote an overall weak cell-matrix adhesion.
Discussion

Muc4/MUC4 expression has been linked to anti-adhesion (Carraway et al., 2000) and to promotion of oncogenesis by facilitating cell motility and anti-apoptotic properties (Komatsu et al., 2000; Moniaux et al., 2007; Ponnusamy et al., 2008). These signaling effects were attributed to the mucin subunit of Muc4 and to its association with the receptor ErbB2. The signaling analysis presented here was aimed at understanding the role of the transmembrane subunit of Muc4 in signaling by using a microarray antibody signaling analysis that examined the effects of a mucin truncated Muc4 expression in A375 human melanoma cells. An understanding of the Muc4 signaling pathways that drive oncogenic potential has been unclear and is sought for in order to better understand the signaling pathways and associated cellular mechanisms that drive these phenotypes, and the dependent signaling relationship between Muc4 and ErbB2.

Analysis of the C-terminal tail of Muc4 suggests that intracellular signaling by Muc4 would be mediated via its dependent, direct contact with ErbB2. The results in chapter 2 of this work show that Muc4 direct association with ErbB2 significantly potentiates ErbB2 phosphorylation, thereby creating several potent binding motifs for cellular signaling components. Moreover, the stability of this complex at the membrane can promote the formation of a signaling hub that can potently transmit sustained intracellular signaling.

One of the hallmarks of neoplastic transformation is the ability of transformed cells to escape the cell-cell and cell-extracellular matrix adhesion requirements while maintaining the ability to reorganize the actin cytoskeleton necessary for motility in a dynamic way. The analyses carried out here indicates that Muc4 negatively regulates the
AJ pathway by interfering with the cadherin-beta catenin complex integrity at the cell membrane, thereby leading to weak cell-cell contacts. This also results in increased beta catenin levels, which act to activate the Wnt signaling pathway. Moreover, changes in the phosphorylation state of FAK indicate that Muc4 promotes dynamic change in FA turnover, a common feature of migrating cells requiring alternative modes of cell-extracellular matrix contact adhesion and release. This effect seems to be mediated by decreased Src activity, which normally plays a major role in the FA maturation process in this pathway.

Overall, this signaling analysis portrays Muc4 as a potent signaling molecule through its direct association with the receptors ErbB2 and ErbB3. The formation of this complex results in intracellular events that indicate a role in decreased cell-cell and cell-matrix association, and promotion of loss of cell polarity. The mechanism by which Muc4 promotes these anti-adhesion effects seems to be mediated indirectly through the formation of large Muc4-ErbB2-ErbB3 complexes at the plasma membrane. These complexes may form micro signaling domains within the plasma membrane that are similar to focal adhesions in structure, in that they can serve as membrane signaling hubs for intracellular signaling molecules. However unlike focal adhesion centers, Muc4-ErbB2-ErbB3 complexes lack in cell-matrix adhesion contact, and instead promote an opposite anti-adhesion effect. This then is hypothesized to lead to the antagonizing action of focal adhesion centers and to the promotion of lose of cell polarity. This hypothesis predicts that increased loss of cell adhesion will be positively correlated with increasing Muc4 expression levels.
The study discussed here is an initial signaling analysis for the action of the Muc4-ERbB2-ErbB3 complex. To further establish these findings, additional validation is required at the molecular and cellular imaging levels for other molecular components that participate in the noted signaling pathways.
CHAPTER FOUR

CONCLUSIONS

This work investigated cellular signaling that results from the direct interaction of two membrane components, the mucin Muc4, and the receptor tyrosine kinase ErbB2. Although each component independently participates in cellular signaling, the Muc4 function in signaling is dependent upon its large extracellular domain, while ErbB2 is dependent upon its intracellular domain. This study shows that through direct and stable interaction, Muc4 and ErbB2 can display potent signaling via a synergistic effect of their signaling potential. This work reinforces the idea that coordinated cellular communication from the “outside-in” requires an intimate interaction between the extracellular and intracellular environments, and specifically suggests that this type of “outside-in” relationship can be mediated through interactions with extracellular structural features that are otherwise incompatible of yielding signaling effects.

In the first part of this study, an analysis of the nature of the Muc4-ErbB2 complex in signaling potential was carried out. This mechanistic analysis helped enhance the previous Muc4-ErbB2 model (Figure 1-4), and also showed that the hypothesis concerning increased signal duration was correct. More specifically, the analysis indicates that Muc4 associates directly with ErbB2 and ErbB3. The association with ErbB3 indicates that ErbB3 may have a structural stability role in the signaling potential of this complex. ErbB2 phosphorylation is potentiated significantly in this complex and is dependent on ErbB2 intrinsic kinase activity, not on tran-activation mechanisms. Most significantly this complex leads to sustained ErbB2 signaling, an important factor in
signaling potential and diversity and in mediating oncogenic potential. The biochemical and signaling analyses suggest that the sustained ErbB2 signaling is not mediated through lack of phosphatase activity acting to down regulate ErbB signals, and point to the possibility that Muc4-ErbB2 signaling potential is mediated via stable extracellular interaction.

A possible rationale for the potent extracellular interaction between Muc4 and ErbB2 can be provided from data on the structures and structure-based models of ErbB2. These indicate that ErbB2 is unique among the other ErbBs in that its extracellular domain is not in the characterized autoinhibited tethered conformation in the absence of ligand, but is rather constitutively in the extended, untethered conformation. This feature renders ErbB2 ready to be dimerized with other Erb receptors, suggesting that the main limiting factor for its activation is the formation of a stable dimer. However, despite the implication that such a dimer should readily form, studies on the individual interaction domains of ErbB receptors indicate that dimer stability is achieved through multiple receptor interactions at the extracellular, transmembrane, and cytoplasmic domains in order to result in activity. Thus dimer association alone will not yield an active dimer until specific spatial association requirements are met, ideally those that are imposed by ligand binding. This notion may also explain the observed lack of ErbB2 homodimers, which are rationalized to fail to form due to electrostatic repulsion within the extracellular dimerization arm of the receptor (Garrett et al., 2003). One exception to ligand-independent activation, or formation of stable dimers/oligomers observed in models and in in vivo studies, is the effect of an increased local concentration of receptors. This exception suggests that ligand-independent stability and activity of
receptor dimers can be achieved in the absence of ligand through increased receptor contacts at the required interfaces. The revised model (Figure 2-8) depicts Muc4 as a membrane component that can impart stability to ErbB2-ErbB3 dimers, resulting in potentiated ErbB2 activity. I suggest that Muc4 expression leads to increased membrane local concentration of ErbB2 and in turn to ErbB2 conformation stability and activity. The conformation stability effect is believed to be mediated through extracellular domain interactions, specifically via the N terminal EGF-like domain of Muc4 as was established in earlier co-immunoprecipitation studies utilizing deletion Muc4 constructs expression (Carraway et al., 1999).

The single pass transmembrane domains of Muc4 and ErbB2 may also play a significant role in promoting this stable association. Experimental evaluations of the effect of this domain on ErbB receptor activity, using mutational analyses and molecular models, indicate that dimerization also occurs within this domain, and that specific alignment of transmembrane helices can result in ligand-independent activity and stability (Mendrola et al., 2002; Samna Soumana et al., 2007; Sharpe et al., 2000). Specifically the presence of the putative GXXXG motif and its variant motifs in the correct orientation play a major role in mediating stable membrane associations (Dawson et al., 2002). All ErbB receptors as well as Muc4 contain this motif, and one future test that can establish the role of this domain in contributing added stability to the Muc4-ErbB2 complex is to construct a molecular model that can predict transmembrane helices dimerization.

One interesting feature regarding the model is that ErbB2 signal potentiation involves ErbB2 intrinsic activity. This suggests that the Muc4 effect does not act as a
mere trapping membrane component that increases the local concentration of ErbB2, but that Muc4 is capable of imparting a stabilizing effect that promotes intracellular kinase activity. In my work, I used a potent ErbB2 inhibitor, Lapatinib, to establish this finding. Additional tests of this Muc4 feature, possibly by assessing ErbB2 phosphorylation in cells expressing the kinase dead ErbB2 construct with a tag, can further test this Muc4 effect on ErbB2 signaling. In addition, an examination of changes in ErbB3 phosphorylation in the complex did not yield a signal, and suggested that despite complex association, ErbB3 may be in the tethered confirmation. Additional tests of the ErbB3 phosphorylation state in this complex should also be expanded to survey other phosphorylation sites with phospho-specific antibodies directed towards other ErbB3 sites. Further establishment of the ErbB3 “inactive” role in the Muc4-ErbB2-ErbB3 complex could suggest that Muc4 interaction with ErbB members in promoting signaling is dependent on association with receptors in the untethered confirmation. This hypothesis is in agreement with the Muc4 effect on the constitutive untethered ErbB2, and with the ability of Muc4 to potentiate ErbB3 phosphorylation only upon ligand treatment (Carraway et al., 1999; Carraway et al., 2003a; Funes et al., 2006), which changes ErbB3 confirmation from the tethered to the untethered conformation. This hypothesis also predicts that Muc4 would be able to potentiate the phosphorylation signal of the other ligand-bound members of this family, i.e. ErbB1 and ErbB4.
In the second part of this study, an analysis of downstream signaling pathways resulting from Muc4-ErbB2 complex formation was carried out. Here, I hypothesized that Muc4 expression would promote ErbB2-mediated signaling pathway effects. The results suggest that ErbB2 cellular effects that are associated with common ErbB2 signaling are potentiated, specifically by promoting anti-adhesion and loss of cell polarity signaling. Surprisingly however, these signaling are mediated by alternative signaling pathways, independent of the MAPK signaling pathway, and independent of the action of Src.

The signaling screen utilized an antibody microarray that surveyed over 650 molecular components participating in various signaling pathways. Several advantages of this screen helped provide an initial view of the signaling pathways that are involved. One advantage is that most of the surveyed targets were analyzed with phospho-specific antibodies, which helped indicate the activity state of the major participating components, kinases and phosphatases. In addition, these phospho-dependent signals provided clues on intracellular localization and specific associations with other signaling components, thus offering multiple clues for the analysis. The large data set output of this screen was also beneficial in providing the ability to examine several pathway components, even when the attributed scores of these were not included in the filtered criteria, thus allowing for flexibility in the analysis. Finally, unlike the “direct-bait” approach that examines signaling pathways through the use of specific signaling pathway antibodies, this approach allowed an overall evaluation of signaling, and signaling cross talks as well.

Anti-adhesion signaling was established through identification of components in the adherens junction (AJ) pathway that exhibit differential signal intensity under Muc4
expression treatment. The Adherens junction pathway promotes signaling that helps maintain cell-cell contact, but its down regulation suggests that cell-cell contacts are weaker and more prone to loss of cell-cell adhesion. The integrity of this pathway is regulated at the molecular level by the cadherin-catenin complex, which links the membrane cadherin to the actin cytoskeleton. The results indicate that the cadherin-catenin complex integrity in the Muc4 expressing cells is compromised.

Additional support for anti-adhesive signaling activity was identified through the focal adhesion pathway. The focal-adhesion signaling pathway mediates the assembly of large specialized structures (focal complexes) that contain many proteins, and facilitates extracellular matrix adhesion links to the actin cytoskeleton. This adhesion is essential for normal cell function; however, it is down regulated in motile cells that require alternating adhesion and release modes of focal complexes. Thus a turnover of mature focal adhesion (FA) helps maintain the smaller and more readily detached focal complexes (FC), and is a hallmark of migrating cells. Distinction between the states of molecular components that are uniquely associated with each adhesion complex type facilitated evaluation of Muc4 effect on this signaling pathway. The analysis indicated that cells expressing Muc4 are subjected to FA to FC turnover, and that the FC state is more prevalent, thus promoting weak cell-matrix adhesion and loss of cell polarity.

To complement the anti-adhesion implications identified in the adherens junction and focal adhesion pathways, a signaling pathway that facilitates the formation of different organizational arrays of the actin filaments in the actin cytoskeleton had to be identified. Potential targets for this role were identified in the screen as the small Rho GTPases Rac1 and RhoA. These GTPases fit the non-canonical action of the planar cell
polarity Wnt signaling pathway that is implicated in cytoskeleton remodeling effects via membrane interaction. The validation analysis provided further support for the role of RhoA in mediating this effect through changes in the levels of the downstream RhoA-activated stress kinase ROCK. Further direct analysis of the role of these GTPases in Muc4 expressing cells is required. Possibly, examination of real time imaging of these molecules in Muc4 expressing cells will help establish their role in Muc4-ErbB2 signaling, and will further highlight their role in promoting cell detachment and motility.

An interesting observation regarding the signaling analysis is that Muc4 expression has also been previously noted in promoting anti-adhesion and loss of cell polarity cellular effects, albeit by unknown molecular mechanisms and signaling pathways. These Muc4 signaling effects have been attributed to the mucin subunit of Muc4, and were shown to depend on the number of tandem repeats that are present in the mucin subunit of Muc4. The signaling analysis carried out here however analyzed samples expressing the mucin-truncated Muc4 protein, therefore suggesting that the Muc4 transmembrane subunit alone can also contribute to anti-adhesive signaling.

Finally, it is important to note that the analysis of the signaling pathways was carried out based on the available information on existing signaling pathways, which is incomplete. In addition, the analysis cannot distinguish between pleiotropy, signaling that is derived from the same receptor, and cross talks, signaling that is derived from different receptors. A distinction between these can become available upon careful identification of intracellular signaling components that can bind specific motifs on the
C-terminal tail of ErbB2, ErbB3 or Muc4 (Table 3-2), and participate in the attributed pathways. One initial search in distinguishing between these two modes of signaling is to identify PDZ domain containing proteins that participate in the above mentioned signaling pathways, since this is the only common domain in all three molecules in the complex.

In summary, the work presented here investigated cellular signaling that results from the direct interaction of two membrane components, the mucin Muc4, and the receptor tyrosine kinase ErbB2. The mechanistic signaling potential of this interaction suggests that despite a lack of apparent structural compatibility, these two membrane proteins can form a complex that is capable of potent signaling. The analysis of activated pathways suggests that through this interaction, the effect of signaling pathways is synergized and in turn modified, representing unique signaling potentiation. The signaling analysis provides an initial overview of the Muc4 dependent molecular mechanisms and signaling pathways.

This work highlights the need to expand our views of membrane protein signaling transduction potentials, and consider additional factors that may modify the membrane domain dynamics.
WORKS CITED


