Regulation of SMC/MUC4 Expression in the Airway

George Theodore
University of Miami, theogengr@yahoo.com

Follow this and additional works at: https://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
Theodore, George, "Regulation of SMC/MUC4 Expression in the Airway" (2010). Open Access Dissertations. 364.
https://scholarlyrepository.miami.edu/oa_dissertations/364

This Open access is brought to you for free and open access by the Electronic Theses and Dissertations at Scholarly Repository. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of Scholarly Repository. For more information, please contact repository.library@miami.edu.
REGULATION OF SMC/MUC4 PROTEIN EXPRESSION IN THE AIRWAY

By

George Theodore

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

May 2009
UNIVERSITY OF MIAMI

A dissertation submitted to the faculty
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

REGULATION OF SMC/MUC4 PROTEIN EXPRESSION IN THE AIRWAY

George Theodore

Approved:

Theodore J. Lampidis, Ph.D.
Professor of Cell Biology and Anatomy

Terri A. Scandura, Ph.D.
Dean of the Graduate School

Kermit L. Carraway, Ph.D.
Professor of Cell Biology and Anatomy
and Biochemistry and Molecular Biology

Vinata B. Lokeshwar, Ph.D.
Professor of Urology and
Cell Biology & Anatomy

Thomas K. Harris, Ph.D.
Associate Professor of Biochemistry
and Molecular Biology
MUC4 is a heterodimeric mucin glycoprotein expressed in the epithelia of tissues. Previous studies in our laboratory demonstrated that MUC4 protein expression is regulated by exogenous growth factors and that MUC4 is found in complex with the receptor tyrosine kinase ErbB2. MUC4 protein expression in airway epithelia was evaluated using molecular biology techniques. The impact of the protein on ErbB2 activation was evaluated post mechanical wounding of airway epithelia, and upon MUC4 RNA silencing. MUC4 levels were increased with exposure to the differentiating agent retinoic acid and decreased upon exposure to epidermal growth factor, a proliferative agent. In the absence of MUC4, ErbB2 phosphorylation was diminished. These results support the hypothesis that MUC4 expression is enhanced during differentiation of epithelia. Furthermore these findings provide evidence for an additional level of ErbB regulation in airway injury and subsequent epithelial wound healing.
DEDICATION

This dissertation is dedicated to my parents Angelo and Angeliki Theodoropoulos.

Σας ευχαριστώ.
Παιδια σας αγαπω και σας σκεπτομαι παντα!

Their loving guidance has helped throughout every aspect of my existence.

My parents should be considered my greatest teachers and the ultimate references for me, the author.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>viii</td>
</tr>
<tr>
<td><strong>CHAPTER I</strong></td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Mucins</td>
<td>1</td>
</tr>
<tr>
<td>Smc/MUC4</td>
<td>5</td>
</tr>
<tr>
<td>Transcriptional regulation</td>
<td>10</td>
</tr>
<tr>
<td>MUC4/ErbB2 Complex</td>
<td>18</td>
</tr>
<tr>
<td><strong>CHAPTER II</strong></td>
<td></td>
</tr>
<tr>
<td>Regulation of MUC4 Protein Expression by Mediators of Wound Healing in</td>
<td>22</td>
</tr>
<tr>
<td>the Airway</td>
<td></td>
</tr>
<tr>
<td>Overview</td>
<td>22</td>
</tr>
<tr>
<td>Introductory Remarks</td>
<td>23</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>28</td>
</tr>
<tr>
<td>Results</td>
<td>32</td>
</tr>
<tr>
<td>Discussion</td>
<td>38</td>
</tr>
<tr>
<td><strong>CHAPTER III</strong></td>
<td></td>
</tr>
<tr>
<td>MUC4 Involvement in ErbB2/ErbB3 Phosphorylation and Signaling in</td>
<td>52</td>
</tr>
<tr>
<td>Response to Mechanical Injury</td>
<td></td>
</tr>
<tr>
<td>Overview</td>
<td>52</td>
</tr>
<tr>
<td>Introductory Remarks</td>
<td>53</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>55</td>
</tr>
<tr>
<td>Results</td>
<td>61</td>
</tr>
</tbody>
</table>
CHAPTER IV
CONCLUSION AND FUTURE DIRECTIONS  81

REFERENCES  84
LIST OF FIGURES

Figure 1. Table of mucin genes. 2
Figure 2. Model of SMC/MUC4. 6
Figure 3. Homology of SMC and MUC4. 7
Figure 4. Model of SMC in the airway. 8
Figure 5. Table of transcription factors involved in mucin gene regulation, common to MUC4 and other mucins. 10
Figure 6. Molecular signaling in mucin regulation 12
Figure 7. Table of tissue distribution and regulative growth factors of airway mucins. 14
Figure 8. Intramembrane interaction of MUC4 and ErbB2. 20
Figure 9. SMC localization in the rat airway. 44
Figure 10. MUC4 localization in the human airway. 45
Figure 11. ECM Regulation of SMC/Muc4 expression. 46
Figure 12. Effect of exogenous growth factors and matrix adhesion on MUC4 protein expression. 47
Figure 13. EGF treatment downregulates MUC4 protein expression in airway. 49
Figure 14. Erk signaling regulates MUC4 protein expression. 50
Figure 15. Expression of Muc4/MUC4 and ErbB2 in airway epithelial cells. 71
Figure 16. Co-localization of MUC4 and ErbB2 in human airway epithelium. 72
Figure 17. Association of MUC4 and ErbB2 in complex in three types of human airway epithelial cells. 73
Figure 18. Effect of scratch wounding of airway cell cultures on ErbB phosphorylation in complexes with MUC4. 75
Figure 19. Downstream pathways affected by scratch wounding of airway cell cultures. 78

Figure 20. Effect of MUC4 knockdown on scratch-wounding induction of ErbB2 and ErbB3 phosphorylation. 79

Figure 21. Model of injury in airway epithelia. 80
ABBREVIATIONS

Ab; antibody
ALI: air liquid interface airway epithelia
ASGP: a transmembrane glycoprotein component found on the surface of the highly metastatic ascites 13762 rat mammary adenocarcinoma cell line
cDNA: complimentary DNA
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
H&E: hematoxylin and eosin staining
IB: immunoblot
IF: immunofluoresence
IFN: interferon factor
IL: interleukin
IP: immunoprecipitation
Smc: sialomucin complex
MAPK: mitogen activated protein kinase
mRNA: messenger ribonucleic acid
Muc: rat mucin
MUC: human mucin
NE: neutrophil elastase
NHBE: normal human tracheobronchial epithelia
PCR: polymerase chain reaction
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA:</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RT:</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SMAD:</td>
<td>homologs of the drosophila protein, mothers against decapentaplegic</td>
</tr>
<tr>
<td>TGFβ:</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TNFα:</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>PKC:</td>
<td>protein kinase C</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION *

Mucins

Mucins are large, highly O-glycosylated proteins which provide protection for epithelial surfaces. Mucins are grouped together because most have similar characteristics. The two major features are the large fraction of serine and threonine residues which are O-glycosylated and the presence of tandem repeats (Carraway, 2000). Some 20 different human mucins (Fig. 1) have been described, primarily from sequence data that provide the first characterization. These genes are not localized on the same chromosome, so their treatment as a gene family is based on biochemical similarities. Mucins are generally divided into secreted and membranous, based both on sequence data and biochemical studies. Some membrane mucins such as SMC/MUC4 can be cleaved within the cell and secreted (Komatsu et al., 2002). Functionally, gel-forming mucins provide hydration, lubrication, transport and protection mechanisms for mucosa. They are produced primarily by specialized cells, goblet cells embedded in epithelia or submucosal glands (Ali and Pearson, 2007).

*Modified excerpt from manuscript; Theodoropoulos G., Carraway KL. 2007 Molecular signaling in the regulation of mucins. J. Cell. Biochem. Dec 1;102(5):1103-16 e.g. to promote cell survival (Komatsu et al., 2001; Raina et al., 2004),
Fig. 1 Table of Human Mucin Genes. Search conducted through NCBI Genomic Biology, Human Genome. Rodent names are shown with a lower case “mucin”. Cell surface mucins are in “bold”.

<table>
<thead>
<tr>
<th>MUCIN</th>
<th>Approved Name</th>
<th>RefSeq ID’s</th>
<th>Chromosome</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td>mucin 1, cell surface assoc.</td>
<td>NM002456</td>
<td>1q22</td>
<td>4582</td>
</tr>
<tr>
<td>MUC2</td>
<td>mucin 2, oligomeric mucus/gel-form.</td>
<td>NM002457</td>
<td>11p15.5</td>
<td>4583</td>
</tr>
<tr>
<td>MUC3</td>
<td>mucin 17, cell surface assoc.</td>
<td>NM001040105</td>
<td>7q22</td>
<td>140453</td>
</tr>
<tr>
<td>MUC3A</td>
<td>mucin 3A, cell surface assoc.</td>
<td>AF113616</td>
<td>7q22</td>
<td>4584</td>
</tr>
<tr>
<td>MUC3B</td>
<td>mucin 3B, cell surface assoc.</td>
<td>AJ291390</td>
<td>7q22</td>
<td>57876</td>
</tr>
<tr>
<td>MUC4</td>
<td>mucin 4, cell surface assoc.</td>
<td>NM018406</td>
<td>3q29</td>
<td>4585</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>mucin 5AC, oligomeric mucus/gel-form.</td>
<td>XM001130382</td>
<td>11p15.5</td>
<td>4586</td>
</tr>
<tr>
<td>MUC5B</td>
<td>mucin 5B, oligomeric mucus/gel-form.</td>
<td>XM001126093</td>
<td>11p15.5</td>
<td>727897</td>
</tr>
<tr>
<td>MUC6</td>
<td>mucin 6, oligomeric mucus/gel-form.</td>
<td>XM290540</td>
<td>11p15.5</td>
<td>4588</td>
</tr>
<tr>
<td>MUC7</td>
<td>mucin 7, secreted</td>
<td>NM152291</td>
<td>4q13.3</td>
<td>4589</td>
</tr>
<tr>
<td>MUC8</td>
<td>mucin 8</td>
<td>U14383</td>
<td>12q24.3</td>
<td>4590</td>
</tr>
<tr>
<td>MUC9</td>
<td>oviductal glycoprotein1, 120kDa</td>
<td>NM002557</td>
<td>1p13.2</td>
<td>5016</td>
</tr>
<tr>
<td>MUC10</td>
<td>RECORD DISCONTINUED</td>
<td>NM0002557</td>
<td>386748</td>
<td></td>
</tr>
<tr>
<td>MUC11</td>
<td>Withdrawn/substituted by MUC12</td>
<td>10071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUC12</td>
<td>mucin 12, cell surface assoc.</td>
<td>XM379904</td>
<td>7q22</td>
<td>10071</td>
</tr>
<tr>
<td>MUC13</td>
<td>mucin 13, cell surface assoc.</td>
<td>NM033049</td>
<td>3q21.2</td>
<td>566671</td>
</tr>
<tr>
<td>MUC14</td>
<td>EMCN, endomucin</td>
<td>NM016242</td>
<td>4q22.1</td>
<td>51705</td>
</tr>
<tr>
<td>MUC15</td>
<td>mucin 15, cell surface assoc.</td>
<td>NM145650</td>
<td>11p14.3</td>
<td>143662</td>
</tr>
<tr>
<td>MUC16</td>
<td>mucin 16, cell surface assoc.</td>
<td>NM024690</td>
<td>19p13.2</td>
<td>94025</td>
</tr>
<tr>
<td>MUC18</td>
<td>MCAM, melanoma celladhesive molecule</td>
<td>X68264</td>
<td>11q23.3</td>
<td>4162</td>
</tr>
<tr>
<td>MUC19</td>
<td>mucin 19, oligomeric</td>
<td>AY236870</td>
<td>12q12</td>
<td>283463</td>
</tr>
<tr>
<td>MUC20</td>
<td>mucin 20, cell surface assoc.</td>
<td>NM152673</td>
<td>3q29</td>
<td>200958</td>
</tr>
</tbody>
</table>
Membrane mucins not only provide the ultimate barrier for the epithelial surface but also stimulate additional protective mechanisms by their involvement in cell signaling (Carraway et al., 2003). Membrane mucins are produced by many epithelia of many tissues and organs. Much of the attention directed toward mucins results from their roles in diseases of the epithelia in which production of the mucins is altered, including carcinomas, which are derived from epithelial cells.

**Biosynthesis**

The synthesis of mucins follows the general scheme of all secreted and cell surface glycoproteins. Extremely large transcripts are translated on endoplasmic reticulum-associated ribosomes and threaded through a channel into the ER lumen. N-glycosides are added co-translationally, and the signal sequence that specifies ER binding is removed by proteolysis. The glycoproteins next undergo a “copy-editing” step to eliminate mis-folded proteins that involves deglucosylation/glucosylation and proteosomal degradation (Helenius and Aebi, 2004). O-glycosylation and further processing of the N-glycosides occurs during the transit of the mucins to the cell surface. In the case of the gel-forming mucins, disulfide formation between subunits and packaging into granules also happens during this transit (Perez-Vilar and Hill, 1999). The soluble form of at least one membrane mucin, Muc4, is also packaged into secretory granules in selected tissues (Rossi et al., 1996; Arango et al., 2001). Although membrane mucins are frequently pictured as perpendicular to the membrane, it is clear that
this orientation is not possible in the transit compartments responsible for glycosylation, since the mucin polypeptides are much longer than the glycosyltransferases which must glycosylate them. In order to account for the extensive glycosylation along the mucin polypeptides and the relatively rigid structure resulting from that glycosylation, the membrane mucins must be parallel to the membrane as they are being glycosylated. This requirement may explain why most, if not all, membrane mucins undergo a cleavage into two subunits early in their transit to the cell surface (Sheng et al., 1990; Hilkens et al., 1992). Perhaps they assume a more perpendicular orientation after they reach the cell surface.

**Distribution**

Mucins exhibit a highly ordered tissue distribution, indicating a tight regulation of their expression. Some, such as the membrane mucins MUC1 and MUC4, are present in multiple tissues. Expression is also developmentally regulated, usually arising at a specific developmental stage and continuing in the adult [Reid and Harris, 1998]. The airway appears to produce the largest variety of mucins (Rose and Voynow, 2006) and provides examples of cell-specific expression. MUC5AC is secreted by goblet cells of the airway luminal epithelium, while MUC5B is secreted by airway glandular epithelium (Rose and Voynow, 2006). Carcinomas, which are derived from epithelial cells, frequently exhibit an altered expression of mucins compared to their normal counterparts (Hollingsworth and Swanson, 2004). For example, over expression of MUC1 is a characteristic of
most breast carcinomas (Gendler, 2001). Dysregulation of mucin expression also frequently accompanies inflammatory responses (Voynow et al., 2006).

Much of the early work was done by analyzing transcripts by Northern blotting or in situ hybridization but mucin protein levels do not always correspond to the transcript levels. Hence protein levels must be evaluated in order to resolve some of the reported discrepancies in the mechanisms of mucin regulation. Such an example is rat Muc4 in the mammary gland, which is post-translationally down-regulated in the virgin gland but not in the lactating gland (Price-Schiavi et al., 1998). Protein expression has not been as widely evaluated in mucin molecules due to a lack of reagents/antibodies. Many groups have made antibodies against tandem repeat sequences. These antibodies frequently do not recognize mature forms of the mucin, in which these sequences are heavily glycosylated (Burchell et al., 2001).

**SMC/MUC4**

Rat SMC/Muc4 is a heterodimeric glycoprotein originally identified and isolated from a mammary ascites tumor (Sherblom et. al. 1980) and is composed of a mucin subunit ASGP-1 (Sherblom Buck et. al. 1980) attached to the membrane by a transmembrane subunit ASGP-2 (Hull et. al. 1990) (Fig. 1). The human homologue MUC4 similarly contains two subunits, called MUC4α and MUC4β, respectively (Moniaux et. al. 1999) (Figs.2 and 3).
Figure. 2 Model of SMC/MUC4. The two subunits ASGP1/MUC4α and ASGP2/MUC4β are depicted. The glycosylated moieties are shown on both subunits as well as the EGF-like domains on ASGP2/MUC4β.

The two subunits are products of a single gene, which is transcribed as a single mRNA of 9 kb in the rat (Sheng et al., 1992) (18-27 kb polymorphic transcript in the human), which is translated into a polypeptide of approx. 300 kDa with extensive N-glycosylation in the C-terminal third of the precursor protein (Sheng et al., 1990). This precursor is then cleaved to its two subunits prior to the extensive O-glycosylation which occurs in the ASGP-1 subunit (N-terminal two-thirds of precursor) (Sheng et al., 1990). The mucin subunit is heavily glycosylated and for this reason it is depicted on a slant more perpendicular to the membrane (Fig. 2).
Figure 3. Homology of SMC and MUC4. SMC or rat Muc4 has a high degree of homology to the human MUC4. **Figure courtesy of Dr. C. Carraway.**

Although it is expressed in many tissues MUC4 was cloned from cDNA (Fig 1.) isolated from the tracheobronchial mucosa. SMC is both membrane bound and secreted and it is highly expressed in the airway epithelium and rat trachea where our laboratory has previously hypothesized that it may be protective (McNeer et al., 1998). The membrane bound and secreted forms can be easily distinguished from one another through the absence of the c-terminus in the secreted form (Fig. 4). Recent evidence (Komatsu et al., 2002) indicates that the cleavage involved in the formation of the soluble form and the two membrane subunits occurs at the same time. This mechanism is intracellular and proteolytic.
The regulation of gel forming mucin expression in the airway is an active area of investigation, but little is known about the membrane mucins. Most investigations are focused on MUC2 and MUC5. Through the development of polyclonal and monoclonal antibodies to MUC4 our laboratory is uniquely well positioned to elucidate the protein expression of this molecule in the airway. We have antibodies to ASGP1 and ASGP2 (intracellular and extracellular epitopes). These antibodies can be used in immunohistochemistry, immunoblotting, immunofluorescence and immunoprecipitation experiments. The antibodies available in our repertoire are as follows:
• **4F12**: an anti-rat monoclonal antibody to ASGP2

• **1G8**: an anti human monoclonal antibody to MUC4β. The location of the epitope on the molecule is illustrated in fig.3.

• **C-pep**: anti-human and rat polyclonal antibody against the c-terminus of ASGP2.

• **HAM1/HCpep**: anti-human polyclonal antibody against the c-terminus of MUC4β.

• **Poly-ASGP2**: anti-rat polyclonal antibody against c-terminus of ASGP2

Even though not much is known at this time about specific Muc4 mediated signaling paths, an antiapoptotic effect has been reported in tumor cells transfected with Muc4 (Jepson et al, 2002). This effect could be significant in pathological conditions since over-expression of MUC4 protein is reported in human lung carcinomas (Hanaoka et al., 2001) as well as in tumors from other tissues. The regulatory events and mechanisms leading to the mis-expression of this protein are not known. The influence of factors known to mediate mucin synthesis such as EGF, RA (Gray et al., 2001), and cigarette smoke (Takeyama et al., 2001) on MUC4 could be further evaluated.
TRANSCRIPTIONAL REGULATION

Promoter regions of mucin genes

Much of the work on mucin regulation has been directed toward transcriptional control. The diversity of gene expression patterns described above can be ascribed to two aspects of transcriptional regulation: specific, unique promoter sequences in the MUC genes and differential expression and regulation of transcriptional factors. Mucin promoter sequences (van Seuningen, 2001; Andrianifahanana, 2006) have been described and served to identify many specific transcription factors involved in MUC gene regulation. Some of these transcription factors are common to multiple mucins (Fig. 5). This table contains transcription factors common to MUC4 and other mucins that have been characterized by analyses of their specific interactions with the defined promoter elements.

**Fig. 5. Table of transcription factors.** These transcription factors are involved in mucin gene regulation that are common to MUC4 and other mucins.

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Mucin gene regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDX family</td>
<td>MUC2, MUC4</td>
</tr>
<tr>
<td>GATA family</td>
<td>MUC1,MUC3/17, MUC4, MUC5B</td>
</tr>
<tr>
<td>HNF</td>
<td>MUC4</td>
</tr>
<tr>
<td>PEA3</td>
<td>Rat Muc4</td>
</tr>
<tr>
<td>Sp1 family</td>
<td>MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC6</td>
</tr>
</tbody>
</table>
Differentiation factors

Mucins are the products of differentiated epithelia and their production is affected by factors that promote differentiation. What is sometimes difficult to discern is whether changes in mucins result only from cellular changes or also from increases in gene expression within those cells. For example, airway epithelia respond to irritants by increasing the number of goblet cells which secrete the gel-forming mucin MUC5AC (Rose et. al., 2006). Chronic goblet cell hyperplasia also occurs in chronic airway diseases such as asthma. Thus, the resulting increase in mucin production can be partially explained by the increase in the terminally differentiated goblet cells. However, studies in cell culture models also suggest that differentiation factors can directly affect mucin gene expression.

The primary differentiation factor studied has been retinoic acid, which acts via specific nuclear receptors RAR and RXR to activate gene transcription (Leid et al., 1992). Retinoic acid contributions to airway cell differentiation depend on its integration with a complex set of environmental factors, including the extracellular matrix (Moghal and Neel, 1998). Normal human bronchial epithelial cells undergo mucosecretory differentiation when grown in the presence of retinoic acid on collagen gel, which is necessary for the induction of the retinoic acid receptor. An important effect of the retinoic acid is to repress signaling through the canonical mitogenic pathway Raf-Erk (Fig. 6). Inhibition of either the upstream receptor tyrosine kinase, the epidermal growth factor receptor, or the Erk kinase MEK could replace the collagen requirement for retinoic acid-
dependent differentiation. What other pathways may be involved is unclear, but studies in some other systems have shown the ratio of the activities of two MAP kinases, p38 and Erk, to be an indicator of differentiation (Nebreda and Porras, 2000).

**Fig. 6 Molecular signaling in mucin regulation**

In the case of the membrane mucin MUC4, retinoic acid induced increased gene expression in the pancreatic tumor cell via its receptor RARα (Choudhury et al., 2000). Surprisingly, the retinoic acid appeared to act via an intermediate TGFβ, which has been shown in other systems to repress Muc4 expression by a post-translational (Price-Schiavi et al., 2000) or transcriptional (Idris and Carraway, 2000) mechanism. Anti-TGFβ blocked both TGFβ- and retinoic acid-induced expression of the MUC4 gene in the pancreatic tumor cells. Moreover, RARα antagonists inhibited the upregulation of both TGFβ-2 and MUC4 transcripts. Thus, TGFβ appears to be able to act on MUC4 by a number of mechanisms depending on cell context.

Phorbol esters serve as both differentiation factors and tumor promoters, depending on cell context, frequently by activating protein kinase C (PKC)
isoforms. Phorbol 12-myristate 13-acetate (PMA) increases the transcription of MUC2 in HM3 colon cancer cells, acting on a 5'-flanking promoter region containing Sp1 and NFκB sites (Lee et al., 2002). Analyses using pharmacological inhibitors and dominant negative effectors implicated activation of the Ras-Erk pathway via PKC. NFκB was also directly implicated. Activated Erk participates in the regulation of several different mucin genes (Figs. 6,7), involving different upstream activators and downstream effectors, representing the variety of mechanisms by which this pathway may participate in cellular regulation.

Butyrate acts as a differentiation agent for a number of cell types, including those of the colon, in which it is produced by anaerobic bacteria. Treatment of the human colon cancer cell line LS174T with butyrate induces mucin expression, increases histone H3 acetylation and activates MUC2 production by stimulating MUC2 gene expression (Hatayama et al., 2007). Cell cycle arrest, but not apoptosis, accompanied this treatment. Moreover, inhibition of MEK blocked MUC2 production, implicating the Erk signaling pathway (Fig. 6). These results suggest an activation of the MUC2 gene by butyrate that involves inhibition of histone deacetylation and Erk phosphorylation.
Figure 7. Table of tissue distribution and regulative growth factors of airway mucins.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
<th>Tissue</th>
<th>Pathway</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>↑</td>
<td>lung</td>
<td>SP1</td>
<td>Transcript.</td>
<td>Kuwahara, 05</td>
</tr>
<tr>
<td>Elastase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>↑</td>
<td>nasal epithel.</td>
<td>TNFR</td>
<td>Transcript.</td>
<td>Shirasaki, 03</td>
</tr>
<tr>
<td>MUC2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>↑</td>
<td>Airway</td>
<td>SP1</td>
<td>Transcript.</td>
<td>Perrais, 02</td>
</tr>
<tr>
<td>IL1</td>
<td>↑</td>
<td>Airway</td>
<td></td>
<td>Transcript.</td>
<td>Kim YD</td>
</tr>
<tr>
<td>MUC4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFTR</td>
<td>↓</td>
<td>Pancreas</td>
<td>?</td>
<td>transc/posttrans.</td>
<td>Singh, 07</td>
</tr>
<tr>
<td>IGF</td>
<td>↑</td>
<td>rat mammary</td>
<td>Erk</td>
<td>trasc./product.</td>
<td>Zhu, 00</td>
</tr>
<tr>
<td>IGF</td>
<td>↑</td>
<td>mammary</td>
<td>Erk</td>
<td>Transcript.</td>
<td>Xiaoyun, 05</td>
</tr>
<tr>
<td>IL4</td>
<td>↑</td>
<td>lung</td>
<td>Jak3</td>
<td>Transcript.</td>
<td>Damera, 06</td>
</tr>
<tr>
<td>IL9</td>
<td>↑</td>
<td>Lung</td>
<td>Jak3</td>
<td>Transcript.</td>
<td>Damera, 06</td>
</tr>
<tr>
<td>Interferonγ and</td>
<td>↑</td>
<td>pancreas</td>
<td>stat1/RAR</td>
<td>Transcript.</td>
<td>Adrianifahana-</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nana, 06</td>
</tr>
<tr>
<td>Leptin</td>
<td>↑</td>
<td>rat colon</td>
<td>PI3K/MAP</td>
<td>Transcript.</td>
<td>El Homsi, 07</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>↑</td>
<td>Airway</td>
<td>?</td>
<td>Transcript.</td>
<td>Fischer, 03</td>
</tr>
<tr>
<td>Elastase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinoic</td>
<td>↑</td>
<td>Pancreas</td>
<td>RAR/Tgfβ</td>
<td>Transcript.</td>
<td>Choudhury, 00</td>
</tr>
<tr>
<td>Acid (RA)</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ</td>
<td>↓</td>
<td>pancreas</td>
<td>smad2/4</td>
<td>Transcript.</td>
<td>Jonckheere, 04</td>
</tr>
<tr>
<td>TGFβ</td>
<td>↓</td>
<td>uterus</td>
<td>?</td>
<td>transc/product.</td>
<td>Idris, 00</td>
</tr>
<tr>
<td>Protein</td>
<td>Action</td>
<td>Location</td>
<td>Pathway</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>--------</td>
<td>----------------</td>
<td>------------------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td>TGFβ</td>
<td>↓</td>
<td>mammary</td>
<td>SMAD Posttranslat.</td>
<td>Price-Schiavi Soto 98,00</td>
<td></td>
</tr>
<tr>
<td>MUC5AC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexa-methasone</td>
<td>↓</td>
<td>Lung tumor</td>
<td>? transc./prodc.</td>
<td>Lu, 05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑</td>
<td>primary lung</td>
<td>? Translat.</td>
<td>Lu, 05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓</td>
<td>lung</td>
<td>GRE Transcript.</td>
<td>Chen Y, 06</td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>↑</td>
<td>Airway</td>
<td>SP1 Transcript.</td>
<td>Perrais, 02</td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>↑</td>
<td>Airway</td>
<td>EGFR activ. transc./prodc.</td>
<td>Casalino, 06</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>↑</td>
<td>Airway</td>
<td>MAPK/Akt trasc./prodc.</td>
<td>Kitazaki, 05</td>
<td></td>
</tr>
<tr>
<td>HAT</td>
<td>↑</td>
<td>Airway</td>
<td>EGFR/Erk Transcript.</td>
<td>Chokki, 04,05</td>
<td></td>
</tr>
<tr>
<td>IL13</td>
<td>↑</td>
<td>Airway</td>
<td>TGFα, EGF Transcript.</td>
<td>Zhen, 07</td>
<td></td>
</tr>
<tr>
<td>IL13</td>
<td>↑</td>
<td>Airway</td>
<td>? Production</td>
<td>Kondo, 02,06</td>
<td></td>
</tr>
<tr>
<td>IL13</td>
<td>↑</td>
<td>Airway</td>
<td>differntiation multiple levels</td>
<td>Yasuo, 06</td>
<td></td>
</tr>
<tr>
<td>IL17</td>
<td>↑</td>
<td>Airway</td>
<td>Erk/MAPK Production</td>
<td>Inoue, 06</td>
<td></td>
</tr>
<tr>
<td>IL1</td>
<td>↑</td>
<td>Airway</td>
<td>Cox2/PKA Production</td>
<td>Gray, 04</td>
<td></td>
</tr>
<tr>
<td>IL1</td>
<td>↑</td>
<td>Airway</td>
<td>Erk/p38 Transcript.</td>
<td>Song, 03</td>
<td></td>
</tr>
<tr>
<td>Neutrophil Elastase</td>
<td>↑</td>
<td>Lung tumor</td>
<td>ROS/NQ O1 multiple levels</td>
<td>Zheng, 07</td>
<td></td>
</tr>
<tr>
<td>Neutrophil Elastase</td>
<td>↑</td>
<td>Airway</td>
<td>Nfkb/MAPK Transcript.</td>
<td>Song, 05</td>
<td></td>
</tr>
<tr>
<td>Neutrophil Elastase</td>
<td>↑</td>
<td>Airway</td>
<td>ROS/PKC transc./prodc.</td>
<td>Shao, 05</td>
<td></td>
</tr>
<tr>
<td>Neutrophil Elastase</td>
<td>↑</td>
<td>Airway</td>
<td>ROS</td>
<td>mRNA stability</td>
<td>Fischer, 02</td>
</tr>
<tr>
<td>---------------------</td>
<td>---</td>
<td>--------</td>
<td>-----</td>
<td>----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Neutrophil Elastase</td>
<td>↑</td>
<td>Airway</td>
<td>TGFα/EGF</td>
<td>Production</td>
<td>Kohri, 02</td>
</tr>
<tr>
<td>NO</td>
<td>↑</td>
<td>Airway</td>
<td>PKC</td>
<td>Transcript.</td>
<td>Song, 07</td>
</tr>
<tr>
<td>PMA</td>
<td>↑</td>
<td>Airway</td>
<td>Ras/MEK/Sp1</td>
<td>transcr./prodc.</td>
<td>Hewson, 04</td>
</tr>
<tr>
<td>Prostaglandin E2</td>
<td>↑</td>
<td>Airway</td>
<td>?</td>
<td>transcr./prodc.</td>
<td>Kook Kim, 06</td>
</tr>
<tr>
<td>TGFα</td>
<td>↑</td>
<td>Airway</td>
<td>e-cadherin/EGF</td>
<td>Production</td>
<td>Kim, 05</td>
</tr>
<tr>
<td>TGFβ2+IL13</td>
<td>↑</td>
<td>Airway</td>
<td>?</td>
<td>transc./prodc.</td>
<td>Chu, 04</td>
</tr>
<tr>
<td>TNFα</td>
<td>↑</td>
<td>Airway</td>
<td>IKKβ</td>
<td>transc./prodc.</td>
<td>Lora, 05</td>
</tr>
<tr>
<td>TNFα</td>
<td>↑</td>
<td>Nasal epithel.</td>
<td>Erk</td>
<td>Transcript.</td>
<td>Young Kim, 04</td>
</tr>
<tr>
<td>TNFα</td>
<td>↑</td>
<td>Airway</td>
<td>Erk/p38</td>
<td>Transcript.</td>
<td>Song, 03</td>
</tr>
</tbody>
</table>

**MUC5B**

<table>
<thead>
<tr>
<th>Neutrophil Elastase</th>
<th>↑</th>
<th>Lung tumor</th>
<th>ROS/NQ O1</th>
<th>Multiple levels</th>
<th>Voynow, 07</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL17</td>
<td>↑</td>
<td>Airway</td>
<td>Jak/Stat/Erk</td>
<td>Transcript.</td>
<td>Chen, 03</td>
</tr>
<tr>
<td>IL6</td>
<td>↑</td>
<td>Airway</td>
<td>Jak/Stat/Erk</td>
<td>Transcript.</td>
<td>Chen, 03</td>
</tr>
<tr>
<td>IL6</td>
<td>↑</td>
<td>Airway</td>
<td>?</td>
<td>?</td>
<td>Smirnova, 01</td>
</tr>
<tr>
<td>PMA</td>
<td>↑</td>
<td>Airway</td>
<td>Ras/Erk</td>
<td>Transcript.</td>
<td>Yuan Chen Wu, 07</td>
</tr>
<tr>
<td>RA</td>
<td>↑</td>
<td>Airway</td>
<td>?</td>
<td>Transcript.</td>
<td>Chen Y, Zhao, 01</td>
</tr>
</tbody>
</table>
**Growth factors and receptors**

Cell surface receptors play a critical role in sensing environmental changes and responding. The epidermal growth factor receptor (EGFR) acts as an important damage sensor in the airway to regulate mucin gene expression (Burgel and Nadel, 2004) (Fig. 7). Two mechanisms appear to be important in activation of the receptor. First, expression of the EGFR is reported to be increased by airway damage (Burgel et al., 2000). Second, EGF, the ligand for the EGFR, is present in airway luminal fluid, but segregated from the receptor by the epithelial polarity barrier (Vermeer et al., 2006). Damage to the epithelium breaks the polarity barrier and permits the ligand-receptor association necessary for activation of the receptor. Airway fluid also contains neuregulin (Vermeer et al., 2003), the ligand for two other members of the EGFR family, ErbB3 and ErbB4. Interestingly, the membrane mucin Muc4 acts as an intramembrane ligand for ErbB2, localizing it in the apical membrane, which can segregate it from forming active heterodimeric complexes with the other receptors until the polarity barrier is broken (Ramsauer et al., 2003,2006; Carraway and Carraway, 2007).

EGFR can be activated by other mechanisms, including transactivation by GPCR via activation of proteases that release EGFR ligands from their membrane precursors, as noted above. Multiple proteases have been reported to be involved in ligand production, either directly or indirectly, including neutrophil elastase, tumor necrosis factor alpha-converting enzyme (TACE) and tissue kallikrein (Nadel et al., 2007). A key aspect is how these proteases are
activated. Oxidant damage, such as that induced by cigarette smoke, stimulates the cellular production of reactive oxygen species (ROS) via NADPH and dual oxidase-1. ROS activate the protease TACE to cleave the precursor for TGFα to release it as an active ligand for EGFR (Nadel et al., 2007). Activated EGFR stimulates *MUC5AC* gene expression via the Erk pathway and NFκB (Nadel and Burgel, 2001).

**MUC4 /ErbB2 Complex**

**ErbB2 signaling in the airway**

The ErbB family (EGFR, ErbB2, ErbB3, ErbB4) of receptor tyrosine kinases has been implicated in proliferation, differentiation, cell death and development (Klapper et al., 2000). Signaling by these four receptors is activated through binding of ligands of the epidermal growth factor family to stimulate receptor phosphorylation via the kinase activity of their cytoplasmic domains (Ullrich et al., 1990). However, ErbB2 has no soluble, high affinity ligand, and ErbB3 has no kinase activity (Klapper et al., 2000). Instead, these two receptors act as a heterodimeric couple to produce a potent signal for cell proliferation and cell survival (Carraway and Cantley, 1994). This signaling capability can contribute substantially in proliferative diseases such as cancer (Klapper et al., 2000).

In a recent paper, Beer et al. were able to predict patient survival in early stage lung adenocarcinoma using gene expression profiles from microarrays (Beer et
al., 2002). The ErbB2 gene had a 92% expression change in tumor vs. normal and a 120% change in expression between stage I and stage III tumor. These data along with a positive relationship between ErbB2 gene expression and poorer patient outcomes indicate that this gene may be significant in tumorigenesis in the lung. As an intramembrane ligand of this molecule, and as a molecule with a of strong expression profile in the airway, MUC4 may have a function in this process.

ErbB2 is necessary for the proper differentiation of airway cells (Fischer et al., 2003) and is activated during airway wound healing (Vermeer et al., 2003). Furthermore the repair process that airway cells undergo in response to inflammation and neutrophil elastase (NE) damage, requires ErbB2 for DNA synthesis during cellular proliferation. Thus, it is very important in differentiated cells to control the activation of ErbB2 and ErbB3. We have proposed that Muc4 contributes to this regulation (Carraway et al., 2002) and inducing ErbB2 phosphorylation.

**MUC4/ ErbB2 interaction in the airway.**

SMC /Muc4 function in the respiratory epithelium is undefined at this time. Muc4 is an intramembrane ligand for the receptor tyrosine kinase ErbB2 (Carraway KL 3\textsuperscript{rd} et al., 1999). This relation has been established in other tissues such as in mammary epithelia (Carraway et al., 2000) and pancreatic cells. Muc4 and
ErbB2 have been found in a heterodimeric complex or in a heterotrimeric complex with ErbB3 (in the presence of neuregulin). Evidence from our laboratory suggests that the trimeric Muc4/ErbB2/ErbB3 complex is formed in the presence of neuregulin (Fig.8) and results in greater phosphorylation of ErbB2 and ErbB3 than the dimeric complexes. These interactions have not been reported or described in the airway.

**Figure 8. Intramembrane interaction of MUC4 and ErbB2.** Effect of neuregulin on the phosphorylation of ErbB2 and ErbB3 with and without MUC4 (figure courtesy of Dr. C. Carraway). ErbB2 is activated when in complex with Muc4 or ErbB3 (in the presence of neuregulin).

Additionally, the functional significance of the MUC4/ErbB2 complex should be further investigated in the airway. In some human carcinomas, specific integrins bind to and modulate signaling by the receptor tyrosine kinase ErbB2 (Falcioni et al., 1997). In addition, new reports found MUC4 over-expression in human lung carcinomas (Hanaoka et al., 2001) and an increase in ErbB2 gene expression in lung tumors (Beer D.G., et al. 2002). The cumulative, synergistic, or even
antagonistic effects of these observations have not been addressed in the airway.

In recent work the effects of neuregulin on the lung have been evaluated (Liu et al., 2002) providing some evidence for a significance of neuregulin signaling in the airway and more specifically the activation of an ErbB2 mediated path. MUC4 is known to affect the phosphorylation and localization of ErbB2 in other polarized epithelia (Ramsauer et al. 2003, 2006). It is possible that MUC4 functions in a recruiting role, transporting more ErbB2 molecules to the apical cellular membrane in epithelia (Ramsauer et al. 2003). It is theorized that MUC4 may contain a signal for apical localization although the specific signal in not known at this time (Carraway, Farooq et al., 2007). This signal may guide the MUC4/ErbB2 complex to the membrane, and localize it there for a prolonged period. Alternative mechanisms for apical localization of glycoproteins in polarized epithelia include N-glycosylation, O-glycosylation, and incorporation into lipid rafts facilitated by acylation (Milligan et al., 1995; Gut et al., 1998; Resh et al., 2004). All these mechanisms can contribute to apical Muc4 localization (Carraway, Farooq et al., 2007) because it is highly N- and O-glycosylated and contains cytoplasmic juxtamembrane cysteines that are appropriately located for palmitoylation (Sheng et al., 1992). The potential signaling outcomes of this phenomenon and their roles in airway conditions or other maladies involving epithelia are not known.
CHAPTER II

Regulation of MUC4 Protein Expression by Mediators of Wound Healing in the Airway*

OVERVIEW

MUC4 is a heterodimeric mucin that is expressed in multiple tissues and on the membrane of airway epithelia as well as secreted into the overlying mucus. In addition to its contribution to the protective mucus layer, MUC4 is the only intramembrane ligand of ErbB2. MUC4 binds to, and modulates the phosphorylation state and cellular localization of this tyrosine kinase. The airway is at constant risk to injury and inflammation in conditions such as asthma. ErbB2 is involved in airway healing upon wounding and in the differentiation of airway epithelia. Although MUC4 has been studied in other tissues, little is known about the conditions and factors that control protein expression in the airway. As a consequence the resultant effects on ErbB2 are also unknown. We investigated the expression of MUC4 in airway cell culture and identified growth factors and cytokines involved in wound healing as mediators of its expression in the airway. Retinoic acid enhanced MUC4 protein expression and EGF decreased MUC4 protein expression. These two effects were not dependant on extracellular matrix (ECM) components, but were ligand dependent responses. Furthermore, Tgfβ down-regulated MUC4 protein expression, a result similar to that we have previously observed in the mammary gland. The EGF effect

*Manuscript to be submitted in Spring 2009
included the activation of Erk and was transcriptional. These studies describe a new tissue specific regulation for MUC4 in the airway and support MUC4 as a molecule that is enhanced by differentiation.

INTRODUCTORY REMARKS

Altered mucin expression is a common characteristic of many airway disorders. MUC4 is expressed in the normal airway (Mc Neer et al., 1998) and miss-regulated at the transcript, and protein levels in the upper airway of different disease conditions (Martinez-Anton et al., 2006). The authors support our own prior finding that MUC4 protein is endogenously expressed in ciliated airway epithelia. More significant is evidence of variance in the regulation of mucins molecules with no specific trend or indication among the other mucins. Hence, although mucins have been considered molecules of similar function, the regulation of each type may be distinct and specific (Theodoropoulos et al., 2007; Durand-Reville et al., 1999). MUC4 expression was previously reported to be up regulated in lung carcinomas (Martinez-Anton et al., 2006). Increased support for an altered state of MUC4 expression in airway related diseased conditions has made the urgency for the elucidation of additional regulative paths more pressing.

The role of ErbB2 in the proliferation of epithelia is widely studied as are the signaling effects of its over-expression in tumor cells. Accordingly, studies have shown that ErbB2 expression is altered in early stage lung adenocarcinomas,
where varying integrin complexes may bind to ErbB2 (Falcioni et al., 1997). These studies relied on data gathered using gene expression/RNA profiles from microarrays (Beer et al., 2002), and were not conclusive with respect to protein expression levels. MUC4 is the only identified intramembrane ligand for this receptor. In mucoepidermoid carcinomas, MUC4 is located throughout the membrane (Handra-Luca et al., 2005) (i.e. not solely apical) and is more highly expressed in low grade, more highly differentiated tumors. In another recent paper, MUC4 expression in mucoepidermoid tumors was found to correlate with a better prognosis (Alos et al., 2005) and expression was localized to the membrane and cytoplasm of these samples. These findings along with a positive relationship between ErbB2 gene expression, and poorer patient outcome indicate that this gene may be significant in tumorigenesis in the lung. It was recently shown that neutrophil elastase up regulates the expression of MUC4 in the airway (Fischer et al., 2003) and that MUC4 is transcriptionally regulated by IL-4, an inflammatory cytokine significant in asthma (Damera et al., 2006). These data demonstrate that the MUC4 and ErbB2 complex may be active during inflammation or other processes.

Our lab proposes that MUC4 is regulated by multiple exogenous growth conditions, factors, and cytokines in airway epithelia. We have focused on the regulation of protein expression and analyzed epidermal growth factor (EGF) which is known to be a proliferative signal, and compared it to retinoic acid (RA), a known differentiating signal in the airway. Vitamin A inhibits the secretion of
EGF in airway cells (Miller et al., 1993). On the other hand, airway signals that promote cellular growth/replication discourage differentiation. In particular EGF increases total cell numbers and the removal of EGF from culture increases the amount of ciliated cells which are a marker of differentiation (Clark et al, 1995). In the airway, EGF is present in the luminal air surface liquid and stimulates the phosphorylation of ErbB1/ EGF receptor (Vermeer et al., 2005). It is proposed by the same group, that EGF is not expressed directly by airway cells, and that EGF stimulation may require depolarization, to re-position and expose EGFR, since they find EGFR expressed more basal to the tight junctions of these cells. The effects of EGFR stimulation and inhibition may be tissue or cell-type specific and thus variable. EGFR could be activated in airway epithelia in an autocrine fashion through the lateral intercellular space when cells experience mechanical stress (Tschumperlin et al., 2004). This stress induced activation further activates the Erk pathway. In more recent work, the EGF receptor (EGFR) has been linked to e-cadherin signaling in the airway. Inhibition of EGFR, upregulated e-cadherin in airway cells (Al Moustafa et al., 1999) and promoted a more differentiated phenotype in airway cell lines. This finding is consistent with other data demonstrating that e-cadherin and EGF regulate mucin expression (Kim et al., 2005). The proposed mechanism for the effect on mucin is through the promotion of recruitment of an EGFR phosphatase through E-cadherin activation. This phosphatase activity down-regulates EGFR phosphorylation.
These two experimental observations lead us to test the effect of EGF on MUC4 protein expression.

Like other wound repair processes, the sequence of events in airway repair involve cell spreading and migration followed by proliferation. The latter presumably to close the wound if it is large, requiring new cells. In the airway, EGF stimulates cell spreading, migration, and the promotion of wound healing (Kim et al., 1998). Also, the inhibition of EGFR in epithelia increases the expression of pro-inflammatory genes (Woodworth et al., 2005). The above mentioned data (Fisher et al., 2005) are consistent with this EGFR data since NE is a pro-inflammatory agent. Hence there is support for altered MUC4 expression during EGF activation, and EGF mediated expression of inflammatory cytokines.

Retinoic acid (RA) has been found to be a morphogen during development and a differentiation factor in airway epithelia. Numerous effects have been described in the airway, including developmental defects in the bronchi of embryos when RA signaling is faulty (Matt et al., 2003). In an earlier paper. From this work, investigators claim that MUC4 RNA expression is RA dependent (Bernacki et al., 1999). These initial observations lead us to believe that RA is a candidate exogenous growth factor that may alter MUC4 protein expression in airway epithelia.

At this time there is a significant void in our knowledge of the consequences, molecular and pathological, of mis-localized and mis-phosphorylated ErbB2 in
the airway or the mechanisms through which such alterations may influence wound healing. Altered MUC4 expression consequently affecting ErbB2 is one potential known mechanism that may generate such an impact. Our long term goal in the laboratory is to uncover the processes by which MUC4 is regulated in the airway, and to determine the effects of this altered expression on ErbB2 and other molecules. As a significant modulator of ErbB2 signaling activity, MUC4 is a protein whose own regulation should be further investigated. There is a general trend in the airway regulation of ErbB phosphorylation by factors involved in tissue injury and healing, and in the regulation of MUC4 expression by inflammatory cytokines (NE, IL, IL4, IL13). Recent work has advanced our understanding of mucin regulation in the airway, but the regulation of MUC4 protein expression by other growth factors and cytokines known to be significant in the airway repair process has not been addressed. In this study we examined the influence of EGF and RA known factors central in airway cell growth, differentiation and wound healing, on the expression of MUC4 protein. We used the widely accepted cell models normal human trachoebronchial epithelia (NHBE), rat tracheal epithelia (RTE), air liquid interface cells (ALI), and the NCI-H292 human airway cell line in our experiments and evaluated changes in protein expression upon growth factor treatment. We find that MUC4 is a molecule whose protein expression is enhanced in more differentiated cells treated with RA, and is downregulated by EGF at the transcript level through a MAPK pathway.
MATERIALS AND METHODS

Cell Culture

NCI-H292 cells were obtained from ATCC, and maintained according to ATCC specifications (RPMI 1640 with 2mM L-glutamine, and 1.5g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum). Normal human tracheobronchial epithelia cells (NHBE) (Cambrex Corp.) were maintained according to the providers’ specifications. Briefly NHBE were grown on plastic in bronchial/tracheal cell basal medium (BEBM), with the following additives: bovine pituitary extract, human epidermal growth factor, hydrocortisone, epinephrine, transferrin, insulin, retinoic acid, thiodothyronine, gentamicin, and amphotericin. ALI culture was maintained as described below. Primary rat tracheal epithelia (RTE) were isolated from Fischer 344 rats and were grown as previously described (Kaartinen et al., 1993). For some experiments, RTE and NHBE were grown on plastic, Type I collagen, laminin, Matrigel, or growth factor reduced Matrigel. Briefly, each extracellular matrix component was deposited into individual wells, allowed to polymerize under sterile conditions, and then cells were plated. Laminin thickness experiments were conducted by adding a volume of laminin solution to each well or double the volume to each well and then plating the cells.
Cell Lysate Preparation

Cells were rinsed twice with PBS and lysed in the plate with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCL, pH 8.0) containing protease and phosphatase inhibitors (Sigma, St. Louis, MO). Cells were scraped from the culture dishes to generate total cell lysates, which were then cleared at 4°C in a microcentrifuge at 14,000 rpm for 10 minutes. The protein concentration of each cell lysate was determined using the Advanced Protein Assay Reagent (Cytoskeleton, Denver CO). Cells grown on Matrigel were isolated using Matrisperse as per the manufacturer’s instructions.

Immunoblotting

Cell lysates and immunoprecipitates were collected as described above. Laemmli buffer containing β-mercaptoethanol (Biorad, Hercules, CA) was added, and the samples were boiled for 5 minutes. For the separation of membranous and hydrophilic proteins the Mem-PER Eukaryotic Protein Extraction Kit (Pierce Corporation) was used as per the manufacturer’s instructions. Equal amounts of proteins in cell lysates were loaded and separated by 8 or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) at 120 V for 1 hour. Proteins were transferred to a nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI) for 1 hour at 100 V or overnight at 15 V, or to a nitrocellulose
membrane (Hybond, Amersham) for 2 hours at 40 V. Immunoblotting procedures were performed according to the protocol for each antibody. Membranes were probed with primary antibodies against: Muc4 4F12 (Rossi et al., 1996) or 1G8 (Zhang et al., 2005). The secondary antibodies used were goat anti-mouse or anti-rabbit (Promega, Madison, WI) immunoglobulins coupled to peroxidase. Membranes were developed using the Supersignal West Pico Chemiluminescence substrate (Pierce Biotechnology, Rockford, IL) and exposed on X-ray film.

**Immunohistochemistry**

Paraffin-embedded, consecutive 5 μm sections of rat trachea and normal human airway were immunostained for SMC (4F12 mAb), MUC4 (1G8 mAb, Zymed) and ErbB2 (Calbiochem ab3) as described previously (Weed et al., 2004; Zhang et al., 2006). Briefly, slides were dried for 1 h in a horizontal position at 58°C in an oven. Tissues were then dewaxed in xylene and rehydrated through an ethanol gradient to water series. After washing in water, the endogenous peroxidases were blocked with a freshly made 3% H₂O₂ solution for 5 min. Slides were then treated in a preheated target retrieval solution (DAKO S1699) for 20 min at 97°C and cooled for 20 min at room temperature. After washing, tissues were stained with the antibodies in an antibody diluent solution with background reduction at 37°C in a humidified chamber for 30 min. A biotinylated secondary antibody was applied to the slides and incubated for 30 min, and conjugated
streptavidin-peroxidase (DAKO K1500) was added for 30 min at room
temperature. Peroxidase activity was detected by incubation with DAKO Liquid
DAB (3’3-diaminobenzidine dehydrate) in substrate-chromogen solution for about
2 min. Slides were rinsed in water, counterstained with hematoxylin for 25
seconds, then rinsed in running water. Slides were then dehydrated in ethanol
and cleared with xylene before mounting (VWR mounting medium). Images were
acquired with an Olympus BX40 microscope (Olympus, Japan). Negative
controls had no primary antibody added.

**Immunofluorescence**

Consecutive 5 μm sections of paraffin-embedded rat trachea were analyzed
using 4F12 mAb, and human sections with 1G8 primary mAb against MUC4β or
Ab3 (Calbiochem) primary mAb against ErbB2. IHC was conducted as described
above. Immunofluoresence immunostaining was used to localize MUC protein
expression in NHBE cells as described above. Laser microscopy was performed
as described below.

**U0126 Treatments**

NCI-H292 cells were grown as described above. Cells were treated with U0126
(Biosource) using our lab protocol (Ramsauer et. al. 2006). Triplicates for each
condition were pooled.
MUC4 RT PCR RNA amplification

RNA was isolated from NCIH292 treated with or without exogenous EGF using Trizol reagent as per the manufacturer’s instructions. Triplicate RNA samples were used for each condition. Sample integrity and subsequent amplifications were conducted at the University of Miami Oncogenomics Core Facility. RNA integrity and concentration was analyzed with the Bioanalyzer 2100, and Nano Drop device. The reverse transcriptase reaction was done with the ABI RT kit. Amplifications used the Light Cycler PCR System (Roche). Primers were designed to amplify MUC4β on the BioProbes software from the Universal Probe Library (Roche Biosciences) and were purchased from Biosearch without any modifications. Amplified products were normalized to the following genes: B2M; beta 2 microglobulin, PBGD; porphobilinogen deaminase, HPRT; hypoxanthine guanine phosphoribosyltransferase, GLOB; globin, GAPDH. Genorm software was used to calculate the geometric mean of the most stable reference genes that we used to normalize. Primers for the housekeeping genes were purchased from a commercial supplier.

RESULTS

MUC4 protein expression in airway epithelial cells

We investigated the expression pattern in tissue by immunostaining various airway tissue samples (Figs.9-10). In the human, the respiratory epithelium of
the trachea is similar to the rest of the bronchial tree and is comprised of tall pseudostratified ciliated columnar cells, goblet cells, stem cells and serous cells. Goblet cells and basal cells are more common in the upper trachea and columnar cells are more prevalent in the lower trachea. Beneath this layer is the vascularized lamina propria and below that the submucosa consisting of seromucinous glands. These cells are surrounded by hyaline cartilage which comprises the trachea. Lower and more distal, the primary bronchus contains less goblet cells.

We performed immunohistochemical protein detection on slides of rat and human airway tissue. SMC was localized to the most luminal epithelia (Fig.9C). ErbB2 was present in the same cells but has a apical and basolateral staining pattern (Fig. 9D). Staining with additional anti-SMC antibodies provided similar staining patterns in the rat trachea (Figs. 9E-H).

MUC4 protein expression was localized to the most apical layers of the pseudostratified airway epithelia (Figs.10 A,B), as was previously observed for MUC4 mRNA (Buisine et al., 1999). These data provided further insight as to the possible functional significance of the resulting localization within the cell. MUC4 was also found on blood vessel endothelia more basal to the luminal airway epithelia (Fig. 10C) (Zhang et al., 2006). Staining of the ALI cell culture model closely resembled the cell pattern found in tissue of normal human airway (Figs.10 E, F). All of the staining patterns were consistent among the various tissues and cells stained for the expression of SMC/MUC4. MUC4 was
expressed in all of the apical airway epithelial cell layers. Taken together these results clearly show MUC4 protein localized to the apical membrane of differentiated airway epithelial cells.

Effects of the extracellular matrix on MUC4 expression

Mucin gene expression is subjected to differential regulation in the lung and in particular MUC4 gene expression is low in poorly differentiated cells (Buisine et al., 1999). SMC is expressed by rat airway cells (McNeer et al., 1998), so to further these findings we evaluated the potential role of exogenous factors and the extracellular matrix in the regulation of SMC/MUC4 protein expression in the airway.

RTE were grown on plastic (less differentiated) and Matrigel (more differentiated), and immunofluorescence stained for MUC4. MUC4 expression was altered and more concentrated in Matrigel (Fig. 11 A) relative to the plastic grown RTE primary cells. Secreted and membranous MUC4 was up-regulated when grown on Matrigel (Figs. 11 B, E) or thicker ECM (Fig. 11C). The growth of the primary RTE cells on plastic diminished MUC4 levels to undetectable levels and was not used further as a culture condition (Fig. 11D). These findings support previous work from our lab demonstrating ECM dependant MUC4 expression in mammary tissue (Price-Shiavi et al., 1998), and the influence of substratum thickness on mucin regulation.
Regulation of MUC4 protein expression by EGF and RA

To determine if RA and EGF regulated MUC4 expression in the airway we stimulated cells with RA and EGF and evaluated protein expression (Fig. 12). We examined extended EGF signaling, such as during a period of wound healing, and proliferation. To stimulate EGFR phosphorylation, and to induce a ligand dependant response, we treated cells with soluble recombinant rat and human EGF. We found that all of the model cells lines (RTE, ALI) were positive for MUC4 protein expression (Fig. 12A, 2B). In addition, RA stimulated MUC4 expression while EGF down-regulated expression. These effects were not ECM dependant since we observed similar findings in plastic and Matrigel grown cells (Figs. 12A, 12B). All cells were treated with basic and defined media (without serum) and the control cells were not treated with RA or EGF. We used the concentration of EGF previously found to enhance proliferation and the mucous phenotype (Guzman et al., 1995) in airway cells and to activate the MAPK path. Furthermore, NCIH292 cells express EGFR without mutations (Janmaat et al., 2005) so the canonical pathway is stimulated with the addition of exogenous EGF in those cells as well.

During injury airway cells activate and express more TGF β1, as it has an expediting effect on the rate of wound repair (Howat et al., 2002). In our lab, we have observed the downregulation of MUC4 by TGFβ1 by posttranslational mechanisms (Soto et al., 2003) in mammary epithelia. In these experiments, the regulation was SMAD mediated and was reversed by IFNγ treatment which up-
regulated the repressor SMAD 7. TGFβ is a multifunctional cytokine whose mediated signaling is implicated in many tissues and conditions. In this current work, we considered TGF β1 as an agent having a similar effect as EGF on airway wound repair and assessed the effect of this cytokine on airway MUC4 expression. Cells were grown on collagen and Matrigel. Upon treating airway cells with TGF β1, MUC4 was down-regulated (Fig. 12B). Relying on our previous data in the mammary epithelia we presume that the fore-mentioned signaling mechanism (Alos et al., 2005) maybe involved in this regulation as well. Furthermore, this inhibitory effect was not ECM dependant as it was observed on both ECM growth conditions. These results suggest that MUC4 is enhanced during airway epithelial differentiation and down-regulated during proliferative periods.

**Activation of EGFR signaling**

To determine the effect of exogenous EGF on airway signaling we investigated downstream signaling. In previous analyses of the MUC4 promoter, investigators had not seen an effect on MUC4 expression upon TNFα treatment, and that EGF activated the receptor to a greater extent (Reddy et al., 1998). Hence TNFα factor was not used in our experiments. As was mentioned above, we utilized a concentration of EGF that was known to enhance proliferation and stimulated MAPK activation (Guzman et al., 1995). As we increased the concentration of
EGF we observed a dose dependent decrease in MUC4 protein (Fig. 13A top). These results raised the issue as to whether a MAPK path was activated in a similar fashion. To address this issue we immunoblotted for activated Erk/MAPK (Fig. 13A below). We found that Erk was activated in a dose dependent manner and that 20ng/mL was sufficient to activate the pathway. In order to assess the activation of the EGF signaling path we IB treated cell lysates for EGF receptor (EGFR) and for activated EGFR (pEGFR). The receptor was activated by EGF as was Erk (Fig. 13B). The U0126 inhibitor was able to inhibit Erk phosphorylation. Furthermore, EGFR was not phosphorylated at detectable levels without the exogenous addition of soluble EGF. U0126 was able to inhibit pErk with and without EGF, and EGFR levels were not affected by the inhibitor. These results demonstrate that our experimental system functioned with specificity and consistency.

**Effects of EGFR signaling on MUC4 expression**

To determine which downstream signaling pathway induced by EGFR phosphorylation diminished MUC4 protein expression, we used pharmacological inhibitors to block various paths. An EGFR blocking antibody (Ab) blocked the activation of EGFR and up-regulated MUC4 expression (Fig.14A).

U0126 the MEK 1/2 inhibitor, inhibits the subsequent phosphorylation of Erk1/2 by MEK. This pathway is known to be activated by EGFR activation, thus we
exposed 292 cells to the inhibitor for 0.5 and 1 hour and then replaced the media with fresh media (Fig. 14B). Protein was collected after 24 hours and immunoblotted. We found that U0126 mediated inhibition of Erk1/2 phosphorylation results in an upregulation of MUC4 protein. Hence the EGF negative regulation was reversed by Erk1/2 inhibition.

Next MUC4 RNA was amplified using real time PCR in order to elucidate the mechanism by which EGF mediates MUC4 expression. We isolated RNA from NCI-H292 cells treated with and without EGF, from multiple experiments, as described above. We analyzed the integrity of the RNA and made cDNA. Then we performed real time quantitative PCR amplification for the MUC4 transcript and numerous control transcripts. We found that MUC4 transcript is decreased by approximately 3.5 times when EGF is added (Fig. 14C). From these data we can discern that MUC4 protein is decreased via a transcriptionally regulated EGF mediated path.

**DISCUSSION**

In this study we examined the impact of exogenous factors and extracellular matrix components, known to be relevant in airway epithelia differentiation and wound healing, on MUC4 protein expression in the airway. Even though it was previously reported that airway cells adhere equally to laminin, fibronectin, and type IV collagen (Rickard et al., 1991) the ECM is known to modulate other
mucins. Some changes to the ECM may occur through remodeling in the airway during smoking or other injury, hence mucin expression could be affected during that time. Through the observations made in culture (Fig.11 A) and the premise that ECM remodeling occurs in some microenvironments in the transition from normal, to injured, to the restored state, there are a myriad of ECM alterations that may specifically alter MUC4 expression in vivo.

Retinoic acid (RA) is a known morphogen and is necessary for the proper differentiation of airway cells in culture (Matt et al., 2003). In the presence of RA, MUC4 mRNA transcription is up regulated, as are higher molecular weight MUC4 transcripts during the differentiation process. Absence of RA during growth and differentiation alters cellular morphology, resulting in a lack of differentiation, and hyperplasia. EGF activates the EGFR and the EGF Ras/MEK/ERK signaling path. In the airway, EGF stimulation enhances the migration of cells in a wounded area. The two growth factors are linked in the airway. In a relevant and recent paper, Miller et. al (Miller et al., 1993) reported that all-trans retinol inhibited the secretion of EGF. The pro-differentiating effects of ATRA and the pro-proliferative effects of EGF may be opposing. Miller found that in the presence of retinol and EGF, growth of NHBE was enhanced modestly. When EGF was omitted though, cell growth was unaffected since the media was rich in other growth factors, but in the presence of retinol and the absence of EGF, cell growth was drastically reduced. Since MUC4 is enhanced in the presence of RA,
we support a model wherein MUC4 functions in differentiation, and not proliferation of normal airway epithelia.

Next we explored the effects of proliferative (EGF) and other signals (TGFβ1). There are multiple sp1 sites present on the MUC4 promoter (Perrais et al., 2001). These sites are the downstream binding sites for the EGFR phosphorylation Ras/MEK/ERK signaling path. Hence the MUC4 gene is a possible target of EGF/Erk1/2 signaling. One example of induced EGFR phosphorylation that may affect MUC4 in airway epithelial cells is by cigarette smoke (Shao et al., 2004). Furthermore the induction of other mucins by the EGFR/Ras/Raf pathway in airway epithelia, has been established (Perrais et al., 2001, Shao et al., 2004) and it is likely that this signaling may alter MUC4 levels in vivo. MUC4 promoter analysis in airway cells, revealed (Perrais et al., 2002) sp1 cis-elements that may be negative or positive regulators of MUC4 expression and that these effects maybe cell type specific.

From our present work we observe that EGF down-regulates MUC4 expression through a MEK1/2 mediated MUC4 inhibition upon EGFR activation (Figs. 12,14). These findings are consistent with the Miller (Miller et al., 1993) paper which linked these two growth factors (EGF and RA) in the airway MUC4 is regulated by the pro-differentiation agent RA and inhibited by the proliferative agent EGF. Even though these effects were ECM independent in culture, it is widely accepted that proper ECM binding promotes differentiation and polarization in the airway. As more cells are moved in proximity to one another
and as more proliferation occurs, there is an increase in cell-cell junctions. Such a scenario can be envisioned during wound repair, and tissue reconstitution. This growth increases e-cadherin binding which in turn inhibits and is affected by EGF mediated signaling (Kim et al., 2005).

TGFβ is also implicated in airway repair and is activated through integrin signaling paths (Neurohr et al., 2006). Integrins are in turn activated during ECM remodeling in injury. TGFβ decreases migration but enhances adhesion of bronchial epithelial cells (Howat et al., 2002). Our experiments demonstrate a matrix independent down-regulation of MUC4 membrane expression upon TGFβ treatment (Fig.12B).

MUC4 may be significant for the proper polarization and differentiation of airway cells. Since the polarized, ciliated airway epithelia form a barrier to the exterior environment, the state of the intercellular complexes is significant in this protective role. Airway cells have distinct cadherin/intracellular expression patterns and form distinct intracellular and cytosolic complexes (Molock et al., 2006). These distinct complexes may account for varying control of expression in different tissues and responses to injury and depolarization.

MUC4 has been localized to the apical domain, and around the junctional complexes of normal cells. Disruption of the junction may lead to altered signaling in these cells via altered receptor access to ligands such as EGF or heregulin. This model has been proposed previously for ErbB2 (Vermeer et al.,
EGFR activation is cyclical, lasting until epithelial integrity is re-established (Vermeer et al., 2005). MUC4 may affect the propensity for the complex to form, and consequently of ErbB1/ ErbB2 heterodimer (Warren et al., 2006) signaling in the airway. As we mentioned earlier, ErbB2 is constantly expressed in the airway (data not shown). Since Erbb2 is also involved in DNA repair mechanisms, it may be up regulated upon injury (Hu et al., 2006). Our lab has reported that MUC4 has antiadhesive qualities, so a temporary decrease in MUC4 may lead to more cell-cell binding, upon injury. This result would aid in the restoration of proper cell contact, polarity and tissue re-composition.

MUC4 protein decreases interaction of ErbB2 with the ErbB2 targeted antibody Herceptin in tumors (Nagy et al., 2005). If this concept is extended to other molecular interactions, we could envision a situation by which MUC4 regulation ultimately determines not only the amount of MUC4/ ErbB2 complexes formed, but also the availability of ErbB2 for other molecular interactions. It is important to note that our lab has observed that MUC4 does not prevent the association of ErbB2 to ErbB3 in the airway epithelia (data not shown). It is also known that Erbb2 may be sequestered in the ER (You et al., 1998) and not localize to the membrane where it is functional. Hence the investigation of MUC4 expression contributes to the understanding of another one of it’s complex roles in influencing the binding availability of ErbB2. This last role being distinct from the others we have mentioned; 1. MUC4 EGF –like domain modulation of ErbB2 phosphorylation (Carraway KL 3rd et al., 1999; Funes et al., 2006 and 2.
Recruitment of ErbB2 to the membrane (Funes et al., 2006; Ramsaeur et al., 2003).

In support of our previous work, we have already shown that MUC4 regulation is affected by multiple factors. Our own work has contributed to the observation that MUC4 transcription is up regulated in more highly differentiated airway cultures in the presence of retinoic acid (Bernacki et al., 1999) by adding that MUC4 protein expression is enhanced during differentiation in culture, and by RA.

In more recent work, MUC4 was found to be transcriptionally regulated in the airway (Martinez-Anton et al., 2006). The regulation of MUC4 is significant and may subsequently influence repair through ErbB2. Here we included relevant factors, ECM, cytokines and growth factors and took a first step in a more comprehensive evaluation of the effects of differentiation and proliferation on MUC4 by dissecting the influences of individual agents. Our results demonstrate that MUC4 protein expression is regulated by multiple exogenous growth factors, and ECM components, confirming previous data from our lab collected from other tissues. This work advances the understanding of MUC4 regulation from promoter analysis to tissue specific protein regulation.
**Fig 9. SMC localization in the rat airway.** Rat tacheal sections were processed for immunohistochemical detection, antibodies and magnifications are specified; 
A. H&E staining, 400X  
B. Negative control, 400X.  
C. Anti-Smc 4F12 Ab, 400X.  
D. Anti-ErbB2 Ab 3, 600X.  
E. Anti-Smc pASGP2 Ab, 200X  
F. Anti-Smc pASGP2 Ab, 400X  
G. Anti-Smc cpep Ab, 200X.  
H. Anti-Smc cpep Ab, 400X.
Fig 10. MUC4 localization in the human airway. A-D. Human airway sections. E-G. Human air-liquid interface (ALI) airway epithelial culture insert sections. 200X Abs used are listed: A. anti-MUC4 Ab 15H10, 200X B. anti-MUC4 Ab 15H10, 400X C. anti-MUC4 Ab 1G8, 400X with blood vessel staining D. Negative control. E. ALI anti-MUC4 Ab 1G8 F. ALI H&E G. ALI negative control.
Fig. 11. ECM Regulation of SMC/Muc4 expression. A. IF staining of RTE grown on plastic and Matrigel. B. IB of membrane and secreted Muc4 from RTE grown on Collagen or Matrigel. C. IB of lysates (5μg) and media from RTE grown on 1X or 2X thick laminin. D. IB of RTE media and lysate grown on plastic or collagen. E. Densitometry results of IB from secreted Muc4 from RTE grown on specified substrata. Triplicates of pooled triplicates were used. 4F12 mAb was used in all IF and IB.
Fig. 12. Effect of exogenous growth factors and matrix adhesion on MUC4 protein expression.  
A. RTE cells grown on Matrigel or growth factor reduced Matrigel were treated with (+) or without (-) RA and cell lysates were IB with 4F12 mAb. Two independent experiments were conducted for each condition.  
B. Comparison of RA, EGF, TGFβ mediated MUC4 protein expression. Cells were grown on the specified substrata and treated with the respective growth factor. Protein was collected from triplicates, pooled and IB. Actin levels were detected and used for protein loading control. RTE cells grown on Matrigel or growth factor reduced Matrigel were treated with (+) or without (-) RA and cell lysates were immunobloted with 4F12 mAb. RTE cells grown on plastic onr Matrigel, and ALI cells grown on collagen were treated with (+) or without (-) EGF and cell lysates were immunobloted for MUC4 and actin. RTE cellular lysates from cells grown on collagen or Matrigel and treated with the following factors: TGFβ 200 pmol/ mL and immunobblotted for MUC4 and actin.
A.

<table>
<thead>
<tr>
<th></th>
<th>- RA</th>
<th>ECM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC4</td>
<td></td>
<td>Matrigel</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>GFR Matrigel</td>
</tr>
<tr>
<td>MUC4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>- +EGF</th>
<th>ECM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC4</td>
<td></td>
<td>Plastic</td>
</tr>
<tr>
<td>MUC4</td>
<td></td>
<td>Matrigel</td>
</tr>
<tr>
<td>MUC4</td>
<td></td>
<td>Collagen</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUC4</td>
<td>- TGFb1</td>
<td>Collagen</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>Matrigel</td>
</tr>
<tr>
<td>MUC4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 13. EGF treatment downregulates MUC4 protein expression in airway cells. NCIH292 cells were treated with EGF cell lysates were immunoblotted for the specified molecules. **A.** EGF treatment downregulates MUC4 protein expression and activates the MAPK signaling pathway. EGF was added to cell cultures and cell lysates were collected and immunoblotted for MUC4, pErk, and actin. **B.** EGF induced activation is inhibited by the potent MAPK (MEK) inhibitor U0126. EGF and UO126, or both were added to cell cultures and lysates were collected and IB for pEGFR, EGFR, pErk, Erk and actin.

### A.

<table>
<thead>
<tr>
<th>ng/mL human EGF</th>
<th>15</th>
<th>60</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho Erk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B. Treatment and lysate collection

<table>
<thead>
<tr>
<th></th>
<th>EGF</th>
<th>u0126</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhosphoEGFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PhosphoERK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 14. Erk signaling regulates MUC4 protein expression. NCIH292 cells were treated with the specified inhibitors and protein was IB with 1G8 anti MUC4 mAb. Actin levels were used for protein loading control. **A.** Inhibition of EGF receptor kinase activity with an anti EGFR Ab (ab), up-regulates MUC4 expression. Cells were treated with and without the Ab and cell lysates were IB. **B.** U0126 mediated MEK inhibition up-regulates MUC4 expression. Cells were exposed to U0126 (MEK inhibitor) for 0.5 and 1 hr., then inhibitor was removed and fresh media was added. Protein was collected after 24hrs. and IB. **C.** EGF treatment transcriptionally regulates MUC4 expression. MUC4 RNA was isolated from cells treated with and without EGF. RTPCR amplification for MUC4 RNA was conducted.
CHAPTER III
MUC4 Involvement in ErbB2/ErbB3 Phosphorylation and Signaling in Response to Mechanical Injury *

OVERVIEW
The receptor tyrosine kinases ErbB2 and ErbB3 are phosphorylated in response to injury of the airway epithelium. Since we have shown that the membrane mucin MUC4 can act as a ligand/modulator for ErbB2, affecting its localization in polarized epithelial cells and its phosphorylation, we questioned whether Muc4 was involved, along with ErbB2 and ErbB3, in the damage response of airway epithelia. To test this hypothesis, we first examined the localization of MUC4 in human airway samples. Both immunocytochemistry and immunofluorescence showed a co-localization of MUC4 and ErbB2 at the airway luminal surface. Sequential immunoprecipitation and immunoblotting from airway cells demonstrated that the MUC4 and ErbB2 are present as a complex in airway epithelial cells. To assess the participation of MUC4 in the damage response, cultures of NCI-H292 or airway cells were scratch-wounded, then analyzed for association of phospho-ErbB2 and -ErbB3 with MUC4 by sequential immunoprecipitation and immunoblotting. Wounded cultures exhibited increased phosphorylation of both receptors in complex with MUC4. Scratch wounding also

increased activation of the downstream pathway through Akt, as predicted from our previous studies on Muc4 effects on ErbB2 and ErbB3. The participation of MUC4 in the phosphorylation response was also indicated by siRNA repression of MUC4 expression, which resulted in diminution of the phosphorylation of ErbB2 and ErbB3. These studies provide a new model for the airway epithelial damage response, in which the MUC4-ErbB2 complex is a key element in the sensor mechanism and phosphorylation of the receptors.

**INTRODUCTORY REMARKS**

Membrane mucins are large, highly glycosylated proteins that are expressed at the luminal surfaces of most wet-surfed epithelia (Carraway, 2000). Although they are primarily recognized for their steric protection of cell surfaces (Komatsu et. al, 1999), recent studies have indicated an additional role in cell signaling functions that regulate cell proliferation, differentiation and death (Carraway et al., 2003). For example, MUC1 is phosphorylated on tyrosines on its cytoplasmic tail to provide docking sites for initiating signaling pathways (Carraway et al., 2003).

Muc4 modulates signaling by acting as a ligand for the receptor tyrosine kinase ErbB2 to influence both its cellular localization (Ramsauer et al., 2003; Funes, et. al. 2006) and its phosphorylation (Carraway KL 3rd et al. 1999; Ramsauer, et. al.
In this way Muc4 can potentially influence all of the signaling pathways driven by ErbB2.

Shortly after completion of the synthesis of the Muc4, it forms a complex with ErbB2, which then transits to the cell surface (Ramsauer et al., 2003). In polarized, cultured epithelial cells, the complex is primarily found at the apical surface (Ramsauer et al., 2003) because the apically-directed Muc4 overrides signals which would otherwise direct ErbB2 to the lateral surface (Borg et al., 2000; Dillon et al., 2002).

In differentiated, polarized cells, Muc4 association with ErbB2 causes the ErbB2 to transit to the apical surface (Ramsauer et al., 2003). In contrast, ErbB3 transits to the lateral surface, co-localized with E-cadherin (Ramsauer et al., 2006). This mechanism effectively segregates the two receptors (Ramsauer et al, 2006; Carraway and Carraway, 2007), preventing their heterodimerization and the downstream signaling that promotes proliferation.

Both Muc4 and the ErbBs have been implicated in the airway. In the rat airway Muc4 is found as a membrane form at the luminal surface of the epithelial cells and as a soluble form in the liquid associated with the luminal surface (McNeer et al., 1998). ErbB2 and ErbB3 are also found in the luminal cells and have been proposed to act in a sensor and repair mechanism on the damaged airway (Carraway and Carraway, 2007; Vermeer et al. 2003). A key observation is that the ErbB3 ligand neuregulin is found in the luminal fluid of the airway epithelium (Vermeer et al. 2003). Thus, it is segregated from its receptor ErbB3 in the
lateral cell surface by the epithelial tight junction barrier. Damage to the epithelium and the barrier allow the ligand to bind its receptor, induce ErbB2-ErbB3 heterodimerization, activate receptor phosphorylation and initiate downstream signaling (Carraway and Carraway, 2007; Vermeer et al. 2003). Based on our previous work, described above, the question then arises whether Muc4 participates in this signaling mechanism, since it was not considered in the previous work on the mechanism for ErbB activation in airway damage (Vermeer et al. 2003). We have addressed this question by examining the localization of Muc4 and ErbB2 in the airway, by establishing their presence together in a complex and by analyzing the participation of Muc4 in the activation of ErbB2 and ErbB3 phosphorylation and downstream signaling in response to damage in an airway epithelial cell model.

MATERIALS AND METHODS

Cell Culture

NCI-H292 cells were obtained from ATCC, and maintained according to ATCC specifications (RPMI 1640 with 2mM L-glutamine, and 1.5g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum). Normal human tracheobronchial epithelia cells (NHBE) (Cambrex Corp.), and air liquid interface cells (ALI) (Mattek Corp.) were maintained according to the providers’ specifications. Briefly NHBE were grown on plastic in bronchial/tracheal cell basal medium (BEBM), with the following
additives: bovine pituitary extract, human epidermal growth factor, hydrocortisone, epinephrine, transferrin, insulin, retinoic acid, thiodothyronine, gentamicin, and amphotericin. ALI cells were previously grown 25 days by the manufacturer and maintained on collagen-coated Millipore filters. In our laboratory, serum free DMEM with various hormones and growth factors (provided by Mattek Corp.) was changed each day in the basal compartment of these ALI cells. Primary rat tracheal epithelia (RTE) were isolated from Fischer 344 rats and were grown as previously described (Kaartinen et al., 1993).

**Cell Lysate Preparation**

Cells were rinsed twice with PBS and lysed in the plate with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCL, pH 8.0) containing protease and phosphatase inhibitors (Sigma, St. Louis, MO). Cells were scraped from the culture dishes to generate total cell lysates, which were then cleared at 4°C in a microcentrifuge at 14,000 rpm for 10 minutes. The protein concentration of each cell lysate was determined using the Advanced Protein Assay Reagent (Cytoskeleton, Denver CO). These lysates were used in immunoprecipitation and/or immunoblotting analyses.
**Immunoprecipitation**

Cell lysates in RIPA buffer, 50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, were utilized (150-200 μg per immunoprecipitation) in the immunoprecipitation with the IP Catch and Release 2.0 (Upstate Biosciences) according to the protocol provided. Briefly, columns were cleared and equal concentrations of protein lysates were added, as was the specific immunoprecipitating antibody: 2 μL of human c-pep anti-MUC4 rabbit polyclonal antibody (from laboratory stocks), 2 μL of Zymed 1G8 ab (provided at 0.5 mg/mL), or of Neomarkers Ab 17 (provided at 200 μg/ml) with 10 μL of affinity ligand provided by the manufacturer, and wash buffer to a total volume of 0.5 mL. Columns were rotated overnight at 4°C, and the immunoprecipitate was collected for immunoblotting. For Figs. 3B and 3C an alternative immunoprecipitation method was utilized. Cell lysates in RIPA buffer, 50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40 (150-200 μg lysate protein per IP) were used with Protein A/G agarose beads (Calbiochem). Equal concentrations of protein lysates were added. Immunoprecipitating antibodies were added at 10 μL each: anti MUC4 4F12 (laboratory stocks of 400 μg /ml), Dako A0485 (provided at 0.5 g/L) or Neomarkers Ab 17, and Ab 21 (provided at 200 μg/ml), and wash buffer to a total volume of 0.5 mL. Beads were rotated overnight at 4°C, and the immunoprecipitate was collected for immunoblotting.
Immunoblotting

Cell lysates and immunoprecipitates were collected as described above and immunoblotted as described in Chapter II. Immunoblotting procedures were performed according to the protocol for each antibody. Membranes were probed with primary antibodies against phospho-Akt 473 (Cell Signaling, Beverly, MA), β-Actin (Sigma, St Louis, MO), ErbB2 Ab3 (Calbiochem), phosphor-Tyr 1248 ErbB2 (Upstate, Lake Placid, NY), anti- ErbB2 ab17 (Neomarkers), phosphor-Tyr 1248 ErbB2 (Ab 18, Neomarkers), human c-ErbB2 (DAKO Cytomation, Carpinteria, CA), ErbB3 (1B2E) and phospho-ErbB3 Tyr 1289 (21D3) (Cell Signaling, Beverly, MA), E-cadherin (BD Biosciences, San Jose, CA), phospho-Thr 202/Tyr 204 p44/p42 MAP Kinase (Cell Signaling, Beverly, MA), Muc4 4F12 (Rossi et. al., 1996) or 1G8 (Zhang et al., 2005) and phospho-p70s6K (Cell Signaling, Beverly, MA). The secondary antibodies used were goat anti-mouse or anti-rabbit (Promega, Madison, WI) immunoglobulins coupled to peroxidase. Membranes were developed using the Supersignal West Pico Chemiluminescence substrate (Pierce Biotechnology, Rockford, IL) and exposed on X-ray film. The intensity of the bands was quantified by digitizing the image (Scion Image, Scion Corporation) from x-ray film.
**Immunohistochemistry**

Paraffin-embedded, consecutive 5 μm sections of normal human airway were immunostained for MUC4 (1G8 mAb, Zymed) and ErbB2 (Calbiochem ab3) as described previously in Chapter II (Weed et al., 2004; Zhang et al., 2006).

**Immunofluorescence**

Consecutive 5 μm sections of paraffin-embedded normal human trachea were analyzed using tyramide signal amplification immunofluorescence (Molecular Probes) with 1G8 primary mAb against MUC4β or ab3 primary mAb against ErbB2. Paraffin was removed, and sections were hydrated with three 3 minute baths in xylene, xylene/ethanol 1:1, ethanol, 95%ethanol, 80%ethanol and water. Sections were then bathed four times, for 10 minutes each, in a freshly made 5 mg/mL solution of sodium borohydride in phosphate-buffered saline (PBS), then rinsed with PBS. Cells were permeabilized in acetone for 15 minutes, then rinsed again with PBS. The sections were quenched in a 3% H₂O₂ solution for 2 hours and further rinsed in PBS. Slides were blocked with 1% blocking reagent (TSA kit, Molecular Probes) for 1.5 hours and rinsed with PBS. Biotin was blocked by incubating with reagent A (Molecular Probes) for 15 min., rinsing with PBS, then incubating with reagent B for 15 min, and rinsing with PBS. Primary antibodies were diluted in Blocking Solution (Molecular Probes), and sections were incubated at 4°C overnight. After rinsing with PBS, sections were incubated with peroxidase-conjugated secondary antibodies (Molecular Probes) diluted in blocking solution for 45 min. The manufacturer’s instructions were followed for
Tyramide amplification. Sections were mounted and laser confocal microscopy was performed with a Zeiss LSM 510 microscope (Carl Zeiss, GmbH Germany) using a 40X objective in the Imaging Core at the Diabetes Research Institute, University of Miami Miller School of Medicine. Immunofluorescence immunostaining was used to localize MUC4 protein expression in ALI cells and colocalize MUC4 and ErbB2 in NHBE cells. Media were removed and cells were washed with PBS, and fixed with 3% paraformaldehyde. Cells were then permeabilized in 0.2% Triton X-100, and blocked with 5% bovine serum albumin. Then primary antibodies were added overnight, and washed three times. Secondary antibody (goat-anti rabbit-texas Red, Molecular Probes) was added, and then washed. DAPI was included, or not, in the subsequent wash, and slides were mounted (Prolong gold medium, Invitrogen Corp.). Laser microscopy was performed as described above.

**Mechanical wounding**

NCI-H292 cells were cultured as indicated above, and wounding was done by scratching cell layers with a pipette tip multiple times in each well, using a cross-hatch pattern of four vertical and four horizontal scratches. Three and 24 hours post-injury, cell lysates were collected as described above. Cell lysates from three wells were pooled for immunoprecipitation and immunoblotting.
**MUC4 siRNA inhibition**

NCI-H292 cells were transfected with 100 nM MUC4 siRNA (Dharmacon, Lafayette, CO) or non-targeted siRNA using transfection reagent 2 (Dharmacon, Lafayette, CO) as per the manufacturer's instructions 48 hours before wounding was performed. Triplicates for each condition were pooled.

**AG825 Treatments**

NCI-H292 cells were grown as described above. Cells were treated with AG825 (Biosource)/ tyrophostin AG825 using our lab protocol (Ramsauer et. al. 2006), or treated with AG825 and injured as described above. Total cell lysates were collected three hours post-injury and analyzed by immunoblot. Triplicates for each condition were pooled.

**RESULTS**

**Muc4/MUC4 and ErbB2 co-expression and co-localization in airway epithelial cells**

Our previous work has shown that Muc4 is expressed at the apical surface of the rat trachea (McNeer et al., 1998). However, Muc4 expression is sometimes lost when cells are placed in culture (Komatsu et al., 1997). Therefore, we have examined the expression of Muc4 and ErbB2 in four different types of airway cell cultures. Figure 15A shows strong expression of both Muc4/MUC4 and ErbB2 in
rat tracheal epithelial (RTE) cells, normal human bronchial epithelial (NHBE) cells and air liquid interface (ALI) cultures. We have never encountered a time during which MUC4 protein expression was lost in these cell lines. NCI-H292 cells are frequently used as a model culture system for the airway epithelium (Kim et al., 2005) and undergo contact growth inhibition (van Schilfgaarde et al., 1995). This culture system forms tight junctions between cells (van Schilfgaarde et al., 1995) and expresses all of the catenins, E-cadherin, ICAM-1 (Molock et al., 2006), and actin. These cells undergo differentiation as they approach confluence, with increased amounts of E-cadherin (Fig. 15B) and the formation of adherens junctions. This differentiation is accompanied by increased expression of both MUC4 and ErbB2 (Fig. 15B).

Our previous results on cultured epithelial cells predict that Muc4 and ErbB2 will be co-localized at airway luminal surfaces (Ramsauer et al., 2003; Ramsauer et al. 2006). To examine this question in the airway, we used both immunohistochemical and immunofluorescence analyses in airway tissues. As shown by immunohistochemistry of human airway sections, MUC4 is located at the apical surfaces of the cells of all three layers of the pseudostratified epithelium (Fig. 16A). ErbB2 is co-localized with the MUC4 in the cells exposed to the luminal surface. The co-localization is further shown by merged immunofluorescence images of Muc4 and ErbB2 in human tissue (Fig. 16B). In this case the extracellular Muc4 in the ciliary layer can clearly be seen above the apical surface of the epithelium, where the Muc4 and ErbB2 are co-localized in the apical membrane. These results raise the question whether the MUC4
observed in the ciliary layer is actually associated with the cilia or only with the periciliary fluid. To address this question, we used fluorescent staining of ALI cultures. As shown in Figure 16C, MUC4 staining of ALI clearly shows the presence of the MUC4 in the cilia and apical membrane. ErbB2 and ErbB3 staining were not observed in the cilia and were limited to the cell membrane. This staining pattern was similar to that seen in human airway tissue (Fig. 16B). Importantly, ErbB2 was associated with the apical surface, while ErbB3 was associated with the lateral surfaces of the cells (Fig. 16C), as we have shown in previous studies. These findings were corroborated by immunohistochemical localization of MUC4 to the apical membrane of ALI cell cultures (data not shown). These combined results also show, in contrast to previous studies (Vermeer et al., 2003), that ErbB2 is present at the luminal surface of the airway. As we have demonstrated previously, the localization of ErbB2 is highly dependent on the antibody used for its detection (Idris et al., 2001). Thus, we have used multiple antibodies to confirm the presence of ErbB2 at the luminal surface. Our studies do not, of course, rule out the possibility that some ErbB2 is also localized to lateral surfaces, as we have shown in mammary epithelia (Price-Schiavi et al., 2005), only that it is present at the apical surface with Muc4.

Our previous studies have also shown that a complex of Muc4 and ErbB2 can be demonstrated in multiple tissues (Carraway et al., 2002). In order to detect complex formation, we immunoprecipitated lysates of rat tracheal epithelial cells with anti-Muc4 Ab. MUC4 binding may alter epitopes on ErbB2 (Idris et al., 2001) due to phosphorylation and binding of signaling complexes. Hence for
immunoprecipitations of the complex, we used both extracellular and intracellular domain-oriented antibodies to the ErbB2 and MUC4 to improve immunoprecipitation efficacy. Immunoblotting of the immunoprecipitates for ErbB2 demonstrated the precipitation of a complex of Muc4 and ErbB2 (Fig. 17A), as we had previously shown for other tissues (Price-Schiavi et al., 2005) and cell types (Carraway KL III et al., 1999). The MUC4-ErbB2 complex was similarly demonstrated in human ALI cultures. In this case the lysates were immunoprecipitated with either anti-ErbB2 or anti-MUC4 and the immunoprecipitates were immunoblotted for ErbB2 (Fig. 17B, left panel). In ALI cells, we also immunoblotted for phospho-ErbB2 (1248) and found the phosphorylated receptor in complex with MUC4 (Fig. 17B, center panel, Ab 18). The presence of ErbB2 in the cell lysate was confirmed via immunoblot as a positive control (Fig. 17B, right panel). We previously showed the specificity of the Ham1/ HCpep anti-MUC4 antibody for membranous MUC4 via immunoblot (data not shown). MUC4-ErbB2 complex was also seen in NCI-H292 cells that were immunoprecipitated for MUC4, followed by immunoblotting for the extracellular domain of ErbB2 (Fig. 17C left (ab20)). Next, we immunoprecipitated MUC4 and ErbB2 and immunoblotted for MUC4 (Fig. 17C right). Thus, immunoprecipitation in all three model cultures detected the MUC4/ErbB2 complex. Lastly we used immunofluorescence to detect the complex in NHBE. The complex was formed and detectable in three day cultures (Fig. 17D). This finding is consistent with previous data confirming that MUC4 and ErbB2 are expressed at this time point in culture (Fig. 15B). Taken together,
these results clearly show that the MUC4/ErbB2 complex is formed in the apical membrane of airway epithelial cells. Our data support a functional complex in which MUC4 may mediate ErbB2 phosphorylation.

**Participation of MUC4 in ErbB2/ErbB3 phosphorylation response to NCI-H292 cell culture damage**

As model systems for airway epithelial cell damage, we used the NCI-H292 cells, a widely used cell model for wounding experiments (Shaykhiev et al., 2008), and ALI cells. Differentiated cultures of NCI-H292 cells (Fig. 15B) were subjected to scratch wounding. After a 3 or 24 hr period the cells were lysed and immunoprecipitated with anti-MUC4. The immunoprecipitates were analyzed by immunoblotting with anti-phospho-ErbB2 and anti-phospho-ErbB3 to determine whether phosphorylated two receptors are associated with MUC4. As shown in Fig. 18A, phosphorylated ErbB2 and ErbB3 in complex with MUC4 was observed at both the 3 and 24 hr periods. In immunoblots utilizing two different anti-MUC4 antibodies, MUC4 was not detectable in the “flow-through” material from these IP’s (Fig. 18A “FT”). These results indicate that HAM1 pAb is efficient in the IP of MUC4. Next, the specificity of the HAM1 Ab for membranous MUC4 was studied. The anti-MUC4 HAM-1pAb was used to detect MUC4 in NCIH292 cellular lysate preparations. S; soluble and M; membrane preparations were subjected to SDS-page electrophoresis and immunoblotted (Fig. 18B left). HAM1 bound to the membrane preparation and not the soluble preparation. MUC4 siRNA inhibition was used to demonstrate the specificity of the Ab in IP (Fig. 18B
Upon siRNA inhibition of MUC4, HAM1 IP and subsequent IB for phospho-ErbB3 resulted in a decrease of detectable phospho-ErbB3.

These experiments indicated the presence of a complex of MUC4 with the phosphorylated ErbBs in wounded cultures and suggested that wounding increases ErbB2 and ErbB3 phosphorylation. We then performed the same wounding experiment on ALI cells. After a 3 hr. period, wounding of ALI cells increased ErbB3 and ErbB2 phosphorylation but did not alter total levels of these molecules, as detected by immunoblotting. Therefore, the same pathway was activated by wounding as in the NCI-H292 cells (Fig. 18C). Three hours post injury, ErbB2 phosphorylation was increased more than two and half times relative to uninjured cells (Fig. 18C bottom). These combined observations agree with previous studies showing increased phosphorylation of these receptors during airway damage (Vermeer et. al., 2003), but also indicate that MUC4 is associated with the complex of receptors in the cell cultures subjected to damage. In contrast to the change in receptor phosphorylation, no change was observed in the levels of MUC4 in the wounded cultures (Fig. 18D). In ALI, we immunoprecipitated total ErbB2 and then immunoblotted for total ErbB2 and total ErbB3 (Fig.18E). As was demonstrated in fig. 18B via IB, total ErbB2 levels are not altered when comparing pre to post injury although phospho-ErbB2 levels were increased post injury. However, we do see an increase in ErbB3 in complex with ErbB2. These IP’s demonstrate that the precipitation is specific for MUC4, and show an enhanced association between ErbB2 and ErbB3 post-injury as compared to pre-injury in ALI cells.
As further evidence for the importance of ErbB phosphorylation in the damage response, we examined effects of culture wounding on pathways downstream of the ErbB2/ErbB3 complex, specifically Erk phosphorylation and the Akt pathway (Funes et. al., 2006; Jepson et al., 2002). No changes were observed in Erk phosphorylation due to the epithelial damage (Fig. 19A). However, phosphorylation of both Akt and p70rs6k (Rsk) from the PI3K/Akt and PI3K/rsk pathways were increased in the wounded cultures (Fig. 19B). The levels of pAkt after injury were two times greater than in non injured cells (Fig. 19C). When these experiments were repeated in the ALI system, the results were similar to data from NCI-H292 cells (Fig. 19D). These results are in accord with our previous studies of the effects of Muc4 on downstream signaling (Jepson et al., 2002).

Work from our laboratory has shown that formation of the MUC4-ErbB2 complex in epithelial cells enhances ErbB2 phosphorylation at position 1248 (Ramsauer et al., 2006). To further test the involvement of MUC4 in the phosphorylation response of the receptors, we knocked down the MUC4 in the NCI-H292 cells with siRNA before inducing damage to the cultures. Using this method, we were able to achieve 80-90% decreases in the levels of MUC4 (Fig. 20A). This MUC4 inhibition did not subsequently decrease the total levels of endogenous ErbB2 receptor (Fig. 20A). As noted in Fig. 20B, loss of MUC4 results in a diminution of the phosphorylation of both ErbB2 and ErbB3 in the NCI-H292 cultures in response to wounding. These findings are consistent with previous work from our lab demonstrating that MUC4 expression upregulates ErbB2 phosphorylation.
without impacting total expression of the tyrosine kinase (Ramsauer et. al., 2003; Funes et al., 2006). The mean relative decrease in ErbB2 phosphorylation upon MUC4 siRNA inhibition was nearly five-fold compared to un-inhibited cells (Fig 20C). We previously showed that although ErbB phosphorylation is altered post injury, total ErbB receptor levels are not affected. To further investigate the role of ErbB2 during the post injury period ErBb2 phosphorylation was partially inhibited by the specific inhibitor AG825 (Fig. 20D). We find that inhibition of ErBb2 phosphorylation diminishes ErbB3 and Akt phosphorylation. Together, these results suggest that scratch wounding activates the Akt signaling path through ErbB2. Next NCI-H292 cells were injured and treated with the inhibitor (Fig. 20E). Phosphorylation of both Akt and Rsk were diminished upon AG825 treatment compared to controls in both wounded and unwounded cultures. These results suggest that Akt and Rsk activation upon mechanical injury was mediated by ErbB2 kinase activity in airway epithelia, although they do not rule out participation by other kinases inhibited by this agent.

DISCUSSION

Previous studies by others introduced a novel model for the response of the airway epithelium to damage via phosphorylation of the receptors ErbB2 and ErbB3 (Vermeer et al., 2003). Damage breaks the tight junction barrier segregating ErbB3 from its ligand neuregulin and induces formation of the ErbB2/ErbB3 receptor heterodimer with its concomitant activation and
phosphorylation. However, this model neglects a possible contribution from the ErbB2 ligand MUC4. We have previously shown that MUC4 modulates the ErbB2/ErbB3 complex in polarized epithelial cells by regulating the localization and phosphorylation of ErbB2 (Ramsauer et al., 2006). In the present studies we provide evidence that MUC4 also participates in the response to airway damage. Three aspects are particularly important. 1) MUC4 and ErbB2 are co-localized and present in a complex at the luminal surface of the airway epithelium. One particularly interesting aspect of that localization is the observation of MUC4 without ErbB2 in the cilia of the airway cells. This result implies that there is a mechanism for transport of the MUC4, but not the MUC4-ErbB2 complex into the ciliary membrane. What function MUC4 serves at the ciliary surface is unknown, but its anti-adhesive properties might suggest a role in preventing interactions between cilia which might hinder ciliary movement. 2) Damage to airway cell cultures increases the phosphorylated ErbB2 and ErbB3 associated with MUC4. The presence of phosphorylated ErbB3 in this complex is particularly important because phosphorylated sites on ErbB3 are involved in activating the phosphatidylinositol 3-kinase pathway upstream of the Akt and Rsk pathways. Similar effects of Muc4 have been observed in other cell types (Jepson et al., 2002). Furthermore, we show that ErbB2 is present on the membrane of ALI cells apically prior to injury (Fig. 16C) although ErbB3 is present on the membrane basolaterally. Although total ErbB2 levels do not change upon injury, the amount of ErbB3 in complex with ErbB2 increases. These changes occur upon the disruption of cell-cell contacts post injury. We do not believe that this
increase in ErbB2/3 complex can be attributed to new ErbB3 protein synthesis since the samples are collected only three hours post injury. 3) Downregulation of MUC4 demonstrates its involvement in the effects on ErbB phosphorylation. Moreover, the effects of the ErbB2 inhibitor establish the role of ErbB2 in the MUC4 activation of the downstream pathways.

These results suggest a new model for the airway damage response (Fig. 21). This model is based on two previous studies, the work by Vermeer et al. (Vermeer et al., 2002) showing ErbB2/3 phosphorylation in response to airway damage and our own work showing ErbB2 and ErbB3 segregation promoted by Muc4 in polarized epithelial cells (Ramsauer et al., 2006).

The model is further supported by our present work demonstrating the presence of ErbB2 with MUC4 at the apical surface of airway epithelia, the presence of ErbB3 at the lateral surface and the increased phosphorylation of both ErbB2 and ErbB3 in complex with MUC4 after damage to an airway epithelial cell culture. In this model damage to the epithelium not only allows access of ErbB3 to its ligand, but also breaks the segregation barrier between the MUC4-ErbB2 on the apical surface and ErbB3 on the lateral surface of the polarized airway epithelial cells, as described in our previous work (Ramsauer et al., 2006). Thus the damage promotes formation of the MUC4-ErbB2-ErbB3 complex, its consequent phosphorylation and activation of the downstream Akt and Rsk pathways.
Figure 15. Expression of Muc4/MUC4 and ErbB2 in airway epithelial cells.
A. Immunoblots of Muc4/MUC4 and ErbB2 in RTE, NHBE and ALI cell cultures.
B. Time course of expression of MUC4 and ErbB2 compared to E-cadherin in NCI-H292 cell cultures growing to confluence and undergoing differentiation.
Figure 16. Co-localization of MUC4 and ErbB2 in human airway epithelium. 

A. Immunohistochemical staining of MUC4 and ErbB2 vs control in human airway tissue. Arrows in control note luminal cell surface below ciliary layer. 

B. Immunofluorescence staining of MUC4 (red) and ErbB2 (green) in human airway tissue. In merged panel, note soluble form of MUC4 in periciliary layer and co-localization of MUC4 and ErbB2 at luminal surface (yellow color). 

C. Confocal microscopy of ALI cell apical surfaces permeabilized and stained with C-pep (anti MUC4). Cilia and membranous staining is visible. (400x) ALI cells permeabilized and stained with Dako pAb (antiErbB2). Membranous staining is visible. Cilia are not stained. (400x). ALI cells were permeabilized and stained with anti-ErbB3 mAb (Cell Signaling). Membranous staining is visible.
Figure 17. Association of MUC4 and ErbB2 in complex in three types of human airway epithelial cells. A. Lysates of NHBE cells were immunoprecipitated (IP) with anti-MUC4 (IP/IB) or control (neg) antibody. The immunoprecipitates were analyzed by IB with anti-ErbB2 Ab. B. Left; Lysates of ALI cell cultures were IP with anti-ErbB2 Dako or anti-MUC4 4F12. The immunoprecipitates were analyzed by IB with anti-ErbB2 antibody. Arrows show ErbB2 bands. Right; Lysates of ALI cells were IP with the anti-MUC4 antibody Hcep/Ham1. Immunoprecipitated samples and cell lysates were then IB with anti-ErbB2 antibody (top), and anti-phospho ErbB2 antibody (bottom). C. Lysates of NCI-H292 cells were IP for; left; MUC4 with anti-MUC4 or control antibody (negative IP). Those immunoprecipitates were IB for ErbB2, as was a total cell lysate control right; (top) MUC4 with the 1G8 mAb, and the polyclonal anti-MUC4 Ab (center) ErbB2 with Neomarkers Ab21, and Dako c-erbB2 Ab (bottom) MUC4 in two reactions with human c-pep Ab. D. Co-localization of ErbB2 and MUC4 in human airway epithelial culture. 3 day culture of NHBE were IF for ErbB2 and MUC4. Merged XY image and merged XZ image.
D.

![Image of ErbB2](image1.png)

![Image of MUC4](image2.png)

![Image of Merge](image3.png)

![Image of Merge XZ Plane](image4.png)
Figure 18. Effect of scratch wounding of airway cell cultures on ErbB phosphorylation in complexes with MUC4. A. (above) NCI-H292 cultures with or without scratch-wounding were kept for 3 or 24 hr. in culture, then lysed for IP with anti-MUC4 or a control antibody. The immunoprecipitates were then immunoblotted with anti-phospho-ErbB2, or anti-phospho-ErbB3. (below) The “flow through” (“FT”) from these IPs was IB with anti-MUC4 mAb (1G8), and anti-MUC4 pAb (HAM1). B. right; Soluble (S) and membrane (M) NCI-H292 cell lysates were immunoblotted for MUC4 with HCpep Ab left; NCI-H292 lysates from control and MUC4 siRNA treated cells was immunoblotted for pErbB3. C. ALI cells with or without scratch wounding were kept for 3 hr. in culture and then were immunoblotted with the listed antibodies. Quantitation of pErbB2 is provided below. D. NCI-H292 cultures with or without scratch-wounding were kept 3 hr., then lysed for IB with anti-MUC4 Ab. E. ALI cells with or without scratch wounding were IP with anti-ErbB2 Ab (Neomarkers Ab 17) and then immunoblotted with anti-ErbB2 Ab (Dako) and anti-ErbB3 Ab (Cell Signaling).

A.
B. MUC4

IP
MUC4

Control MUC4 si

pErbB3

C. No Injury Injury

pErbB3

ErbB3

pErbB2

ErbB2

Actin

pErbB2 normalized arbitrary units of expression

No injury Injury

300
200
100
0
D. 

MUC4

E. 

IP ErbB2

ErbB2

ErbB3
Figure 19. Downstream pathways affected by scratch wounding of airway cell cultures. A. NCI-H292 cell cultures with or without scratch-wounding were kept for 3 hr, then lysed for immunoblotting with anti-phospho-Erk. B. NCI-H292 cultures with or without scratch wounding were kept for 3 hr, then lysed for immunoblotting with anti-phospho-Akt and anti-phospho-p70s6k. C. Quantitation of normalized pAkt with and without scratch wounding. D. ALI cultures with or without scratch wounding were kept for 3 hr, then lysed for immunoblotting with anti-phospho-Akt, Akt, and actin.
Figure 20. Effect of MUC4 knockdown on scratch-wounding induction of ErbB2 and ErbB3 phosphorylation. A. NCI-H292 cells were treated with MUC4 siRNA or control RNA and analyzed for MUC4 and ErbB2 (Ab3) by immunoblotting. B. NCI-H292 cultures treated with MUC4 siRNA or control RNA were scratch-wounded as in Fig. 5 and analyzed for phospho-ErbB2 and phospho-ErbB3 by immunoblotting. C. Quantitation of normalized pErbB2 expression with and without MUC4siRNA inhibition. Results are shown as fold induction over pErbB2 expression without MUC4siRNA inhibition. D. NCI-H292 cells were exposed to multiple mechanical wounds, with or without treatment with ErbB2 inhibitor AG825 (3.5 μM), and cell lysates were immunoblotted for phospho-ErbB2 and –ErbB3. E. NCI-H292 cells with or without wounding and the ErbB2 inhibitor AG825 (7.0 μM/AG 7.0) were immunoblotted for phospho-Akt and –Rsk (Rsk).
Figure 21. Model of injury in airway epithelia. Intact pseudo-stratified airway epithelia are polarized. The apical side of the cells borders the airway lumen and has cilia protruding into the lumen. Our data support a model where MUC4 is in an apical complex with ErbB2, although we do not exclude the presence of lateral ErbB2. Upon mechanical injury, the tight junctions between cells are disrupted resulting in depolarized cells. This lack of polarity leads to the formation of an MUC4/ErbB2/ErbB3 complex which is phosphorylated and activated downstream cell signaling.
CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

This work establishes MUC4 as a membrane bound and secreted mucin in rat and human airway epithelia. MUC4 was also found to be expressed on the cilia of airway epithelia, a new finding never previously recorded. The previously supported role for MUC4 in ErbB2 signaling in the breast and pancreas has been extended to the airway with similar findings. In addition in this study we have shown for the first time the formation of the MUC4/ErbB2 complex in an entact human system. Furthermore, MUC4 is now shown to be relevant in the airway wound healing process through the formation of the MUC4/ErbB2 and MUC4/ErbB2/ErbB3 complex. This role is consistent with previous notions that mucins serve a protective role in luminal epithelia.

The levels of MUC4 protein expression in different pathological conditions of the airway should be further evaluated. This may be achieved by comparing normal cells to others. One feasible comparison could be normal airway vs. cystic fibrosis airway epithelia. Altered levels of MUC4 expression, from baseline, can be correlated to levels of EGF and RA. These results may provide clinical significance to these in vitro findings. This finding could be further compared to activated/deactivated Erb pathways in either the normal or pathological condition. In the future, a model or set of experiments which evaluates all of these relevant candidates and their combined or even synergistic outcomes on MUC4 protein
expression would be of great interest. We expect that due to its recently elucidated role as a modulator of ErbB2 and as a potential sole signaling agent (Pino et al., 2006), MUC4 may be a target for intervention and therapeutics in the future. Through our own elucidation of factors that regulate the molecule we have contributed to this becoming a possibility.

MUC4 may affect the ability of ErbB2 to heterodimerize with other receptor molecules. One such molecule is the EGF receptor (EGFR) itself. ErbB2 and EGFR form homo- and hetero-dimers with each other, and the formation of these complexes varies in different cell types. So far we have observed MUC4/ErbB2/ErbB3 complexes but have not addressed the presence of EGFR in such complexes. It has been demonstrated that the presence of MUC4 decreases the amount of Herceptin antibody the binds ErbB2 (Nagy et al. 2005). Hence the proximity of the large MUC4α subunit to ErbB2 may also affect EGFR/ErbB2 complex formation. These experiments can be conducted with the use of si-RNA inhibition of MUC4 and IP of ErbB2 or EGFR and then IB for the detection of complex. Modification of ErbB2/EGFR complexes will affect subsequent downstream signaling.

MUC4 mediated signaling independent of the ErbB’s in another possibility that should be investigated. Candidate genes and proteins for this potential signaling consequence can be identified through a high throughput technique. This could be a microarray for proteins or oligonucleotides followed up by IB of gene products whose levels are significantly changed.
The impact of MUC4 on wound healing should be further evaluated by measuring wound healing response rates in the presence or absence of MUC4 and ErbB2 \textit{in vivo}. These types of experiments are frequently conducted in laboratories that study the airway by measuring airway cell growth and migration in filling the wounded area. In this work we have optimized the siRNA inhibition of MUC4 and this method can be applied to future investigations of wound closure. \textit{In vitro}, MUC levels may be compared in pathological conditions that result in airway injury such as asthma, cystic fibrosis, or chronic obstructive pulmonary disorder. In addition, the presence or absence of MUC4 may impact the extent to which cells are injured. Investigation of a relevant molecular signaling path may be insightful in measuring such alterations.
REFERENCES


O'Connor JC, Julian J, Lim SD, Carson DD. 2005. MUC1 expression in human prostate cancer cell lines and primary tumors. Prostate Cancer Prostatic Dis. 8:36-44.


