BASIC MECHANISM OF AIRWAY EPITHELIAL REPAIR: ROLE OF IL-13 AND EGFR GLYCOSYLATION

by

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ABSTRACT

Epithelial regeneration following injury is crucial for restoring epithelial function to its normal state. Epidermal Growth Factor Receptor (EGFR) has an essential role in maintenance and repair of epithelial tissues. Glycosylated structures attached to many glycoproteins including EGFR can modulate protein function. The main goals of my doctoral research project have been to investigate: (1) the basic mechanism of airway epithelial repair, focusing on the EGFR and IL-13 pathways and (2) the role of Sialyl-Lewis x (sLe^x) and sLe^x decoration of EGFR in airway epithelial repair. In chapter II, we showed that injured Airway Epithelial Cells (AEC) release EGFR ligands (EGF and HB-EGF) and IL-13 during repair. Moreover, for the first time, we demonstrated that IL-13 plays an important role in epithelial repair, and that its effect is mediated through release of HB-EGF and activation of EGFR. We found that the reparative effects of IL-13 on AEC are mediated via IL-13Rα2. This receptor was thought to act only as a decoy receptor until recently. In chapter III, we demonstrated an important role for a specific fucose-containing carbohydrate structure, sLe^x, in bronchial epithelial repair. In chapter IV we showed that sLe^x decorates EGFR on Primary Normal Human Bronchial Epithelial (NHBE) cells and that EGFR decoration with sLe^x increases after mechanical injury. Fucosyltransferase IV (FucT-IV) showed to mediate sLe^x synthesis in AEC and demonstrated a temporal expression during epithelial repair. Using small interfering RNA (siRNA) and blocking antibody for sLe^x we found that sLe^x has an important role in modulating EGFR activity during epithelial repair.

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LIST OF ABBREVIATIONS

Ab	antibody
ADAM	A disintegrin and metalloprotease
AEC	airway epithelial cell
AHBE	asthmatic human bronchial epithelial
ALI	air- liquid interface
AlloA	allomyrina dichotoma agglutinin
AR	amphiregulin
Asn	asparagine
Asp	aspartic acid
BSA	bovine serum albumin
BTC	betacellulin
CM	conditioned medium
Con A	conconavalin A
CPA	cicer arietinum agglutinin
CR	cysteine rich
DMEM	Dulbecco's Modified Eagle Media
DNA	deoxyribonucleic acid
ECM	extra-cellular matrix
EDTA	ethylenediamine tetra-acetate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EPR	epiregulin
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ERK	extracellular signal-regulated kinase
ESL-1	E-selectin ligand 1
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fuc	fucose
FucT	fucosyltransferase
FucTi	fucosyltransferase inhibitor
FX	GDP-4-keto-6-deoxymannose 3,5-epimerase, 4-reductase
GAG	glycosaminoglycan
GalNAc	*N*-acetylgalactosamine
GalT	galactosyltransferase
GDP	guanosine diphosphate
Glc	glucose
Glcase	glucosidases
GlcNAc	*N*-acetylglucosamine
GlcNAc-PT	GlcNAc-1-phosphate transferase
GM-CSF	granulocyte and macrophage colony stimulating factor

GnT	*N*-acetylglucosaminyltransferase
GPCR	G-protein-coupled receptors
GPI	glycophosphatidylinositol
HA	hyaluronan
HB-EGF	heparin-binding EGF-like growth factor
HGF	hepatocyte growth factor
ICAM	intracellular adhesion molecule
IFN-γ	interferon γ
IL	interleukin
KGF	keratinocyte growth factor
KO	knockout
LAD II	leukocyte adhesion deficiency type II
Le^a	Lewis A structure
Le^b	Lewis B structure
Le^x	Lewis X structure
Le^y	Lewis Y structure
LPS	lipopolysaccharide
M-6-P	mannose-6-phosphate
mAb	monoclonal antibody
Man	mannose
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MHC	major histocompatbility complex
MIG	monokine-induced by γ interferon
MMP	matrix metalloproteinase
NHBE	normal human bronchial epithelium
NK	natural killer cell
NRG	neuregulins
O-Fuc-1	O-fucosyltransferase-1
OST	oligosaccharyltransferase
PBS	phosphate buffered saline
Phe	phenylalanine
PI3K	phosphoinositide-3 kinase
PKC	protein kinase C
PLCγ	phospholipase Cγ
ppGalNAcT	polypeptide *N*-acetylgalactosamine transferase
Pro	proline
PTB	phosphotyrosine-binding
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SA	sialic acid
SEM	standard error of the mean
Ser	serine
SFM	serum free medium
SH2	Src homology 2
SSEA-1	stage-specific embryonic antigens

SV	simian virus
sLe^x	sialyl Lewis X structure
ST3GalT	α2,3 sialyltransferase
STAT	signal transducer and activation of transcription
ST-HSC	short-term hematopoietic stem cell
TFF2	trefoil factor 2
TGF-α	transforming growth factor α
TGF-β	transforming growth factor β
TGN	trans-Golgi network
Th2	T helper 2 cell
Thr	threonine
TM	transmembrane
TNF-α	tumor necrosis factor α
TSR	thrombospondin type repeat
WGA	Wheat germ agglutinin

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COAUTHORSHIP STATEMENT

Chapter II

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Chapter III

Chapter IV is a modified version of a paper published in *The American Journal of Physiology. Lung Cellular and Molecular Physiology* [Allahverdian S, Wojcik KR, Dorscheid DR. Airway epithelial wound repair: role of carbohydrate sialyl Lewis x. Am J Physiol Lung Cell Mol Physiol. 2006 Oct;291(4):L828-36]. KR Wojcik performed the experiments for Fig. III.5 and Fig. III.6. I analyzed this data in combination with the remainder of the figures that were solely performed by myself.

Chapter IV

B.Wong provided the confocal image for the Fig. IV.1 panel A. A.Wang aided in performing the experiments for the Fig. IV.1 panel B and Fig. IV.2.

CHAPTER I. INTRODUCTION*

I.1 Overview

The interaction of the human body with its environment is complex. As a result of constant insults and challenges, our body needs protection. The exposed surfaces of our bodies are covered with a sheet of closely packed epithelial cells that acts as a physical barrier against the external environment. The strength of the barrier is a result of cell structure and cell linkages. Epithelial cells are tightly linked together and to the underlying protein matrix via cell-cell and cell-matrix connections using specialized cellular proteins. In addition to barrier properties, the epithelium participates in the essential functions of secretion, absorption, transport of ions and fluid, diffusion and sensation. After injury, the resulting inflammatory responses are essential to initial repair of the damaged epithelial layer. If repair does not occur, inflammation persists. Molecular mechanisms of epithelial repair are not entirely understood. Likewise, mechanisms for persistence of epithelial injury or inflammation remain largely unknown.

Several proteins essential for normal cell physiology, such as membrane bound receptors for growth factors and cytokines, are glycosylated (1, 2). These carbohydrate structures known as glycans modulate the function of proteins and lipids that they are attached to and can be involved in cell-cell and cell-matrix interactions (3-7). It has been shown that cell surface glycoconjugates have essential roles in cell adhesion (5), migration (8), proliferation (9) and growth potential (10). There are at present many

* The materials presented in this chapter, in part, were presented in a review paper as mentioned in LIST OF PUBLICATIONS, PRESENTATIONS, AND AWARDS and cited as Allahverdian et al. Carbohydrates and epithelial repair - more than just post-translational modification. Curr Drug Targets. 2006 May;7(5):597-606.

disease processes for which treatments are sub-optimal and new therapies will and can only be developed once a more complete understanding of the pathological process is developed. The understanding of the role for carbohydrates in the reparative processes of injured epithelia and how, if dysregulated, they may permit the development of disease is one such area.

I.2 Anatomy of the lower respiratory tract

The respiratory tract is divided into upper and lower portions. The upper respiratory tract extends from the nose to the larynx and the lower from the larynx to the alveoli. In the lower respiratory tract, the trachea and bronchi are a continual system of muscular air conducting tubes, followed by a distal region of gas-exchanging structure formed of alveoli. The tracheobronchial tree is divided into cartilaginous airways, or bronchi, and noncartilaginous membranous airways, or bronchioles. The trachea, bronchi, and bronchioles, including the terminal bronchioles, are conventionally classified as conducting airways. The respiratory bronchioles, alveolar ducts, and alveoli participate in gas exchange and are classified as the terminal respiratory unit, also called acinus (Fig.I.1).

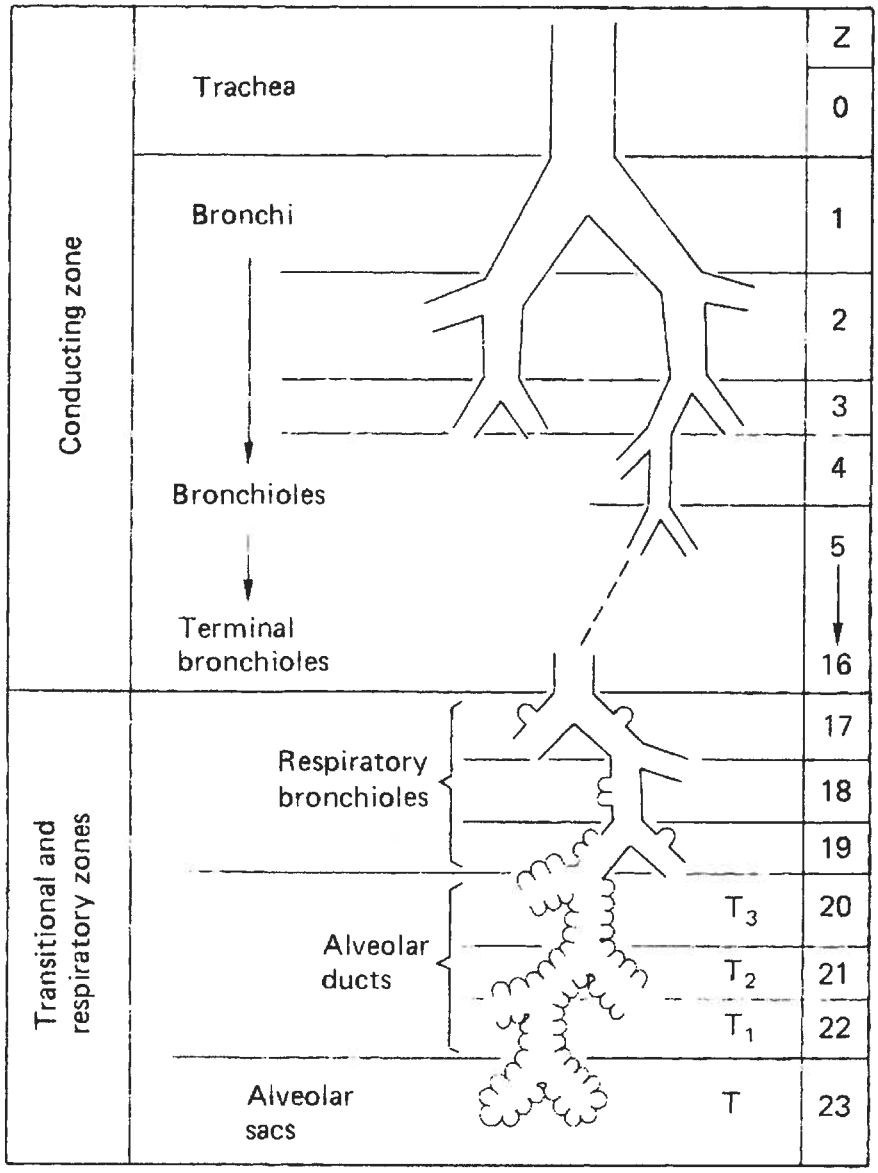

Figure I.1. Lower respiratory tract system. Airway branching in human lung by regularized dichotomy from trachea (generation Z=0) to alveolar ducts and sacs (generations 20 to 23). The first 16 generations are purely conducting; transitional airways lead into the respiratory zone made of alveoli. Adopted from Fishman AP. Fishman's Pulmonary Disease and Disorders. McGraw-Hill; 1998.

I.2.1 Bronchial wall

The bronchial airways vary in width and length throughout the lung, and the structure of their wall varies according to their size. From the proximal to the distal part of the bronchial tree, there is a progressive decrease in the thickness of the respiratory epithelium, the number of the basal cells, the number of submucosal glands, and the number of the cartilage plates. Schematically, the bronchial wall is composed of three major portions: mucosa, submucosa, and muscularis. The mucosa consists of a pseudostratified epithelium supported by a basement membrane and an underlying lamina propria composed of collagen, elastin fibers, blood vessels, lymphatic channels and nerves. The submucosa is composed mainly of a network of elastic fibers. The thickness of the submucosa decreases as the bronchi become smaller. In large bronchi, the submucosa contains abundant mucous and serous glands, which are not observed in bronchioles. The muscularis is a layer of circular smooth muscle cells lying above or between cartilage plates. The mucosa, submucosa, and muscularis are surrounded by adventitia containing connective tissue, bronchial vessels, lymphatics, and nerves. Immune cells such a lymphocytes and mast cells, as well as macrophages and polymorphonuclears, are also present in the bronchial wall (Fig.I.2).

All of the components of the airway wall participate in the airways response to environmental factors and close interaction between them is required; however, the principal role is played by the epithelial lining cells.

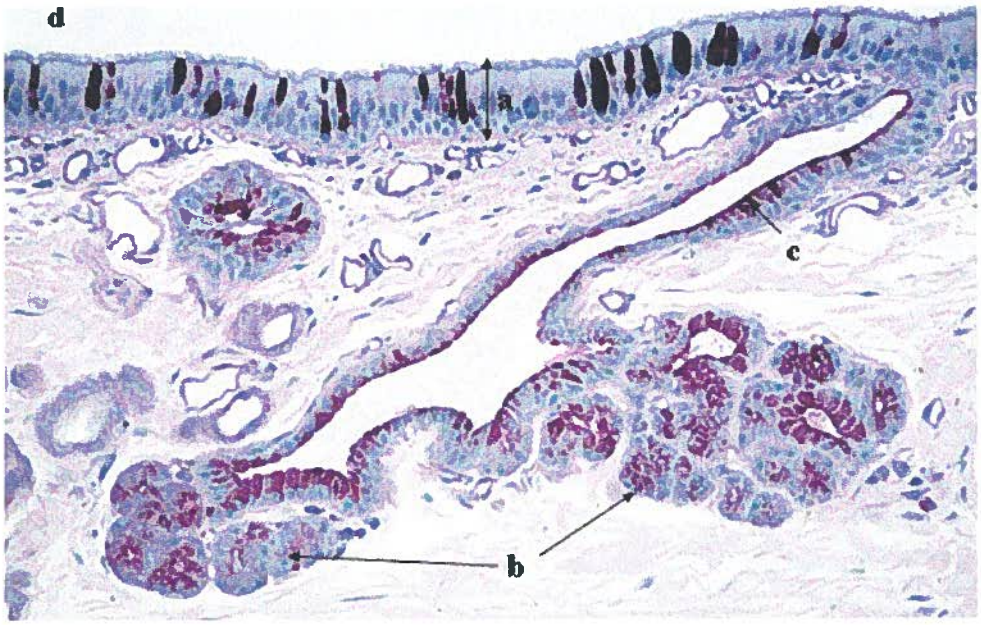

Figure I.2. Histological section of a normal human intrapulmonary bronchus. The bronchial wall consists of a pseudostratified columnar epithelium (a) submucosal serous and mucous glands (b) and collecting glandular ducts (c). Airway lumen is shown as (d). Adopted from Histology Learning System. http://www.bu.edu/histology/

I.2.2 Bronchial epithelium

Bronchial epithelium is a pseudostratified epithelium (i.e., all cells are attached to the basement membrane but not all reach the airway lumen), with the majority of the cells a columnar shape. The thickness of the epithelial lining gradually decreases with the size of the bronchus. It consists of a single layer of cells of columnar shape in the terminal bronchioles and of columnar or cuboidal shape in the respiratory bronchioles. This epithelium is composed of a heterogeneous cell population. At least eight morphologically distinct epithelial cell types are present in human respiratory epithelium. Based on ultrastructural, functional and biochemical criteria these cells may be classified into three categories: ciliated, secretory and basal (Fig.I.3). The distribution and

proportion of these cell types throughout the respiratory tract vary according to the level of bronchial branching.

Columnar ciliated epithelial cells: Ciliated epithelial cells are the predominant cell type within the airways, accounting for over 50% of all epithelial cells (11). Typically, ciliated epithelial cells possess up to 300 cilia/cell and numerous mitochondria immediately beneath the apical surface, highlighting the primary role of these cells, namely the directional transport of mucus from the lung to the throat. Ciliated cells are terminally differenciated cells. They are susceptible to injury from inhaled irritants but are unable to divide (12).

Secterory cells include three principal cell types: mucous, serous and Clara cells. *Mucous cells (goblet cells):* Mucous cells are characterized by membrane-bound electron-lucent acidic--mucin granules, secreted to trap foreign objects in the airway lumen (13). Production of the correct amount and composition of mucus to create the correct viscoelasticity are important for efficient mucociliary clearance. Goblet cells are known to proliferate under irritative exposure, and goblet cell hyperplasia can progressively replace the ciliated epithelium. These cells are thought to be capable of self-renewal and may also differentiate into ciliated epithelial cells. *Serous cells:* Serous cells morphologically resemble mucous cells, although ultrastructurally their granule content is electron-dense, rather than electron-lucent. Until recently, these cells had only been described in rodent airways. However, two populations of these relatively rare cells have been observed in the small airways of the human lung (14). *Clara cells:* In humans, Clara cells are located in large (bronchial) and small (bronchiolar) airways. The cells contain electron-dense granules, thought to produce bronchiolar surfactant and are also

characterized by agranular endoplasmic reticulum in the apical cytoplasm and granular endoplasmic reticulum basally. In addition to their secretory role, Clara cells are believed to metabolize xenobiotic compounds by the action of p450 mono-oxygenases and may also produce specific antiproteases such as secretory leukocyte protease inhibitor. More recent evidence suggests that these cells play an important stem cell role, serving as a resident progenitor for both ciliated and mucus-secreting cells (15).

Basal cells: Basal cells are ubiquitous in the conducting epithelium, although the number of these cells decreases with airway size. There is a direct correlation between the thickness of the epithelium and the number of basal cells as well as the percentage of columnar cell attachment to the basement membrane via the basal cell. Similar to the skin, the basal cell is thought to be the primary stem cell, giving rise to the mucous and ciliated epithelial cells (16). In smaller airways, where basal cells are sparse or absent, Clara cells perform the primary stem cell role. In addition to their progenitor and structural roles, basal cells are also thought to secrete a number of bioactive molecules including neutral endopeptidase, 15-lipoxygenase products and cytokines.

Neuroendocrine cells: The neuroendocrine cells, also referred to as Kultschitzki cells, are scattered along the conducting airways. This cell is usually found isolated in the epithelium, or can be present in small cell clusters. These cells secrete a variety of biogenic amines and peptides, which are thought to play an important role in foetal lung growth and airway function.

Intermediate cells: These cells usually situated in midposition in the bronchial epithelium, above the basal cell layers, have neither cilia nor mucous granules and cannot be clearly classified by light or electron microscopy.

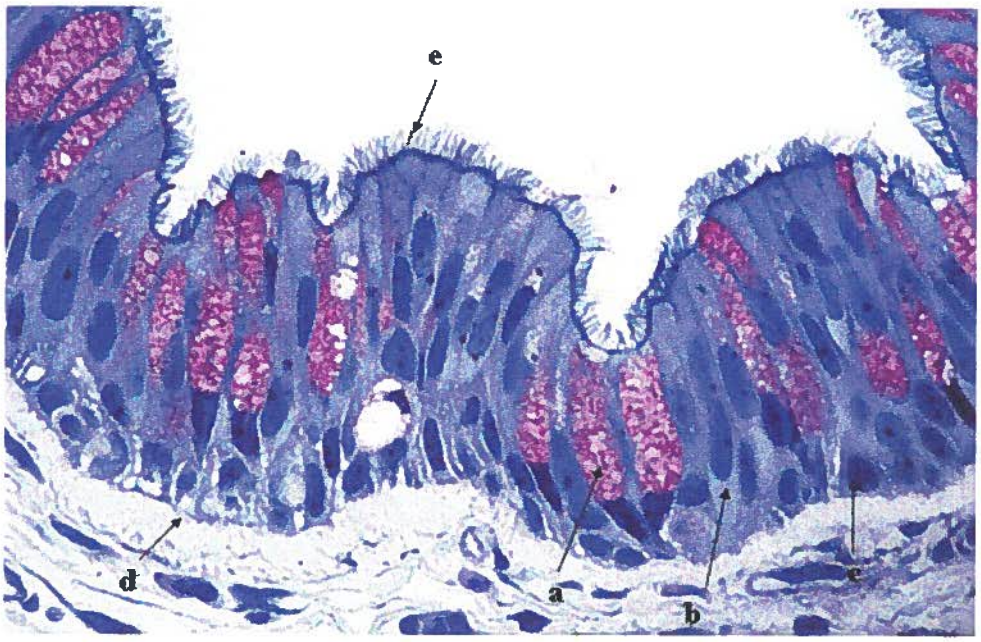

Figure I.3. Human bronchial epithelium. The pseudostratified epithelium is composed of mucous goblet cells (a), columnar ciliated cells (b) with cilia (e), and basal cells (c) resting on the basement membrane (d). Adopted from Histology Learning System. http://www.bu.edu/histology/

I.2.3 Bronchial epithelium in asthma

Asthma is a disease best characterized by a chronic inflammatory process of the entire airway. It is widely accepted that the airway epithelium of asthmatics is abnormal. Epithelial damage and shedding, sub-epithelial fibrosis, and goblet cell hyperplasia and metaplasia are important features of the remodeled airway in asthma (17). Bronchial biopsy studies from patients with asthma have demonstrated physical damage of the columnar cell layer. Moreover, these studies have provided some evidence for injury through the expression of cell stressors such as heat shock protein (HSP) 70 (18) and activation of the caspase enzyme cascade involved in apoptosis in asthma (19, 20). Epithelial fragility in asthma is not confined to the lower airways since disrupted

desmosome formation has also been shown in nasal polyps (21). It has also been shown that the barrier function of the airways epithelium in asthma is impaired (22).

In asthmatic children collagen deposition in the lamina reticularis rather than eosinophil infiltration has been recognized as a consistent feature of the disease (23). Asthmatic epithelium expresses abnormal level of several pro-inflammatory transcription factors such as NF-κB, AP-1, STAT-1, and STAT-6 (24-26). Recent work by Kicic *et al* has described significant intrinsic biochemical and functional differences between healthy and asthmatic bronchial epithelial cells (27). These differences were maintained through repeated passages suggesting that asthmatic epithelium functions abnormally even in the absence of inflammation. This indicates a possible primary defect within the asthmatic epithelium.

I.3 Epithelial injury and repair

Providing the interface between organism and external milieu, epithelial cells are in constant contact with environmental stimuli and therefore are frequently damaged. For example, the epithelial layer or epidermis of the skin tissue is an interface with such constant aggression that may result in the loss of the epithelial layer and its integrity (28). Another example is the epithelial layer of the human cornea, which consists of five to seven layers of cells and provides the eye with its first line of defense against noxious environmental agents and trauma (29). The epithelium lining the gastrointestinal tract is exposed to luminal acid, proteolytic enzymes and noxious ingested agents that can destroy its integrity (30). The airways of mammalian lungs are lined by a similar protective barrier of epithelial cells. This epithelial layer is continuously exposed to

gaseous and particulate components of the inhaled air including pollutants, allergens and virus particles (31). Allergens with proteolytic activity, such as Der P 1 from the house dust mite and proteases from pollen, disrupt mucosal integrity through direct extracellular cleavage of intracellular tight junctions (32, 33). Moreover, exposure to urban particulate-matter air-pollution and respiratory viruses is associated with effects on cell survival/apoptosis pathways within the airways (34, 35). While an intact epithelium prevents passive movement of environmental agents by the peri-cellular route as a result of intact tight junctions, damaged epithelium, consisting of the loss of columnar epithelial cells, impairs the effectiveness of this barrier. Thus allowing antigen or other stimuli access to the underlying bronchial tissue (36). Damaged epithelium may contribute substantially to inflammation, bronchoconstriction and, oedema seen in asthma and a number of other respiratory diseases (24).

Epithelial regeneration following injury is crucial for restoring epithelial function to its normal state and involves an orderly progression of events to reestablish the integrity of the injured tissue. The study of wound repair in epithelial cells of organ systems such as the cornea, skin, lung and gastrointestinal tract has become relevant to study the associated phenotype changes. Although the repair process has common elements among various epithelia, there are differences based on their diverse functions.

I.3.1 Nature of airway epithelial injury

The airways are subject to attack by many pollutants, both chemical and microbiological. With the exception of rare cases of pulmonary injury from poisons transmitted through the blood stream or from direct inhalation of liquid material,

pollutants generally reach the lung through the conducting airways in the form of an aerosol.

Gaseous pollutants

In addition to the respiratory gases, inspired air may contain a variety of gaseous contaminants, including CO, nitrous oxides, ozone, sulfur anhydride, and organic solvents. Their penetration and toxicity depend on their partial pressure in the mixture, as well as on their diffusibility, solubility, and affinity for hemoglobin. Thus the target zones vary according to the gas inhaled, e.g., the selective action of NO_2 on the terminal bronchioles as opposed to the rapid absorption of SO_2 in the upper airways.

Particulate pollutants

Airborne particles are either liquid (mists) or solid (dust or smoke) and organic or inorganic in nature. Depending on the kind of disease they are capable of inducing, they are characterized as infectious, allergenic, or physicochemical (toxic, mechanic, etc.). Infectious particles consist of microorganisms suspended in droplets and are produced mostly by infected subjects when coughing, sneezing, or speaking. Allergic particles include substrates of animal, vegetable (pollen), microorganism (bacteria, fungi, algae), or industrial (beryllium) origin. Physicochemical contaminants may be of natural (erosion, volcanic eruption, etc.) or man-made (certain industries working with silica, asbestos, metals, etc.) origin.

Different mechanisms of response are used by the lung against aerosol penetration including mechanical, immunological, and enzymological. The principal means of mechanical defense is mucociliary clearance. Bronchial secretions create a protective film, which interposes itself between the respiratory epithelium and the pollutants.

Expulsion of the insoluble particles is performed by coughing and mucociliary clearance which transports them towards the aerodigestive junction and enables their elimination by expectoration or swallowing into the digestive tract. Immune defenses are either humoral or cellular. Humoral mechanisms depend on the locally (secretory IgA) or systemically (IgA and IgG) derived immunoglobulin antibodies. Moreover, mucus contains transferrin, lysozymes, and surfactant whose opsonizing action and role in phagocytosis are well known. Cellular mechanisms include phagosytosis and increased production of cytokines like TNF-α. The enzymatic defense systems are involved in the transformation of inhaled substances and mostly include protease-antiprotease system.

I.3.2 Cellular events during airway epithelial repair

The basic mechanism of airway epithelial repair is not fully understood. Much of what is known about epithelial repair comes from either studies in which the repair process are followed histologically over time, or from cell-culture models. Following epithelial removal plasma promptly exudes into the injured site from the underlying vasculature to cover the denuded basement membrane, (Fig. I.4). Cells in and around the wound migrate rapidly to seal the injured epithelium and restore the physical barrier lost in the shedding of epithelial columnar cells (37, 38). Extravasation of plasma to the site of epithelial injury facilitates the binding of serum proteins to their cellular receptor and may play a role in stimulating repair of the damaged epithelium (39). Erjefalt *et al.* (37) described airway epithelial repair processes in an *in vivo* model after injury. The authors demonstrated that following injury, secretory and ciliated cells at the edge of the wound "de-differentiate", flatten and migrate rapidly over the denuded basement membrane to

cover the area of damage. However, significant proliferation of new epithelial cells does not generally occur until after the migration is complete and the wound has sealed. *In vivo* studies have shown that the steps involved in epithelial repair are more complex. Regeneration of hamster tracheal epithelium after mechanical injury has been shown to begin by spreading and migration of viable cells at the wound margins, proliferation and active mitosis, and squamous metaplasia, followed by progressive redifferentiation with the emergence of preciliated cells, and a final step of ciliogenesis and complete regeneration of a pseudostratified mucociliary epithelium (40).

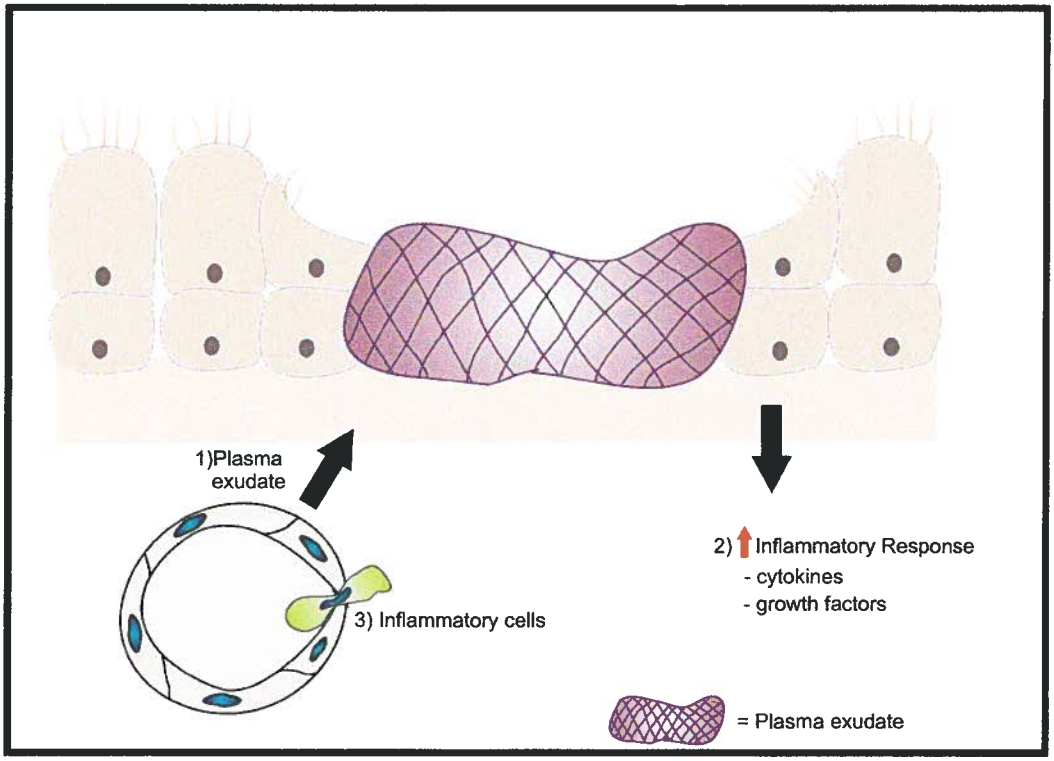

Figure I.4. Early and late response following epithelial injury. Immediately following epithelial loss, plasma exudate accumulates in the airway lumen, providing a protective protein cap and barrier (1). Present in the plasma are serum glycoproteins, cytokines and growth factors that in turn, induce a response in the neighboring resident epithelial cells (2). Inflammatory cells are then recruited to the site of injury to remove cellular debris and participate in the repair (3). Similar signals may recruit distant cells no resident to the epithelium to participate in the repair in addition to the contribution of resident cells. The cells would include epithelial progenitor or stem cells. Modified from Erjefalt and Persson (41).

The re-epithelialization pattern of the airway cells in two-dimensional (2-D) cell culture has been well described by several authors. These studies have demonstrated a similar wound healing process of spreading of epithelial cells at the margin followed by migration of distant cells into the damaged region and, finally, proliferation of new epithelial cells (37, 42, 43).

I.3.2.1 Cell Migration

Because injury often leads to desquamation of cells from the epithelium, the migration of neighboring cells is an important component of the repair process. Within minutes after injury, epithelial cells from neighboring areas migrate to the denuded area. This process is termed restitution. Many disorders characterized by impaired epithelial repair or re-epithelialization are not a result of inadequate proliferation but a loss or inefficient cell migration over the denuded site (44, 45).

Cell migration requires a coordinated, highly complex series of events that involves the formation of cellular projections in the direction of movement, attachment at the leading edge and detachment and contraction of the trailing end of the cell. In the skin two distinct cellular mechanisms contribute to the repair of wounded epidermis. One is re-epithelialization, which is achieved through a combination of migration and enhanced mitosis of keratinocytes proximal to the wound edge. The other is a fibroblast-mediated, centripetal contraction of the newly formed connective tissue bed beneath the wound site, pulling the edges of the wound together (28). Gastrointestinal mucosal repair after injury consists of epithelial cell migration from the crypts and subsequent proliferation. Cell migration appears to play a greater role than cell proliferation in the

early phase of restitution (30). Corneal epithelial repair after injury is one of the most rapid repair processes in the whole body and takes less than 24 hours (29). It has been shown that initial healing of the corneal epithelium takes place by the migration of adjacent cells to the injured area. Failure of epithelium to migrate to the wound surface or the failure of migrated epithelium to remain adherent to the substratum may result in severe clinical problems, leading to the development of persistent epithelial defects and corneal ulceration (46). Using an *in vitro* model, Zahm *et al.* found that closure of small, denuded areas of nasal epithelium occurred by migration of epithelial cells at an average speed of about 0.1μm/min (43). However, it has been shown that under *in vivo* conditions epithelial cells migrate much faster (several μm/min) (47). Rickards and co-workers examined migration of bovine bronchial epithelial cells *in vitro* and observed that extracellular matrix macromolecules, in particular fibronectin, increase the migration of epithelial cells (48). Bronchial epithelial cells produce fibronectin and it has been suggested that epithelial derived fibronectin is an important factor in recruitment of epithelial cells from the wound margin.

I.3.2.2 Proliferation

Although much of the evidence suggests cell migration is key to epithelial repair, it may also be facilitated through proliferation of epithelial cells to fill the region of damaged epithelium. Several studies have focused on the role of epidermal growth factor (EGF) and its family of receptors on epithelial repair (49, 50). EGF, a well known mitogen for epithelial cells, has been used to stimulate epithelial wound healing in guinea pigs as well as human airway cell monolayers *in vitro* (51). Epidermal growth factor-

receptor (EGFR) is known to be up-regulated upon the creation of a wound on airway epithelial monolayers in culture and correlated to the damaged areas of epithelium (50). Increased tyrosine phosphorylation of the EGFR has also been observed after mechanical injury even in the absence of exogenous ligand (50).

I.3.2.3 Differentiation

To restore the function of the epithelium, proliferating cells undergo differentiation into the cell types of interest. This differentiation is affected by the type of injury and the cell types required for protection from further insult. For example, lipopolysaccharide (LPS) instillation causes extensive inflammation and proliferation followed by massive mucous cells metaplasia (52). Alternatively, exposure to high concentrations of cigarette smoke for an extended time changes the mucociliary epithelium lining the proximal septum of the nose into a squamous metaplasia (53). It will be crucial to identify the local chemical signals released in tissues that can activate such differentiation pathways. In general, all findings agree that proliferation must stop before cells can differentiate to carry out the appropriate function. How the population of proliferating epithelial cells decides which cells should become mucous-producing, ciliated, or another type of cell is still a puzzle to airway biologists. Errors during such a regeneration process may be associated with tissue damage and metaplastic changes in chronic diseases, including asthma and chronic bronchitis (54).

I.3.2.4 Role of resident and progenitor stem cells in epithelial repair

The mechanisms discussed so far relate to resident epithelial cells. However, distant or recruited cells may utilize similar processes of migration to participate in the repair of damaged epithelial structures. In the gastrointestinal tract, the existence of a progenitor called the intestinal stem cell, which can give rise to all cells of epithelial lineage has been described (55-57). It is believed that the intestinal epithelial stem cell resides in the crypt base. Similarly, in airways, researchers have identified airway epithelial progenitor cells localized to systematically arrayed gland ducts in the upper trachea and to distinct foci in the glandless distal trachea (58). These distant foci of epithelial progenitor cells tended to be in proximity to the cartilage-intercartilage junctions, where blood vessels typically penetrate toward the epithelium. Clara-cell secretary protein 10-secreting cells, basal and parabasal cells and a population of mucus-secreting cells have also been identified as potential progenitor cells (16, 59, 60). The potential role of distant progenitor cells in epithelia is only now coming into view. As with the resident cells participating in repair, common mechanisms are likely required for the recruited cells to migrate and repair sites of injured tissue.

I.3.2.5 Epithelial-Mesenchymal Transition (EMT)

The epithelium, previously considered to be terminally differentiated, has the ability to differentiate into fibroblasts (61, 62). Epithelial-mesenchymal transition (EMT) occurs normally during early fetal development where there is a communication between epithelial and mesenchymal cells (63). EMT also occurs in some adult tissues during tumor invasion, following epithelial stress such as inflammation, or as a part of wound

repair (61, 62, 64). During EMT, epithelial cells undergo molecular reprogramming and develop a new set of biochemical and genetic instructions. They gain mesenchymal cell properties including motility, express mesenchymal markers such as α-smooth muscle actin, and secrete collagens I and III instead of collagen type IV (65). Growth factors such as IGF-II, FGF-2, EGF, and in particular TGF-β have been shown to induce EMT (66-68). Whether EMT occurs in human airways, particularly during epithelial damage and repair and the remodeling identified in asthma, is unknown.

I.3.3 Molecular events during epithelial repair

The cellular and molecular factors involved in epithelial wound repair, regeneration, and complete redifferentiation are numerous and closely interacting. Cell migration involves protrusion of the plasma membrane (lamellipodium extension) at the leading edge of the cell, which implies cytoskeleton reorganization. Cell movement also implies the formation of new sites of adhesion to the extra cellular matrix (ECM) at the front of the cells but also the release of adhesion sites at the back of the cells. This necessarily implies a coordinated sequence of events involving the following: contraction of the actin and actin-myosin cytoskeleton and interaction with ECM proteins and matrix metaloproteinases (MMPs), with regulation between MMPs and their inhibitors. Furthermore, production of ECM by the airway epithelial cells during the migration process requires a signalling pathway through specific receptors on the airway cell surface. In spreading and migrating airway epithelial cells, the polymerization of G actin to F (filamentous) actin leads to an accumulation of F actin in the lamellipodia of the dedifferentiated and flattened basal cells, which form adhesive contacts with the ECM.

I.3.3.1 Cytokines and growth factors mediating epithelial repair

The activation of chemokines, interleukins, and growth factors has been frequently described during the early inflammatory and chemotactic response of the airway epithelium. These factors are secreted by mesenchymal cells, endothelial cells, inflammatory cells, and epithelial cells during injury and repair. Transforming growth factor (TGF)-β1 modulates the composition of the provisional matrix over which the epithelial cells migrate and has been shown to increase *in vitro* airway wound repair via MMP-2 upregulation (69). In a normal, well-differentiated airway epithelium, EGF is expressed at the apical domain and is separated by tight junctions from its receptor, EGFR, which is localized at the basolateral domain (70, 71). A critical role of signaling mediated by EGFR in repairing damaged epithelium has been well demonstrated in many epithelial systems (72-74). During wound repair, trefoiled peptides, such as TFF2, exhibit a synergistic effect with EGF and enhance epithelial migration by activation of the protein kinase-C and extracellular signal-regulated kinase (ERK) signalling pathways (75). Airway epithelial migration is also induced by other growth factors and mitogen peptides, including insulin, insulin-like growth factors, hepatocyte growth factor (HGF), and keratinocyte growth factor (KGF) (76-78). KGF and HGF may enhance wound repair by acting as a chemotactic or a growth-stimulating factor that in turn may stimulate the synthesis of ECM and facilitate the interaction with MMPs through specific cell receptors (79, 80).

I.3.4 Bronchial epithelial repair in asthma

Detailed cellular and ultrastructural examination of bronchial biopsies and bronchoalveolar lavage fluid has provided evidence for epithelial damage, even in mild asthma (81, 82). This excessive epithelial damage can arise as a result of an enhanced susceptibility to injury and/or an inadequate repair response. An increased susceptibility to oxidant-induced damage and apoptosis in asthmatic epithelium compared to normal epithelium has been demonstrated (19). Moreover, there is increasing evidence to support that normal epithelial repair is compromised in asthma (27). As a consequence of the chronic epithelial injury, the epithelium maintains a repair phenotype responsible for increased production of proinflammatory mediators and profibrogenic growth factors (50, 83, 84), potentially leading to the chronic airway inflammation and remodeling that contributes to all long-term morbidity in asthma (Fig. I.5).

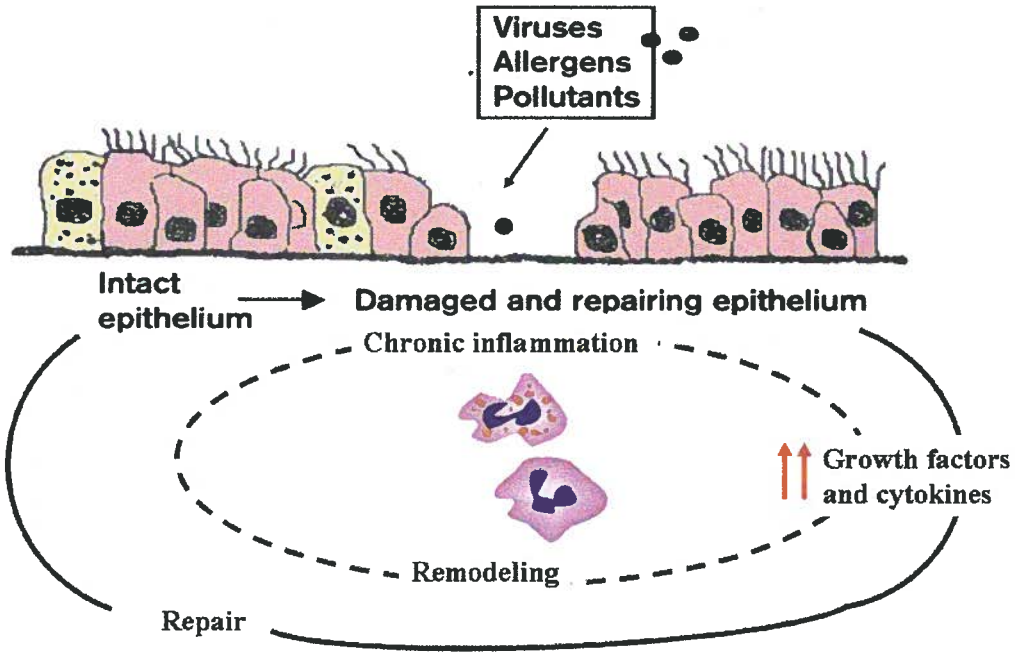

Figure I.5. The relationship between epithelial injury, airway inflammation and, remodeling. Epithelial damage can arise as a result of an enhanced susceptibility to injury or an inadequate repair response, or a combination of both. As a consequence, the epithelium maintains a repair phenotype responsible for increased production of proinflammatory mediators and profibrogenic growth factors resulting in chronic inflammation and remodeling. Modified from Davies DE (31).

I.4 The ErbB receptors and their cognate ligands

The epidermal growth factor receptor (EGFR; ErbB1) is a member of the tyrosine kinase receptor family, which includes HER2/neu (ErbB2), ErbB3, and ErbB4. All proteins of this family have an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase-containing domain (85).The intracellular tyrosine kinase domain of ErbB receptors is highly conserved although the kinase domain of ErbB-3 contains substitutions of critical amino acids and therefore lacks kinase activity (86). In contrast, the extracellular domains are less conserved among the four receptors, suggesting that they have different specificity in ligand binding (85, 87, 88). The extracellular domain of each ErbB receptor consists of four subdomains (I–IV).

Subdomains I and III (also called L1 and L2) are important for ligand binding, whereas subdomains II and IV are cystein rich domains (also called CR1 and CR2) (Fig. I.6).

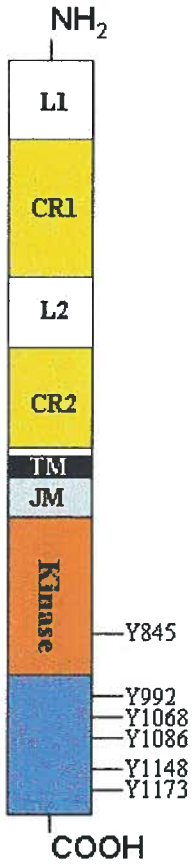

Figure I.6. Structural motifs of the EGF receptor. The extracellular domain of EGFR includes two cysteine-rich (CR) domains and two discontinuous ligand-binding domains (L) which are different but overlapping for the various ligands. The transmembrane (TM) stretch separates the glycosylated extracellular domain from the intracellular regions. This latter includes the tyrosine kinase domain as well as the autophosphorylated tyrosines (Y).

The cytoplasmic domain contains tyrosine kinase and autophosphorylation domains. Five autophosphorylation sites have been identified in the EGFR: three major (Tyr1068, Tyr1148, and Tyr1173) and two minor (Tyr992 and Tyr1086) (89, 90) (Fig. I.6). These sites bind a variety of downstream signaling proteins which contain SH2 domains. Binding of these or other signaling proteins to the receptor and/or their

phosphorylation result in transmission of subsequent signaling events that culminate in DNA synthesis and cell division. Other tyrosine phosphorylation sites include Tyr845 which is located in the catalytic domain. Phosphorylation of EGFR at Tyr845 in the kinase domain is implicated in stabilizing the activation loop, maintaining the active state for the enzyme and providing a binding surface for substrate proteins (91, 92). c-Src is involved in phosphorylation of EGFR at Tyr845 (93). Phosphorylation of EGFR at specific serine and threonine residues attenuates EGFR kinase activity.

EGFR ligands

ErbB receptors are activated by binding to growth factors of the EGF-family that are produced by the same cells that express ErbB receptors (autocrine secretion) or by surrounding cells (paracrine secretion) (85, 88). With respect to ErbB-receptor binding, EGF-related growth factors can be divided into three groups (88, 94). The first group includes EGF, transforming growth factor α (TGF-α) and amphiregulin (AR) which bind specifically to the EGFR. The second group includes betacellulin (BTC), heparin-binding growth factor (HB-EGF) and epiregulin (EPR), which show dual specificity by binding both EGFR and ErbB-4. The third group is composed of the neuregulins (NRGs) and can be divided in two subgroups based upon their capacity to bind ErbB-3 and ErbB-4 (NRG-1 and NRG-2) or only ErbB-4 (NRG-3 and NRG-4) (95-97). None of the EGF family of peptides binds ErbB-2 (Fig. I.7).

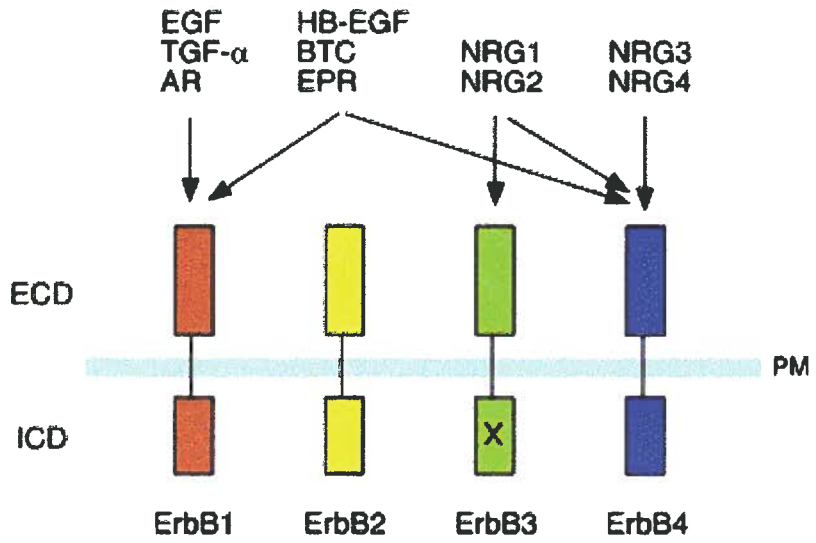

Figure I.7. Binding specificities of members of the ErbB receptor family to EGF ligands. EGF family ligands are separated into four categories according to their specificities for members of the ErbB receptor family. ErbB2 has no ligand. ErbB3 is deficient in kinase activity (X).

EGFR function

EGFR serves a central role as a primary regulator of epithelial cell function, having the ability to induce a wide variety of responses, ranging from induction of DNA synthesis, alterations in cell adhesion and motility, and regulation of differentiated cell function (98). The EGFR family of receptor tyrosine kinases plays a crucial role in the development of the nervous system, cardiovascular system, and the mammary gland, mediating activities that are various and often opposite: proliferation, differentiation, and apoptosis. The ability to promote different cellular responses appears to be as a result of having a complex protein network which acts through and activates several pathways. EGFR and the EGF-family of growth factors have also a central role in the pathogenesis and progression of different carcinoma types (99).

I.4.1 EGFR activation

Binding of ligands to the extracellular domain of the ErbB receptor family induces the formation of receptor homo- or heterodimers, and subsequent activation of the intrinsic tyrosine kinase domain (85) (Fig. I.8). As a result, a number of tyrosine residues at the C terminal end of the ErbB molecules become phosphorylated. The phosphorylated tyrosine residues serve as docking sites for an array of signaling molecules that contain Src homology 2 (SH2) domains or phosphotyrosine-binding (PTB) domains. The 2 major signaling pathways activated by ErbB receptors are the MAPK and

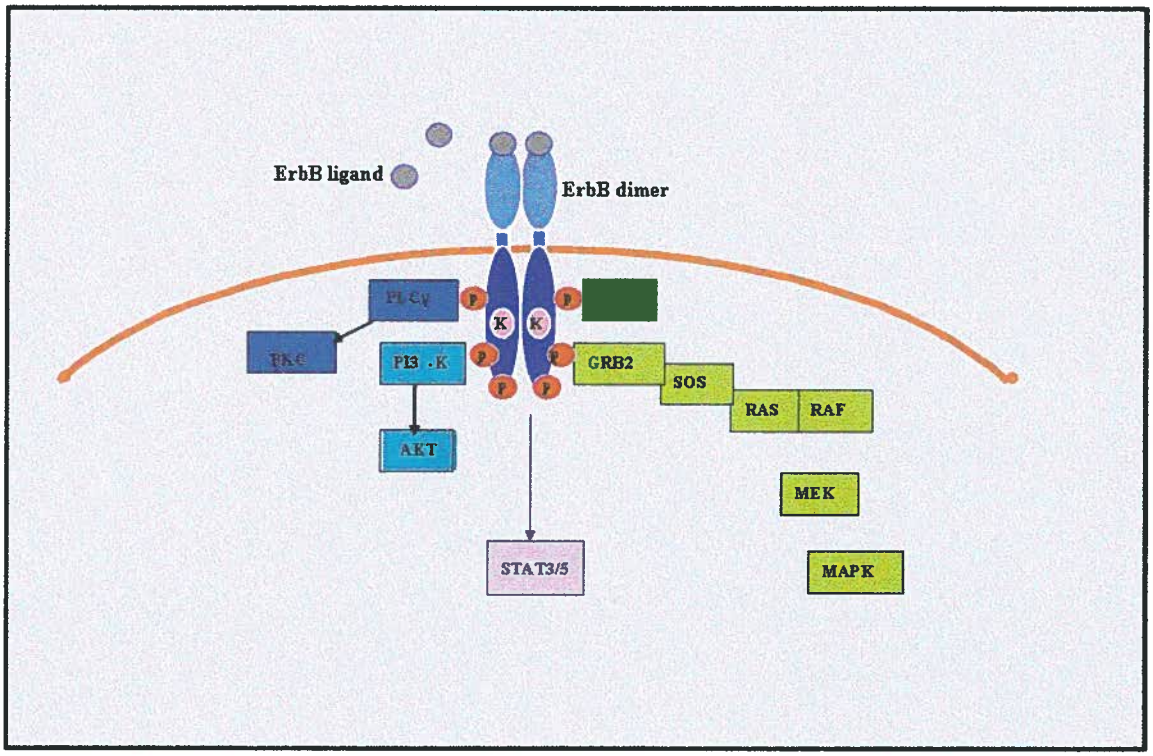

Figure I.8. The EGFR signalling pathways. Binding of ligands to the extracellular domain of ErbB receptors results in receptor dimerization, tyrosine kinase activation and *trans*-phosphorylation (P). The activated ErbB receptors are able to interact with different signaling molecules that transmit the signal in the cell. This in turn results in activation of transcription factors and modulation of the cell cycle, growth, apoptosis, and angiogenic processes.

PI3K-AKT pathways. The general paradigm is that the specific combination of ErbB receptors in the dimer defines the downstream signaling network as well as the intensity and the duration of the stimulation. For example, heterodimers that contain ErbB3 favor activation of the PI3K pathway (100).

ErbB mediated signaling are initiated when activated ErbB tyrosine kinase receptors recruit signaling proteins, such as Shc, Grb7, Grb2, Crk, Nck, the phospholipase Cγ (PLCγ), the intracellular kinases Src and PI3K, the protein tyrosine phosphatases SHP1 and SHP2 and the Cbl E3 ubiquitin ligase (Fig. I.8) (101, 102). All ErbB ligands and receptors induce activation of the *ras*/*raf*/MEK/MAPK pathway through either Grb2 or Shc adaptor proteins (103, 104). ErbB receptors also activate PI3K by recruitment of the p85 regulatory subunit to the activated receptors. Finally, ErbB receptors activate various transcription factors such as c-*fos*, c-*Jun*, c-myc, signal transducer and activator(s) of transcription (STAT), NF-kB, zinc finger transcription factor and Ets family members (105, 106).

Different factors might affect the type and the duration of ErbB signaling. The identity of the ligand, composition of the receptor complex and specific structural determinants of the receptors will determine the engagement of specific signaling pathways. The impaired internalization of ErbB-2 leads to prolonged signaling of receptor complexes that contain this receptor, whereas dimers containing ErbB-3 or ErbB-4 can directly activate PI3K. However, signaling is also affected by ligands. For example, the interaction between EGF and EGFR is stable at endosomal pH and results in lysosomal degradation (107). On the contrary, interaction of TGF-α and NRG-1 with their respective receptors is pH sensitive, resulting in dissociation in the endosome and

recycling to the cell membrane of receptors that will be available for a new cycle of activation by ligands (108).

I.4.2 EGFR Glycosylation

Each of the ErbB proteins is an N-linked glycoprotein; about 20% of the mass of these proteins is carbohydrate. The extracellular domain of EGFR is a 70.9 kDa single polypeptide having 10 to 11 potential N-glycosylation sites occupied with complex-type and high-mannose-type oligosaccharides in a ratio of approximately 2:1. No evidence was found for O-linked oligosaccharides (109-111).

The carbohydrate antigens expressed on the EGFR has been investigated using the human epidermoid carcinoma A431 cell line because it has a high density of receptors ($2X10^6$ receptors/cell) and secretes a soluble EGFR that represents the extracellular domain of the membrane-bound receptor. Glycosylation pattern of EGFR using the above model has recognized fucose containing structures such as Le^x, Le^y, sLe^x, and blood group A and H antigens on complex-type oligosaccharides (112, 113).

When glycosylation of the EGFR is blocked by tunicamycin during synthesis in cell culture, the EGFR protein kinase activity is only 30% of that of the control receptor (109). This treatment inhibits the attachment of carbohydrate, and the molecular weight is decreased from 170,000 to 140,000. However, when carbohydrate is cleaved enzymatically from the mature receptor, protein kinase activity is not diminished. These studies indicate that glycosylation facilitates proper folding of the full-length receptor to generate an active EGF-binding conformation. N-glycosylation also defines localization of EGFR to specific domain of plasma membrane which could facilitate association of

the receptor with other molecules and its transactivation (114). While core glycosylation is necessary for proper folding of the receptor, terminal modifications of the N-glycans has been shown to modulate EGFR function. Using lectins which bind to specific carbohydrate structures, it has been shown that binding of lectins to the EGFR can modulate receptor function (115, 116). Modification of receptor N-glycans can also regulate receptor trafficking and duration of cell surface residency (117). Labeling studies with blood group A reactive anti-EGFR monoclonal antibodies and various lectins have revealed that A431 cultures are heterogeneous with respect to blood group A expression. Interestingly, EGFR from blood group A positive A431 cells shows different affinity for EGF, a reduced tyrosine kinase activity, and slower turn over compared to blood group A negative cells. This finding suggests a possible involvement of GalNAc residue(s) in determining EGFR affinity, protein-tyrosine kinase activity and turnover in A431 cells (118). Wang *et al.* has recently shown that core fucosylation of N-glycans is required for the binding of the EGF to its receptor, whereas no effect was observed for the expression levels of EGFR on the cell surface (119).

I.4.3. Role of EGFR in Airway Epithelial Repair

The role of EGFR activation in mediating epithelial repair has been shown in various cell types in vitro including keratinocytes (74), corneal (73), and intestinal epithelial cells (72). Autocrine activation of EGFR, at least in part, plays an essential role in mediating the key events during epithelial wound healing (72, 73). Studies of the response to acute lung injury have shown that EGFR expression is induced in type II alveolar cells after endotoxin instillation into rat lung (53). Similarly, expression of

EGFR is increased in bronchiolar epithelial cells, alveolar septal cells, and alveolar macrophages after bleomycin-induced lung injury (120) and in bronchiolar cells after exposure to naphthalene (121). These data suggest a role for EGFR in the repair of the large airways. An increased EGFR immunoreactivity has been demonstrated in areas of damaged epithelium where the columnar epithelium had been shed to leave a single layer of basal cells (98). Moreover, a rapid damage-induced phosphorylation of the EGFR in airway epithelial cells grown in monolayer, irrespective of the presence of exogenous ligand, has been shown (50). This suggests that EGFR activation is intrinsic to the repair process and may occur by release of an autocrine ligand.

I.4.4 EGFR ligands in epithelial repair

The EGF family is composed of seven members including EGF, transforming growth factor alpha (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin and, neuregulins. The EGF ligands bind to the erbBs with a variety degree of specificity. Members of the EGF family play an essential role in maintenance and repair of epithelial tissues by virtue of their ability to stimulate cell migration, proliferation, differentiation, and survival (98). A direct role for EGF, HB-EGF, and TGF-α in cutaneous wound healing is already well established (122, 123) and some therapeutic potential has been suggested from their ability to cause a dose-dependent enhancement of epithelialization after topical application (124).

Bronchial epithelial cells produce several ligands for EGFR, including EGF, TGF-α, HB-EGF and amphiregulin (70). An induction in the expression and release of EGFR ligands by airway epithelial cells in response to different stimuli such as cigarette smoke

extract (125), compressive (126) and oxidative stress (127) has been demonstrated. Although the effect of exogenous EGF in acceleration of airway epithelial repair has been demonstrated (50), the role for endogenous ligand(s), released by damaged or adjacent epithelium, in activation of EGFR and their role in epithelial repair remain to be determined.

I.4.5 EGFR in asthmatic bronchial epithelium

Epithelial damage and shedding are important features of asthma (17). This excessive epithelial damage can arise as a result of an enhanced susceptibility to injury and/or an inadequate repair response. EGFR has been shown to be over-expressed in bronchial epithelium in both mild and severe asthma (50, 98, 128). Although there are very high levels of EGFR expression in the bronchial epithelium in asthma, the level of receptor expression in the epithelium is disproportionate to the level of proliferation seen in the epithelial cells measured using markers of nuclear proliferation. This differs from psoriasis (129) or lung cancer (130) where there is an appropriate proliferative response to the level of EGFR expression. Lack of proliferative response in asthma in spite of excessive expression of EGFR has been so far attributed to the high expression of p21, a cyclin kinase inhibitor, in asthmatic epithelium (50). A recent study by Semlali *et al.* showed that baseline as well as EGF stimulated EGFR activation is lower in asthmatic than normal cells (131). Therefore, it is possible that EGFR-mediated epithelial repair might be dysregulated in asthma. An aberrant repair process occurring at the mucosal surface may trigger a cascade of events deeper within the sub-mucosa, leading to direct

effects on the amount and behavior of airway smooth muscle and sub-mucosal glands, an increased deposition of ECM and ultimately airway wall remodeling.

One of the mechanisms by which EGFR-mediated epithelial repair might be handicapped in asthma is ligand availability (17). Polosa *et al.* showed that expression of EGFR ligands in asthmatic bronchial epithelium is not different from that of normal subjects (49). This finding excludes ligand absence as a cause for lower EGFR activity in asthma, however, it does not rule out the possibility of having a non-functional receptor and/or ligand or impaired ligand binding in asthma.

I.5 HB-EGF

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF family of growth factors, exerts its biological activity through activation of the EGFR and other ErbB receptors. HB-EGF is initially synthesized as a transmembrane protein, similar to other members of the EGF family of growth factors. The membrane-anchored form of HB-EGF (proHB-EGF) is composed of a pro domain followed by heparin-binding, EGF-like, juxtamembrane, transmembrane and cytoplasmic domains (132). Subsequently, proHB-EGF is cleaved at the cell surface by a protease to yield the soluble form of HB-EGF (sHB-EGF) using a mechanism known as ectodomain shedding. sHB-EGF is a potent mitogen and chemoattractant for a number of different cell types (133). Studies of mice expressing non-cleavable HB-EGF have indicated that the major functions of HB-EGF are mediated by the soluble form (134). proHB-EGF functions as the sole receptor for diphtheria toxin (135). Moreover, both proHB-EGF and the cytoplasmic domain of proHB-EGF play some roles in HB-EGF function.

Ectodomain shedding from proHB-EGF is critical for HB-EGF activity and can be stimulated by various physiological and pharmacological stimuli such as calcium ionophores (136), and GPCR ligands (137). Cellular stresses caused by inflammatory cytokines, reactive oxygen and osmotic shock can also induce ectodomain shedding of HB-EGF (138). The stimuli for ectodomain shedding activate intracellular signaling molecules, including protein kinase C, Ras, ERK and p38 MAPK. Activation of these molecules results in proteolytic cleavage of proHB-EGF by the ADAM family or other metalloproteases (Fig. I.9).

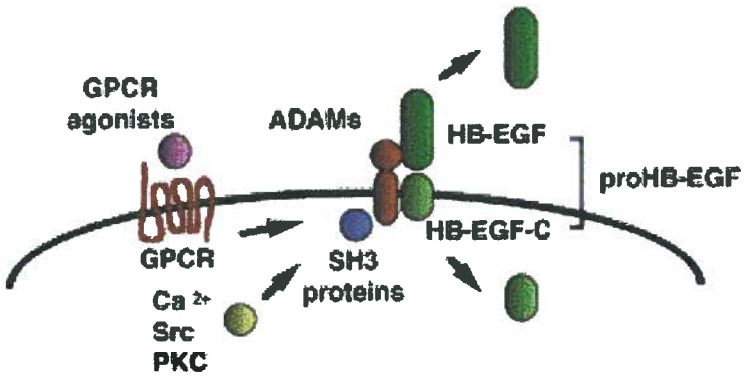

Figure I .9. HB-EGF ectodomain shedding . Disintegrin and metalloprotease (ADAM) proteins are activated by various stimuli including wounding, ion influx, G-protein coupled receptor (GPCR) signaling, growth factor and cytokine signaling, protein kinase C (PKC) activation, and binding of cytoplasmic interactive proteins. EGFR ligand molecules are proteolytically cleaved by specific metalloprotease-activity of ADAMs, resulting in the production of soluble ligands and stimulation of EGFR in autocrine and paracrine manners.

HB-EGF gene expression has been demonstrated in a variety of tissues, predominantly the lung, heart, brain and skeletal muscle (139). HB-EGF participates in a variety of physiological and pathological processes (133), including wound healing (140), eyelid formation (141), blastocyst implantation (142) and atherosclerosis (143). Recently, HB-EGF knockout mice have been established (144, 145). HB-EGF null mice show quite

severe phenotypes and most of the animals die at the neonatal stage. The survivors display enlarged hearts, hypertrophic cardiomyocytes, and abnormal cardiac valves. Moreover, mice expressing an uncleavable form of the HB-EGF develop severe heart failure and enlarged heart valves, phenotypes similar to those in HB-EGF null mice (134). These results indicate that ectodomain shedding of proHB-EGF is essential for HB-EGF function in vivo. HB-EGF gene expression is significantly elevated in many human cancers. Several line of evidence have indicated that HB-EGF plays a key role in the acquisition of malignant phenotypes including tumorigenicity, invasion and, metastasis. HB-EGF has also been shown to have strong activity for enhancing cell motility (146). In eyelid formation in the mouse embryo and skin wound healing, HB-EGF plays a role in cell motility, but not in cell proliferation (140, 141).

I.6 IL-13

The Th2-type cytokine, interleukin-13 is a 17-kDa glycoprotein which has been shown to play a critical role in the pathogenesis of bronchial asthma (147, 148). Although IL-13 is predominantly produced by Th2-polarized $CD4^+$ T cells, it is also produced by a variety of cell types including both Th1 $CD4^+$ T cells, $CD8^+$ T cells, and natural killer T cells (149). Recent studies also suggest that IL-13 is produced by numerous non-T-cell populations that are particularly important in the allergic response such as mast cells, basophils, and eosinophils (147). It has also been shown that IgE-sensitized human airway smooth muscle (ASM) cells produce IL-13 (150). Bronchial epithelial cells synthesize IL-13 and it has been shown that this production increases in response to different stimuli such as SO_2 (151), diesel exhaust (152) and epithelial injury (153).

In hematopoietic cells IL-13 stimulates IgE synthesis by human B cells (154) and induces CD23 and class II major histocompatibility complex antigen expression on monocytes (155). IL-13 has also been reported to have direct effects on eosinophils, including promoting eosinophil survival, activation, and recruitment (156, 157). IL-13 has important functions on non-hematopoietic cells, including endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. In endothelial cells IL-13 is a potent inducer of vascular cell adhesion molecule 1 (158). IL-13 enhances proliferation and cholinergic-induced contractions of smooth muscle cells in vitro (150, 158). It also induces fibroblast growth (159) and type I collagen synthesis in human dermal fibroblasts (160). In epithelial cells IL-13 is a potent inducer of growth factors (161, 162) and chemokine expression (163). It also induces epithelial cell proliferation (161, 164), alters mucociliary differentiation (165), and results in mucin production and goblet cell metaplasia (148, 166-168).

I.6.1 IL-13 receptors

The effects of IL-13 are mediated by a complex receptor system that includes IL-4 receptor α (IL-4Rα), IL-13 receptor α1 (IL-13Rα1) and IL-13 receptor α2 (IL-13Rα2). IL-13Rα1 and IL-13Rα2 are members of the hematopoietin receptor superfamily and bind IL-13 but not IL-4. IL-13Rα1 binds IL-13 with low affinity; however, it can also combine with IL-4Rα to form a high-affinity IL-13 binding complex. In contrast, IL-13Rα2 alone binds IL-13 with high affinity. The IL-13R (IL-4R/IL-13Rα1) is widely expressed on both hematopoietic and nonhematopoietic cells except human and mouse T cells and mouse B cells (169). The IL-13Rα2 chain has been shown to be present in a

variety of tissues such as the testis, brain, liver, thymus, ASM, and airway epithelial cells (147).

Expression of IL-13 receptors in the respiratory system has been well documented. IL-13Rα1 is constitutively expressed on human bronchial epithelial cells (170, 171). Expression of IL-13Rα2 was nearly absent at baseline in mouse lung, but when stimulated by allergens or Th2 cytokines (IL-4 and IL-13), expression of IL-13Rα2 was markedly induced (172). Bleomycin-induced IL-13-mediated lung fibrosis increased IL-13Rα2 expression in airway epithelial cells, whereas expression of IL-13Rα1 remained unchanged (162, 173). In *in vitro* experiments, IL-14 and IL-13 each induce expression of IL-13Rα2 in human airway epithelial cells (170, 171), fibroblasts (174), and keratinocytes (175). In contrast, IL-13Rα1 was expressed constitutively and was not up-regulated by cytokines in human fibroblasts and keratinocytes (175, 176). Lysophosphatidic acid, a bioactive phospholipid, also induced IL-13Rα2 expression, but had no effect on IL-13Rα1 expression (177). TNF-α and IL-4 synergistically up-regulated the expression of IL-13Rα2 on human fibroblasts by inducing gene expression and mobilizing IL-13Rα2 from large intracellular pools to the cell surface (176). These data indicate that Th2 cytokines can induce IL-13Rα2 expression and promote mobilization to the cell surface, and have synergistic effects with other inflammatory cytokines.

I.6.2 IL-13 signaling

Ligation of the IL-13Rα1/IL-4R receptor complex by IL-13 results in the activation of a variety of signal transduction pathways including Jak–signal transducer and activator of transcription (STAT) pathway and specifically STAT6. Jaks are tyrosine

kinases that each contains a true catalytic domain and a pseudokinase domain. There are four Jaks: Jak1, Jak2, Jak3, and Tyk2. Jak1, Jak2, and Tyk2 are ubiquitously expressed, whereas Jak3 expression is limited to hematopoietic cells (178). IL-13 results in activation of Jak1 and Tyk2 in hematopoietic and nonhematopoietic cells. Activation of Jaks results in phosphorylation of the cytoplasmic tyrosines in IL-4Rα, leading to the recruitment of STAT6 to the receptor, followed by STAT6 phosphorylation and activation. Activated STAT6 dimers then translocate to the nucleus, bind specific canonic DNA elements, and initiate transcription of downstream genes (Fig. I.10). STAT6 has been shown to be critical for several IL-13/IL-4-mediated processes such as class switching of B cells (179) and the development of the allergic phenotype (180). IL-13 binding of the IL-13/IL-4 receptor complex also results in tyrosine phosphorylation of the insulin receptor substrate followed by activation of a number of signaling molecules including phosphoinositide 3 kinase, Grb2, and Shc. Stimulation of these pathways is thought to be important in cell proliferation and growth (147).

IL-13Rα2 has been considered for a long time as a decoy receptor which does not contribute to IL-13 signaling. The notion that this receptor had no signaling function arose from the fact that it has a short cytoplasmic tail that does not bind Jaks or STATs and that cells bearing this receptor do not activate STAT6 (166, 181). IL-13Rα2 exists in three compartments, cytoplasmic, surface membrane, and soluble. The majority of the IL-13Rα2 protein exists in intracellular pools which can be rapidly mobilized to the cell surface by IFN-γ (181). Membrane IL-13Rα2 can continually release into the medium in a soluble form which has been shown to inhibit IL-13 responses (166). A new study by Andrews *et al* has demonstrated that while the recombinant soluble form of IL-13Rα2

blocks only the effects of IL-13, the transmembrane form of this receptor could become associated with IL-4Rα and attenuate both IL-13 and IL-4 responses (174). Interestingly, recent investigations have suggested that IL-13Rα2 might act as a signaling receptor as well as a decoy receptor. Dienger *et al* showed that IL-13Rα2 KO mice have attenuated rather than enhanced allergic airway responses, suggesting that under some circumstances, IL-13Rα2 may contribute to IL-13 signaling (147). A recent study has demonstrated that signaling through the IL-13Rα2 mediates TGF-β production in macrophages. In this study investigators showed that IL-13Rα2 signals through AP-1 complex (162) (Fig.I.10).

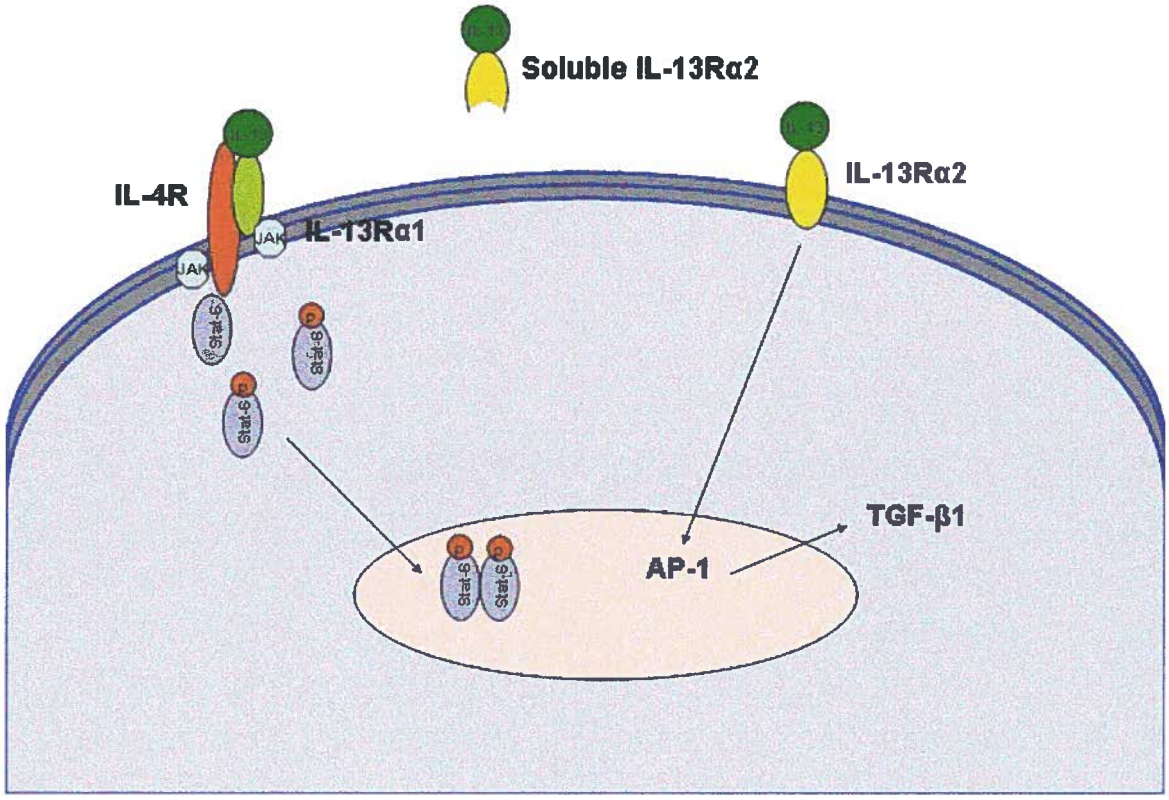

Figure I.10. Schematic representation of IL-13 receptors and signaling pathways. The functional IL-13 receptor consists of a heterodimeric complex composed of the IL-4Rα and IL-13Rα1 chains. IL-13 and IL-4 both can induce heterodimerization of the IL-4Rα and IL-13Rα1 chains, and dimerization of both chains induces phosphorylation and activation of Jak. Activated Jak thus phosphorylate tyrosine residues of the IL-4Ra chain. Signal Transducers and Activators of Transcription 6 (STAT6) is attached with phosphorylated tyrosine via Src homology domains and phosphorylated by Jak. Phosphorylated STAT6 proteins dimerize and translocate to the nucleus where they bind to specific DNA sequences. IL-13Rα2 has a short cytoplasmic domain and exists in soluble form as well as membrane bound form. Soluble IL-13Rα2 binds to IL-13 with high affinity and blocks the effects of IL-13 signaling. Recent studies show that IL-13 binds to IL-13Rα2 and activates AP-1 to induce secretion of TGF-β.

I.7 Glycosylated structures

Glycans, or carbohydrates, are the most-abundant and structurally diverse biopolymers formed in nature comprising a major component of the outer cell surface. Numerous pathways and enzymatic activities involved in glycan biosynthesis generate this diversity in the secretory pathway. The majority of carbohydrates present in cells are associated with proteins or lipids but they can also be found detached from other molecules. There are six major classes of mammalian glycans. The two main groups include the N-glycans and the O-glycans. Other recognized glycans include glycolipids, glycosaminoglycans (GAGs), GPI-anchored glycans and hyaluronan (182) (Fig.I.11).

Carbohydrate structures are not primary gene products. They are synthesized by a series of gene encoded glycosyltransferases within the endoplasmic reticulum (ER) and Golgi apparatus. As nascent peptides are being synthesized in the ER and Golgi apparatus, these glycosyltransferases add monosaccharides one at a time to specific positions on specific precursors. In addition, glycosidases remove specific carbohydrate residues during this process to further regulate the complexity of the synthesized carbohydrate chain and thus modifying the carbohydrate portion of the glycoconjugates. The activity and specificity of these enzymes determine the glycosylation patterns of proteins and lipids.

Relative to nucleotides and proteins, carbohydrates are very complex in structure. Each monosaccharide can theoretically generate an α or a β linkage to any one of the carbon or oxygen positions within the sugar moiety of another monosaccharide in the chain. Three nucleotide bases or amino acids can only generate six variations while three hexoses could produce (depending on which factors are considered) anywhere from 1,056

to 27,648 unique trisaccharides. As the number of units in the polymer increases, this difference in complexity becomes even greater. For example, a hexasaccharide with six hexoses could have more than 1 trillion possible combinations. This complexity gives carbohydrates an immense potential for encoding biological information and thus regulating biological functions.

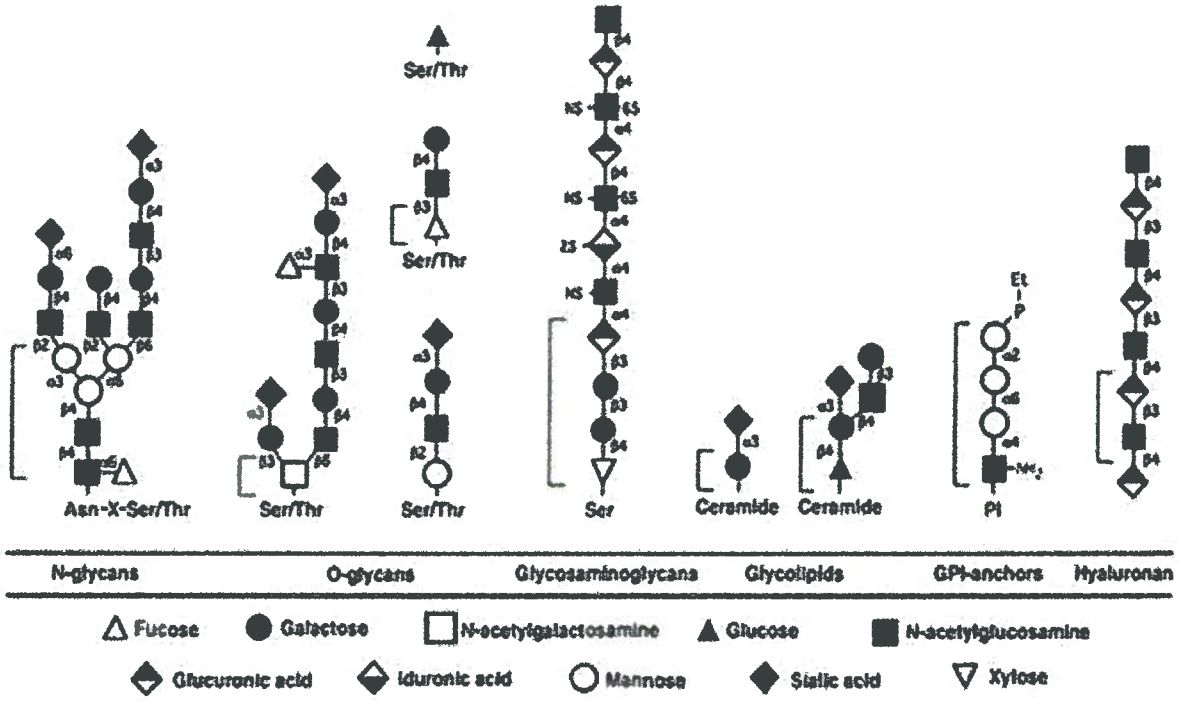

Figure I.11. The six different classes of mammalian glycans. Representative examples of each distinct class of mammalian glycans are schematically illustrated. A bracket adjacent to each representative glycan identifies the part that is generally common to that particular glycan class. In N-glycans the reducing terminal N-acetylglucosamine (GlcNAc) is linked to the amide group of asparagines but in O-glycans oligosaccharides are linked to the hydroxyl groups of threonine or serine residue via an N-acetylgalactosamine (GalNAc). Et-P denotes the ethanolamine phosphate moiety linked to the COOH-terminus (not shown) of GPI-modified proteins. PI denotes the phosphatidylinositol moiety and its fatty acyl group (not shown) that together promote membrane anchoring by insertion into the plasma membrane lipid bilayer. (Adapted from Lowe *et al.* (174).

The timing and pattern of the glycosyl-modification can determine protein folding, storage, membrane localization and ultimately function. Glycosylation of a pre-formed protein in response to an external signal can also regulate acutely a protein's function (183). Glycosylation of lipids can alter membrane rigidity as well as the function of membrane proteins (184).

I.7.1 N-glycans

All N-linked oligosaccharides are linked to Asn in the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid besides Pro and Asp. N-glycans can be subdivided into three distinct groups called high mannose type, hybrid type, and complex type. All of these three types contain a common pentasaccharide core structure, Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc→Asn (contained within dotted box in Fig.I.12).

In mature glycoproteins, N-linked glycosylation is structurally diverse varying in the number and size of branches among cell types, tissues and species. But when they are first added in the ER to asparagine residues within the Asn-X-Ser/Thr consensus sequence on growing polypeptides, the N-glycans are homogeneous and relatively simple. Processing of the N-linked oligosaccharide moieties starts while the proteins are still in the ER, and continues after they arrive in the Golgi apparatus. The processing in the ER introduces only limited diversity that is shared with all glycoproteins but once the proteins arrive in the Golgi, structural diversity is generated. In the early secretory pathway, the N-glycans have a common role in promoting protein folding, quality

control, and certain sorting events. Later in the Golgi, enzymes introduce much diversity which prepares the mature protein for more novel and diverse functions (185).

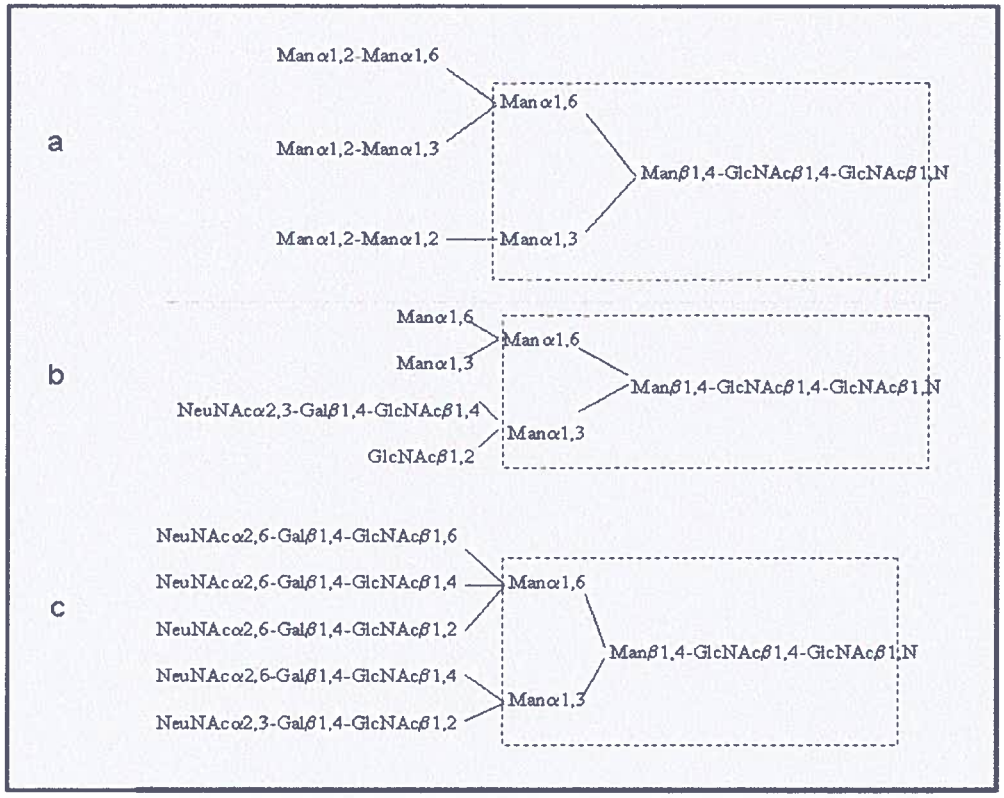

Figure I.12. Three different types of N-glycans. (a) High mannose-type oligosaccharides; (b) hybrid-type oligosaccharides; (c) pentantennary complex-type. The structures encircled by dotted lines represent the "core" structure that is common in all different types of N-glycans.

I.7.2 N-glycan synthesis

Biosynthesis of all N-linked oligosaccharides begins in the rough ER with addition of a large preformed oligosaccharide precursor. This precursor is a 14-saccharide core ($Glc_3Man_9GlcNAc_2$) unit which is assembled as a membrane bound dolichylpyrophosphate precursor by a series of enzymes, beginning with the enzyme

UDP-GlcNAc: dolichol phosphate GlcNAc-1-phosphate transferase (GlcNAc-PT). GlcNAc-PT is essential to N-glycan assembly and plays a critical role in protein folding of many membrane-associated and secreted polypeptides. Tunicamycin is a GlcNAc analogue that competitively inhibits GlcNAc-PT function. The entire core oligosaccharide is transferred en bloc from the dolichol carrier to an asparagine residue on a nascent polypeptide, a reaction catalyzed by oligosaccharide-protein transferase (OST).

The three glucose residues, which are the last residues added in synthesis of $(Glc)_3(Man)_9(GlcNAc)_2$ on the dolichol carrier, appear to act as a signal that the oligosaccharide is complete and ready to be transferred to a protein. Immediately after the oligosaccharide is transferred to a nascent polypeptide, glucosidases (Glcase I and II) quickly remove the two terminal glucose residues. Removal of the third glucose, however, is associated with proper glycoprotein folding and contributes to the ER retention time of that glycoprotein. Folding is monitored by a glucosyltransferase as well as a molecular chaperone, calnexin. This glucosyltransferase acts as a sensor that detects improperly folded proteins by adding a terminal glucose residue. Calnexin, a lectin, selectively binds to reglucosylated $Glc_1Man_{7-9}(GlcNAc)_2$ oligosaccharides and helps retain the glycoprotein in the ER until it is properly folded. This is one of the most important functions of N-glycans (186, 187). Occasionally proteins spontaneously dissociate from calnexin and immediately are deglucosylated; if they then fold properly, they will not be reglucosylated nor rebind to the lectin, and will pass to the Golgi.

Biologists traditionally have considered the series of flattened and spherical sacs (cisternae) composing the Golgi complex as a single organelle. However, the cis, medial,

and trans cisternae of the Golgi contain different sets of enzymes that introduce different modifications to secretory and membrane proteins; thus each region in effect functions as a distinct organelle. Following glucose trimming of the properly folded glycoprotein, N-glycans become available for glycosidase reactions in the ER and Golgi. ER and Golgi (cis) mannosidases remove mannose residues. Further along in the Golgi, the glycan chains undergo further trimming of mannoses and, in many cases, new sugars are added to produce complex glycans and some high-mannose glycans that have escaped terminal glycosylation. The structures undergo further diversification with the action of GlcNAcTs and mannosidases. GlcNAcT-I adds GlcNAc to the high-mannose structure ($Man_5GlcNAc_2$-Asn) and α-mannosidase II removes two mannose residues in the medial Golgi, resulting in a "hybrid" N-glycan ($GlcNAc_1Man_3GlcNAc_2$-Asn). "Complex" N-glycans are then produced with the activity of other GlcNAcTs (GlcNAcT-II, III, IV, V and VI) that add on GlcNAc to mannose residues, as well as fucosyltransferases that add fucose to the core GlcNAc (Fig. I.13). As N-glycans transit through the medial- and trans- Golgi, they become substrates for glycosyltransferases localized toward the end of the assembly line, such as sialyltransferases and sulfotransferases, which add increasing diversity to N-glycans.

Oligosaccharides attached to glycoproteins have many functions beyond protein folding, which take place along the secretory pathway in the cell. They protect the mature proteins from proteolysis, target lysosomal enzymes to lysosomes and prevent their secretion, and participate in cell-cell interactions.

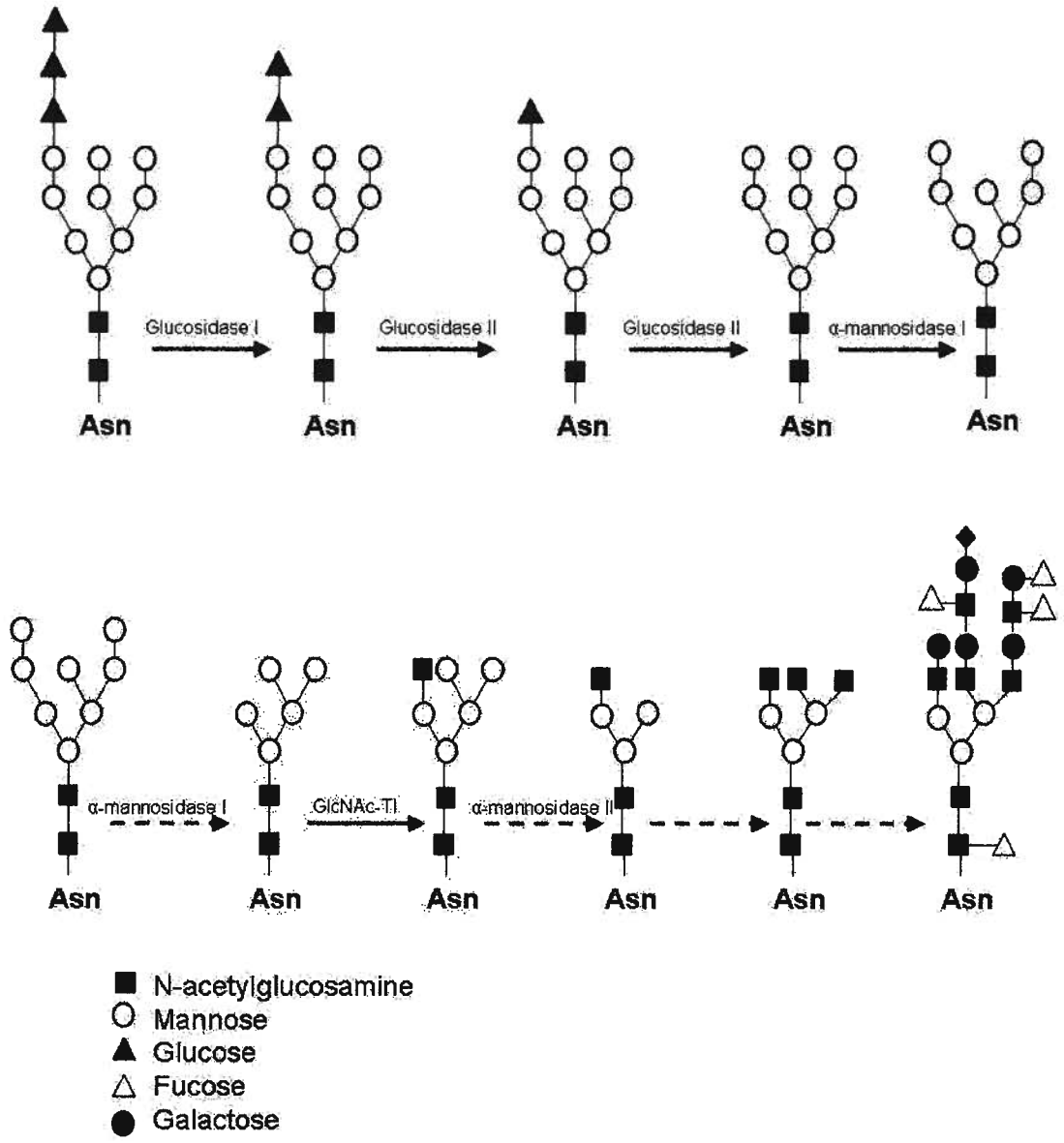

Figure I.13. Mammalian N-glycan synthesis. Biosynthetic events localized to the cytosol, endoplasmic reticulum (ER) lumen and the lumen of the Golgi apparatus. The general scheme for mammalian N-glycan synthesis starts with dolichol pyrophosphate (Dol-P-P) and ends with a final multiantennary structure (*bottom right*). This product is only one representative example of the numerous distinct N-glycans that exist in mammals.

I.8 Fucosylated glycans

Fucose is a monosaccharide that is a common component of many N- and O-linked glycans and glycolipids produced by mammalian cells. Fucose-containing glycans have important roles in blood transfusion reactions, selectin-mediated leukocyte-endothelial adhesion, host–microbe interactions, and numerous ontogenic events, including signaling events by the Notch receptor family (188). Alterations in the expression of fucosylated oligosaccharides have also been observed in several pathological processes, including cancer and atherosclerosis. Fucose deficiency is accompanied by a complex set of phenotypes both in humans with leukocyte adhesion deficiency type II (LAD II) (189) and in a recently generated strain of mice with a conditional defect in fucosylated glycan expression (190).

I.8.1 Lewis blood group antigens

The blood group antigens are a family of cell surface carbohydrate structures and were originally discovered on the surface of erythrocytes. However, expression of these antigens is not limited to erythrocytes and they can be found in different tissues and organs. Unlike the ABH antigens, the Lewis antigens on erythrocytes are passively acquired from glycolipid substances in the serum that are synthesized at an unknown site. Depending on the core disaccharide linkage, Lewis carbohydrate antigens are classified as either type 1 (Galβ1-3GlcNAc) or type 2 (Galβ1-4GlcNAc) structures (Fig. I.14). Type 1 structures such as the Le^a and Le^b blood group antigens are monofucosylated and difucosylated oligosaccharides, respectively. Type 2 structures include Le^x and Le^y

antigens that are analogous to Le^a and Le^b in containing one and two fucose residues, respectively (Fig. I.14).

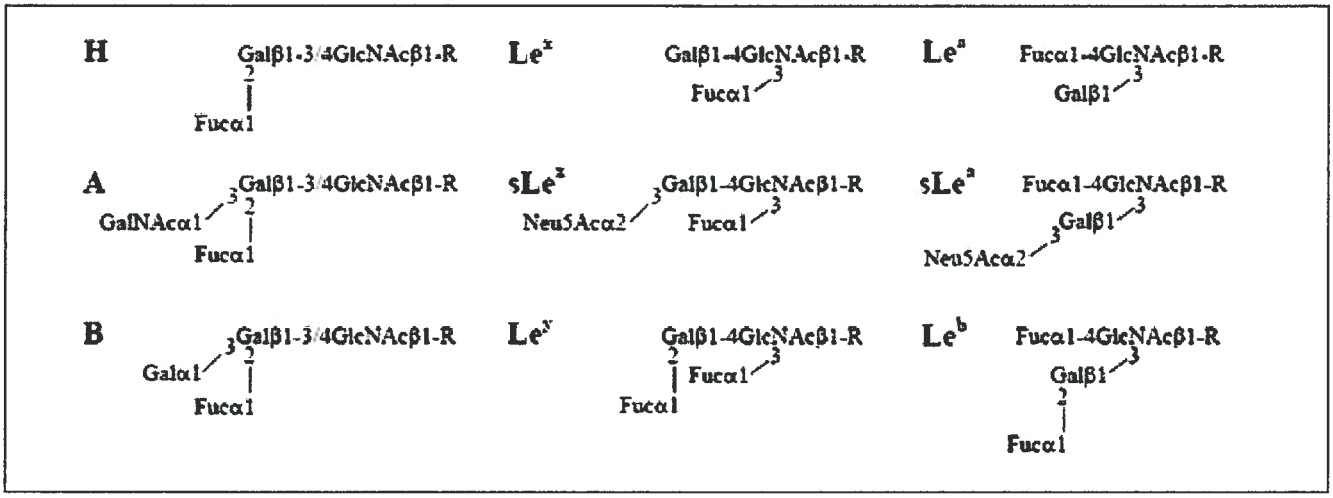

Figure I.14. Schematic representations of blood group A, B, O (H) and type 1 and 2 Lewis carbohydrate determinants. Almost every individual contains α1→2 fucosyltransferase, whereas the presence of A or B blood antigens depends on the genomes of each individual. Blood cells express only type II chains while other tissues such as epithelial layers express type I chains as well.

Although structurally related, the Le^x and Le^y antigens are expressed in only a few cell types, for example leukocytes and certain epithelial cells. They are not found associated with the red blood cell membrane and therefore do not represent authentic blood group antigens (191). The stage-specific embryonic antigens (SSEA-1) famous in developmental biology is the Le^x antigen (192). The sialyl Le^x (sLe^x) is known as a ligand of E-selectin and is important in lymphocyte homing (193-195) . Much of the recent interest in these antigens has resulted from the observation that they undergo specific changes during tissue embryonic development and malignant transformation and are involved in cell-cell interaction.

1.8.2 Sialyl Lewis X (sLe^x)

Some members of the Lewis blood group antigen family have functional relevance in the context of selectin-dependent leukocyte and tumor cell adhesion processes. The relevant members include especially the sialylated and/or sulfated members represented by the sialyl Le^x tetrasaccharide and its sulfated variants. These molecules provide essential contributions to the glycoproteins and glycolipids that function as selectin counter-receptors on leukocytes and tumor cells (196). Immunohistochemical studies on tumor specimens show that Lewis X/A structures are frequently overexpressed in carcinomas, being carried on glycosphingolipids as well as on N- and O-glycans (197, 198). Indeed, sLe^x and sLe^a were first identified as tumor antigens. The expression of these antigens by epithelial carcinomas consistently correlates with tumor progression, metastatic spread, and poor prognosis in humans. sLe^x also forms a critical component of E-selectin ligands and is important in proper immune response (5, 10) . E-selectin binds to the sLe^x structure that is present on neutrophils in the blood stream, and helps to mediate the extravasation of these cells into the surrounding tissue during inflammation.

1.8.3 Fucosyltransferases

A set of glycosyltransferases is responsible for the biosynthesis of Lewis antigens. The last step in their synthesis, fucosylation, is catalysed by specific fucosyltransferases. A fucosyltransferase is an enzyme that transfers an L-fucose sugar from a GDP-fucose (Guanosine diphosphate-fucose) donor substrate to an acceptor substrate including oligosaccharides, glycoproteins, and glycolipids. Based on the site of fucose addition,

FucT are classified into α1,2, α1,3/4, α1,6, and O-FucT. The former three subfamilies of enzymes in eukaryotic organisms are type II transmembrane Golgi-anchored proteins containing an N-terminal cytoplasmic tail, a transmembrane domain, and an extended stem region followed by a large globular C-terminal catalytic domain facing the Golgi lumen. O-FucTs, however, are endoplasmic reticulum (ER)-localized soluble proteins and catalyze O-fucosylation in the ER (199).

Thirteen fucosyltransferase genes have thus far been identified in the human genome (Table I). FUT1 (H enzyme) and FUT2 (Se enzyme) are α(1,2) fucosyltransferases catalyzing the transfer of fucose towards the galactose (Gal) residue of type 1 (Gal β1,3GlcNAc) and type 2 (Gal β1,4GlcNAc) chains, resulting in the synthesis of H-type 1 and H- type 2 chains, respectively. FUT1 (H) determines the expression of O-type antigen (H antigen) of the ABO blood group system on erythrocytes, whereas FUT2 (Se) determines it in saliva, i.e. secretor status (200, 201). Genes FUT3-FUT7 and FUT9 encode six α1,3/4 FucTs, abbreviated Fuc-TIII–VII and Fuc-TIX, all of which have α1,3 activity, but FUT3 and FUT5 also possess α1,4 activity. FucTs are involved in the last steps of synthesis of Lewis blood antigens and Lewis-related carbohydrate antigens (i.e., Le^x, Le^y, Le^a, Le^b, sialyl-Le^x, and sialyl-Le^a).

FucTs III, V, and VI have a high degree of sequence similarity and do not appear to have an essential biological role because not all humans have functional forms of these enzymes. FucTs IV, VII, and IX are less similar both to each other as well as to the other FucTs. Gene knockout studies of FucT IV and VII have demonstrated that these enzymes are essential for normal leukocyte trafficking and function (202). Human α1,6 FucT, encoded by FUT8, is widely expressed in mammalian tissues (203, 204) and directs

addition of fucose to asparagine-linked GlcNAc moieties, a common feature of N-linked glycan core structures (205). It has been discovered that fucose can also be transferred directly to the hydroxyl group of Ser and Thr residues of glycoprotein acceptors that contain either the epidermal growth factor (EGF) (206, 207) or the thrombospondin type repeat (TSR) (208, 209) sequences. These reactions are carried out by O-FucTs called OFUT1 and OFUT2. Finally, though not yet validated by functional studies, two additional putative α (1,3)-fucosyltransferase genes, FUT10 and FUT11 have been identified in the human genome by comparison with fucosyltransferase sequences in the *Drosophila melanogaster* genome (210). The function and the acceptor specificity of FUT10 and FUT11 have not yet been defined.

Table I.1. Fucosyltransferase family

Common name(s)	HUGO name	Ref seq/GenBank accession no	Linkage	Epitope Synthesized
H blood group α2fucosyltransferase	FUT1	NM_000148.1	α1,2	H type1 and 2
Secretor (Se) blood group α2fucosyltransferase	FUT2	NM_000511.1	α1,2	H type1 and 2
Fuc-TIII Lewis-type α3/4fucosyltransferase	FUT3	NM_000149.1	α1,3/4	Le^a, Le^b, sLe^a, Le^x, Le^y, sLe^x
Fuc-TIV Myeloid-type α3fucosyltransferase	FUT4	NM_002033.1	α1,3	Le^x, Le^y, sLe^x
Fuc-TV α3fucosyltransferase	FUT5	NM_002034.1	α1,3	Le^x, Le^y, sLe^x
Fuc-TVI Plasma-type α3fucosyltransferase	FUT6	NM_000150.1	α1,3	Le^x, Le^y, sLe^x
Fuc-TVII α3fucosyltransferase	FUT7	NM_004479.1	α1,3	Le^x, Le^y, sLe^x
Fuc-TVIII α6fucosyltransferase	FUT8	NM_004480.1	α1,6	Core fucosylation
Fuc-TIX α3fucosyltransferase	FUT9	NM_006581.1	α1,3	Le^x, Le^y
Fuc-TX α3fucosyltransferase	FUT10	NM_032664.2	α1,3	Unknown
Fuc-TXI α3fucosyltransferase	FUT11	NM_173540.1	α1,3	Unknown
Polypeptide O-fucosyltransferase	POFUT1	NM_015352.1		Fuc_{α}Serine and Fuc_{α} Threonine, within EGF repeats
Polypeptide O-fucosyltransferase	POFUT2	NM_015227.1		Unknown

I.9 Role of glycosylated structures in health and disease

Glycoconjugates mediate a wide variety of events in cell-cell and cell-matrix interactions crucial to the function of a complex multi-cellular organism and are involved in a variety of normal conditions. Disease states can result from either the deficiency in a particular glycosyltransferase or the ability to utilize simple monosaccharides. Either of these abnormalities will permit the generation of inappropriate glycoproteins or lipids.

Glycoconjugates are major players in inflammation. Leukocyte rolling on the endothelium is an initial and essential step in extravasation and inflammation which is mediated by the interaction of selectins with their carbohydrate ligand (211). Selectins are a class of type I membrane-bound C-type lectins expressed in vascular endothelium and on circulating leukocytes. So far, three selectins have been identified: L-selectin, expressed on all leukocytes; E-selectin, expressed by cytokine-activated endothelial cells; and P-selectin, expressed on platelets and Weibel-Palade bodies of endothelial cells. It has been shown that proper glycosylation of selectin ligands including α1,3-fucosylation and terminal α2,3-sialylation is necessary for selectin-ligand interaction (195, 212). Discovery of an uncommon genetic disorder provides support for the importance of carbohydrates in initiation of an inflammatory response. Leukocyte adhesion deficiency type II (LAD-II) is caused by a deficiency in the supply of fucose and therefore a sharp reduction in cell surface fucosylated glycans. This disorder is characterized by recurrent infections and persistent leukocytosis due to a diminished ability of leukocytes to roll on endothelium and traffic to inflammatory sites *in vivo*. The disorder is a result of defective selectin ligand biosynthesis (189). Because of the critical role of glycoconjugates in selectin-selectin ligand interaction, it could be inferred that they contribute to the

development of pathological processes involving inflammation such as atherosclerosis, ischemic reperfusion injury and certain skin disorders.

Cell surface glycans mediate host-microbe interactions. Adhesion of pathogenic organisms to the cells and mucosal surfaces of the host is a necessary step for most of infectious diseases. In many cases, this adhesion is mediated by lectins present on the surface of the infectious organism that bind to complementary carbohydrates of glycoproteins or glycolipids on the surface of the host tissues. *Helicobacter Pylori*, the causative agent of peptic ulcer, binds sialic acid-containing glycoconjugates on the surface of gastric epithelium (213). *Pseudomonas aeruginosa,* the major pathogen in the airways of patients suffering from cystic fibrosis, binds to sLe^x and sLe^x related structures on the airway epithelium (214, 215). The role of cell surface glycans in adherence and infectivity of influenza and HIV-1 virus has been well documented (216, 217). Many soluble carbohydrates that bind to bacterial and viral receptors have been employed as anti-adhesive agents to block the adhesion of the microorganisms to the cells and prevent infection (218-220).

Cell surface glycans are involved in development. Carbohydrate chains of glycolipids and glycoproteins on the cell surface undergo a complex sequence of changes during embryogenesis. There are numerous studies that have demonstrated the involvement of glycans in ontogenic events. The Lewis X (Le^x) carbohydrate structure, α1,3- fucosyl-*N*-acetyllactosamine, known as stage-specific embryonic antigen-1 (SSEA-1) or CD15 antigen is expressed during early embryogenesis where is considered to function as a cell–cell interaction ligand in the compaction process (192). Liu *et al.* reported a stage-specific expression of fucosyltransferases in mouse embryos (221).

Temporal and region-specific expression of Le^x in the developing brain has been documented which suggests that Le^x plays important role in neuronal development (222-224).

Cell surface glycans are involved in malignant transformation and metastasis. Malignant transformation is highly associated with alteration of cell surface glycosylation, which indicates carbohydrates may play a role in malignant transformation. Moreover, it has been suggested that cell surface carbohydrates determine the ability of malignant cells to form distant metastasis in various anatomical locations (197, 198, 225-227). Increased sialylation and β1-6-linked branching of complex-type N-glycans has been consistently observed in both human and murine tumor cells which appears to be associated with enhanced metastatic potential (225, 226). Gessner *et al.* (228) showed an increased activity of α2,6-sialyltransferase in tumor tissue and particularly in metastasizing tumors. An enhanced epithelial expression of sLe^x and sialyl-Lewis A (sLe^a) in primary and metastatic carcinoma lesions has been reported (197, 198). Modification of oligosaccharides of the cell surface glycoproteins by transfection of genes encoding various glycosyltransferases in sense and antisense orientation provides direct evidence that changes in cell surface carbohydrates are important for the metastatic behavior of tumor cells (229-232).

I.9.1 Role of cell surface glycoconjugates in epithelial repair

The molecular events that initiate, mediate, and regulate different processes involved in epithelial repair have not been fully elucidated, but a number of studies have suggested that glycoconjugates attached to proteins within the plasma membrane of

epithelial cells play a central role in these events. The tremendous capability of carbohydrates to store biological information and their essential role in mediating cellular events make their synthesis, regulation and degradation mechanisms important in the study of diseases.

Many proteins essential for normal cell physiology including adhesion molecules, cell surface receptors, enzymes and, hormones are glycosylated (1, 2). Alteration in the glycosylation pattern of many glycoproteins leads to changes in their function. It has been shown that impaired glycosylation of receptors often leads to abnormal intracellular trafficking, ligand binding and downstream signal transduction ability (3, 4, 6, 233). Tsuda *et al.* demonstrated that removal of sialic acid from erythropoietin leads to loss of its *in vivo* activity (234). Complex carbohydrate structures attached to cell surface proteins and lipids have functional roles in cell motility (8), adhesion (5), proliferation (9), and growth potential (10) in several cell types. It also has been demonstrated that certain apical cell-surface carbohydrates are altered during cellular differentiation (235). Thus, many cellular functions are regulated and dependent upon glycoproteins. Because of the role of glycosylated structures in cell-cell and cell-matrix interaction, it is not surprising that there is a growing interest to explore the role for and regulation of these cell-surface carbohydrates in epithelial repair.

Lectins are naturally occurring proteins that can be isolated from a variety of plants and animals. Each lectin binds a to specific sugar moiety. As such, lectins are exquisitely selective tools to identify or block specific glycoconjugate motifs and have been extensively employed to study the role of cell-surface sugars and complex carbohydrates in cellular function (236, 237).

A variety of approaches have been employed to unravel the role(s) of carbohydrates in the multiple steps of the repair process. Some studies have investigated the expression pattern of different carbohydrates after injury using lectins as probes. Using three methods for localizing or quantifying lectin-binding sites Gipson *et al.* compared cell-surface of normal and migrating corneal epithelium of the rat. In their study they found that cell-surface of migrating epithelia express different sugar moieties relative to cell membranes of stratified stationary epithelia. There was a dramatic increase in concanavalin A (ConA) and wheat germ agglutinin (WGA) binding on migrating cells relative to stationary cells. The authors also showed that migrating cells have an increase in glycoprotein production determined by an increase in the incorporation of radiolabelled leucine and glucosamine. In addition they found that N-glycosylation of epithelial cells is necessary for epithelial cell migration (238, 239). Sweatt *et al.* demonstrated an increase in N-acetylgalactosamine in cell-surface glycoconjugates at the site of epithelial injury in pig cornea (240). Our laboratory has previously characterized cell-surface glycosylation in non-secretory cells of central human airway epithelium and airway epithelial cell lines utilizing lectin-binding patterns (241). In this study it was shown that galactose- or galactosamine-specific lectins labeled basal epithelial cells and cell lines derived from basal cells. Lectins specific for several different carbohydrate structures bound columnar epithelial cells, and certain fucose-specific lectins labeled subsets of the airway epithelial cells. The cellular specificity of these differences suggests they may be relevant in various cellular functions. We also demonstrated that following mechanical injury of guinea pig tracheal epithelium, glycosylation profiles in the repairing epithelium change over time (242). These changes may represent either the

expression of one or more new glycoproteins, or changes in glycosylation of constitutive proteins, required for activation or a change in cell function needed for repair to proceed. It has been shown that injury of the respiratory epithelium enhances *P. aeruginosa* adhesion and it has been speculated that changes of cell surface glycoconjugates related to wound repair, cell migration and/or spreading may favor *P. aeruginosa* adhesion (243).

We examined the functional role of cell surface carbohydrates in an *in vitro* model of wound repair after mechanical injury of human airway epithelial cells. Our results demonstrated that N-glycosylated glycoproteins, particularly those with a terminal fucose residue, are essential in the adhesion and migration of airway epithelial cells and facilitate closure of epithelial wounds in monolayer culture (244). Recently we studied the role of a fucose containing tetrasaccharide, sLe^x [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc], in airway epithelial repair. We observed increased presentation of sLe^x in areas of epithelial damage relative to areas of intact epithelium. In an *in vitro* model of bronchial epithelial repair we have demonstrated an increase in cell surface sLe^x following mechanical injury of airway epithelial cell monolayers. Moreover we have shown that inhibition of sLe^x, in our model of epithelial repair completely prevents epithelial repair (245).

The role of cell surface carbohydrates in repair process was studied by blocking specific carbohydrate moieties with a variety of lectins. It has been shown previously by Donaldson *et al.* that the plant lectin concanavalin A (ConA), which binds glucose and mannose moieties, can inhibit migration of newt epidermal cells (246). Gipson *et al.* cultured rat corneas with 3 mm central epithelial abrasions in the presence of four plant lectins. In this study the authors demonstrated that blocking glucose, mannose, and

glucosamine sites on corneal epithelial cell surfaces and/ or the epithelial basement membrane reversibly slows or inhibits epithelial migration (236). Using the same culture model of bronchial epithelial repair, our laboratory has demonstrated that following mechanical wounding of intact monolayers, the lectins *Allomyrina dichotoma* (AlloA) and *Cicer arietinum* agglutinin (CPA) differentially stain human airway epithelial cells in damaged areas relative to the staining of intact epithelial monolayers. While AlloA positive staining cells are those that appear to be migrating from areas distant to the wound and accumulating in the wound, CPA positive staining cells are restricted to the leading edge of the wound. Moreover, the addition of the above lectins following mechanical wounding inhibited the repair. These results suggest that AlloA and CPA bind specific carbohydrate structures involved in normal epithelial repair (39). Further work is being carried out to determine the identity of the relevant proteins associated with these carbohydrate ligands.

In a study by Trinkaus-Randall *et al.*, the effect of specific carbohydrate moieties of the basal lamina on the attachment and spreading of rabbit corneal epithelial cells was studied. Corneal epithelial basal cells were plated onto freshly denuded basal lamina and three lectins WGA, ConA and RCA were used to block specific sugar moieties in the basal lamina. This study showed that lectin binding of glucose, mannose, and galactose moieties on the basal lamina significantly altered the extent of cellular spreading, while the binding of glucosamine inhibited attachment. This study demonstrated that alteration of specific sugar moieties on the native basal lamina dramatically affects the ability of basal cells of corneal epithelium to attach or spread (7). Using an in vitro model of airway epithelial repair Adam *et al.* demonstrate that lectin WGA which binds to *N*-acetyl

glucosamine residues inhibits the repair of epithelial damage without altering cell viability, while other *N*-acetyl glucosamine binding lectins do not affect the repair process (247).

These studies clearly demonstrate the critical role of carbohydrates in the process of epithelial repair. However, carbohydrates must either modify a protein or lipid to regulate its function or participate in binding to a specific receptor to effect the desired action. As such, the biological role of glycans can be broadly divided into two groups. One group depends on the structural and modulatory properties of glycans and the other relies on specific recognition of glycan structures (generally receptor proteins or lectins).

I.9.2 How carbohydrates mediate cell-cell interaction and migration

The glycans attached to matrix molecules such as collagens and proteoglycans are important for the maintenance of tissue structure, porosity, and integrity. Such molecules also contain binding sites for specific types of glycans, which in turn help with the overall organization of the matrix. Glycans are also involved in the proper folding of newly synthesized polypeptides in the ER and /or in the subsequent maintenance of protein solubility and conformation (1). In this manner, altered glycosylation of matrix proteins may change adhesion properties and the potential of cells to migrate. This is due to either an altered conformation of the extracellular matrix or a change in the carbohydrate ligands available to bind.

Glycosylation of cell surface receptors has tremendous effects on the receptor function. Modification of receptor N-glycans can modulate ligand binding to the receptor (118, 119), regulate receptor trafficking and duration of cell surface residency (117), and

alter receptor localization (114). Direct association between changes in glycosylation of cell surface receptors including EGFR and cellular events involved in epithelial repair such as migration and proliferation remains to be identified.

The specificity of carbohydrate interactions allows for the high degree of selectivity found within the cell. Whether it is in regulating receptor activation, cell-cell or cell-matrix interactions or cell migration, defects in these processes may result in abnormalities and altered phenotypes. Their understanding will provide new avenues for research and therapies.

I.10 Rationale, hypothesis, and specific aims

The overarching hypothesis in this dissertation is: EGFR has a central role in airway epithelial repair and both release of EGFR ligands and IL-13 from injured AEC monolayers and posttranslational modifications of the receptor are necessary for epithelial repair. We tested the above hypothesis through the following specific aims: (1) To determine the basic mechanism of airway epithelial repair, focusing on endogenous mediators released by injured airway epithelium and explore how their interactions affect epithelial repair, (2) to explore the role of Sialyl-Lewis x (sLe^x) and regulation by the activity of FucT-IV in airway epithelial repair and (3) to elucidate the role of sLe^x decoration of EGFR in airway epithelial repair.

I.10.1 To determine the basic mechanism of airway epithelial repair focusing on endogenous mediators released by injured airway epithelium and explore how their interactions affect epithelial repair.

Bronchial epithelial cells produce a diverse array of pro-inflammatory mediators, growth factors and cytokines in response to environmental challenges (125-127, 152) and are actively involved in different stages of epithelial repair. Among these epithelial-derived factors, ligands for the epidermal growth factor receptor (EGFR), the EGF family, are particularly important. Although the effect of exogenous EGF in acceleration of airway epithelial repair has been demonstrated (50), the role for endogenous ligand(s), released by damaged or adjacent epithelium, in activation of EGFR and their role in epithelial repair remains to be determined.

IL-13 is a Th2 like cytokine which has a postulated central role in pathogenesis of asthma. Several studies have shown some degree of relationship between IL-13 and EGFR pathway in bronchial epithelial cells (161, 167, 168). While the majority of studies have focused on the role of IL-13 in mediating different features of asthma, the role of IL-13 in normal airway epithelial repair and its relationship with EGFR in this context needs to be determined.

In this part of my study I used a culture model of epithelial injury and repair to investigate the response of AEC to mechanical injury. Our hypothesis was that AEC release EGFR ligands including HB-EGF and the cytokine IL-13 in response to injury and that IL-13 contributes to the normal reparative response of AEC through HB-EGF and the EGFR pathway.

I.10.2 To identify the role of glycoconjugate sLe^x in airway epithelial repair.

Oligosaccharides on cell surface proteins and lipids have functional roles in cell adhesion (5), migration (8), proliferation (9) and growth potential (10). Molecular events that initiate, mediate, and regulate different processes involved in epithelial repair have not been fully elucidated, however, a number of studies have suggested that glycoconjugates attached to proteins within the plasma membrane of epithelial cells play a central role in these events (236, 240, 246, 247). Our laboratory has previously determined the pattern of cell surface glycosylation in normal human airway epithelial cells (241). These data have shown that glycosylation profiles in airway epithelium change over time during repair of a wound created by mechanical injury (242). Our data also suggested that cell surface N-glycosylation has a functional role in airway epithelial cell adhesion and migration and that N-glycosylation with terminal fucosylation plays an essential role in the complex process of repair by coordination of certain cell-cell functions (244). While previous studies in epithelial repair suggested a role for glycoconjugates, none of these studies specified an oligosaccharide structure(s) to be involved in repair.

Sialyl Lewis x, (sLe^x), is a fucose containing tetrasaccharide [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc] which has been recognized as a ligand for E-selectin and therefore has an important role in lymphocyte trafficking (5, 10). In the present study, we examined the novel role of sLe^x in bronchial epithelial repair and its regulation by FucT-IV.

I.10.3 To determine the role of sLe^x decoration of the EGFR in modulation of receptor function during airway epithelial repair.

Many proteins essential for normal cell physiology including adhesion molecules, cell surface receptors, enzymes and, hormones are glycosylated (1, 2). Alteration in the glycosylation pattern of many glycoproteins leads to changes in their function (4, 234). In the previous chapter we demonstrated important roles for carbohydrate sLe^x in airway epithelial repair (245). sLe^x has been identified as a tumor specific antigen (197, 198). Although the general idea is that sLe^x promotes tumor cell motility through interaction with endothelial E-selectins, another unexplored possibility is that sLe^x may be carried by specific receptors which control cell motility.

EGFR is a glycoprotein with 11 potential N-glycosylation sites in its extracellular domain including fucose containing structures such as Le^x, Le^y, and blood group A and H antigens (109, 112, 113). Glycosylation of EGFR has been shown to modulate its function (114, 118, 119). In this section we examined whether sLe^x decorates EGFR and if it does, how it can modulate receptor function during epithelial repair.

I.11 References

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CHAPTER II. SECRETION OF IL-13 BY AIRWAY EPITHELIAL CELLS ENHANCES REPAIR VIA HEPARIN-BINDING EGF-LIKE GROWTH FACTOR (HB-EGF)*

II.1 Summary

Inappropriate repair following injury to the epithelium generates persistent activation which may contribute to airway remodeling. In the present study we hypothesized that IL-13 is a normal mediator of airway epithelial repair. Mechanical injury of confluent airway epithelial cell (AEC) monolayers induced expression and release of IL-13 in a time-dependent manner coordinate with repair. Neutralizing of IL-13 secreted from injured epithelial cells by shIL-13Rα2.FC significantly reduced epithelial repair. Moreover, exogenous IL-13 enhanced epithelial repair and induced Epidermal Growth Factor Receptor (EGFR) phosphorylation. We examined secretion of two EGFR ligands, EGF and HB-EGF, after mechanical injury. Our data showed a sequential release of the EGF and HB-EGF by AEC after injury. Interestingly, we found that IL-13 induces HB-EGF, but not EGF, synthesis and release from AEC. IL-13 induced EGFR phosphorylation and the IL-13 reparative effect on AEC are mediated via HB-EGF. Finally, we demonstrated that inhibition of EGFR tyrosine kinase activity by tyrphostin AG1478 increases IL-13 release after injury, suggesting negative feedback between EGFR and IL-13 during repair. Our data, for the first time, showed that IL-13 plays an important role in epithelial repair, and that its effect is mediated through the autocrine release of HB-EGF and activation of EGFR. Dysregulation of EGFR-

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phosphorylation may contribute to a persistent repair phenotype and chronically increased IL-13 release, and in turn result in airway remodeling.

II.2 Introduction

The epithelial layer of airways is continuously exposed to gaseous and particulate components of the inhaled air and therefore is frequently damaged. Rapid regeneration following injury is crucial for restoring epithelial function to its normal state and involves an orderly progression of events. Bronchial epithelial cells can produce a diverse array of pro-inflammatory mediators, growth factors and cytokines in response to environmental challenges (1-6) and are actively involved in different stages of epithelial repair. Among these the ligands for the epidermal growth factor receptor (EGFR) are particularly important. Although the effect of exogenous EGF in acceleration of airway epithelial repair has been demonstrated (7, 8), the role for endogenous ligand(s), released by damaged or adjacent epithelium, in activation of EGFR and their role in epithelial repair remains to be determined.

IL-13 is a Th2 like cytokine which has been considered as a central mediator of airway remodeling in asthma. Bronchial biopsy specimens and BAL cells from allergic individuals with asthma show elevated expression of IL-13 compared to control subjects (9, 10). Several studies have shown some degree of relationship between IL-13 and EGFR pathway in which EGFR activation is necessary for IL-13-mediated mucin production and goblet cell metaplasia in airway epithelium (11, 12). IL-13 has also been shown to indirectly activate EGFR via production of TGF-α (13). While the majority of studies have focused on the role of IL-13 in mediating different features of asthma, the

role of IL-13 in normal airway epithelial repair and its relationship with EGFR in this context needs to be determined.

In the present study we used a culture model of epithelial injury and repair to investigate the response of AEC to mechanical injury. Our hypothesis was that IL-13 contributes to the normal reparative response of AEC. Our data, for the first time, showed that AEC produce and release significant quantities of IL-13 in response to mechanical injury which is necessary for epithelial repair. We also found a temporal release of EGF and HB-EGF by AEC after mechanical injury. We showed that IL-13 increases EGFR phosphorylation and enhances epithelial repair through autocrine/paracrine release of HB-EGF. Interestingly, AEC release more IL-13 when EGFR-phosphorylation is blocked. It is possible that the pathological effects of IL-13 occur as a result of persistent excessive IL-13 release in response to incomplete repair. These findings have important implications for understanding basic mechanisms of epithelial repair and remodeling.

II.3 Materials and Methods

II.3.1 Cell culture.

1HAEo$^-$ cells are an SV40-transformed normal human airway epithelial cells that have been characterized previously (14). Well-differentiated human bronchial epithelial cell cultures (EpiAirway^{TM}, air–liquid interface, ALI) were supplied by MatTek Co. (Ashland, MA).

II.3.2 RNA isolation and reverse transcription polymerase chain reaction.

RNA was extracted from 1HAEo$^-$ cells using the TRIzol reagent (GIBCO/BRL), according to the manufacturer's protocol. Conventional PCR was performed using primers specific for IL-13 and β-actin and 2 μl of the synthesized cDNA strand. Specific primers for IL-13 were synthesized by Sigma-Genosys according to published sequences (15): sense, 5'-CTC CTC AAT CCT CTC CTG TT-3'; antisense, 5'-GTT GAA CCG TCC CTC GCG AAA-3'. The samples were amplified in a thermal cycler for 40 cycles, consisting of 1 minute of denaturation at 95°C, 1 minute of annealing at 59°C, and 1 minute of extension at 72°C.

II.3.3 Monolayer wound repair assay.

We have established this method previously (8, 16). Briefly, 1HAEo$^-$ cells were grown in 6-well plates and then placed in the serum free medium (SFM) upon confluency. Circular wounds (~2.0 mm^2) were made in the confluent monolayer using a rubber stylet (4 wounds per well). In each experiment, one well was used as a negative control with no

treatment. Wounds were imaged 0, 8 and 24 hr after wound creation using a Nikon Eclipse TE200 inverted scope equipped with a Nikon Coolpix E995. Corresponding wound areas were determined using ImagePro Plus and the remaining wound areas calculated as a percentage of area at time 0.

II.3.4 Preparation of protein extracts and immunoblotting.

To determine protein expression of HB-EGF and IL-13 by bronchial epithelial cells after mechanical injury, confluent monolayers of 1HAEo$^-$ cells, were subjected to multiple linear injuries (7X7 linear scratches in each well) using a rubber stylet. Monolayers with no scratch wounds were used as the control. Protein cell lysates were collected at different time points after injury. In other experiments, confluent monolayers of 1HAEo$^-$ cells were treated with IL-13 (10 ng/ml) and protein lysates were collected.

II.3.5 Enzyme-linked immunosorbent assay (ELISA).

Confluent monolayers of 1HAEo$^-$ cells were mechanically injured as described previously. Injured monolayers were washed to remove cell debris and the medium was replaced with fresh SFM. Supernatants were collected at different time points after injury, centrifuged to remove cell debris, and frozen prior to analysis. HB-EGF, IL-13 and EGF levels were measured using a modified indirect ELISA method developed in our laboratory. Briefly, serial dilutions of human recombinant HB-EGF, EGF, and IL-13 and the supernatants were coated onto the 96-well Immulon 2HB plates (Thermo Labsystems) and incubated overnight at 4°C. After blocking with 1% BSA in PBS+0.05% Tween-20, monoclonal anti-human HB-EGF, EGF, and IL-13 (1 μg/ml)

(Catalog # AF-259-NA, MAB236, MAB2131 respectively, R&D Systems, Minneapolis, MN, USA) was added to each well and incubated overnight at 4°C. After washing with 0.05% Tween-20 in PBS the secondary HRP-conjugated anti-goat or anti-mouse were added and incubated for 60 min at 37°C. A color reaction was then developed with TMB for 10 min at room temperature. Following the addition of stop solution, absorbance was measured (450 nm test wavelength, 595 nm reference wavelength) on a microplate reader (Bio-Rad). Standard curves ranged from 500 to 0.05 ng/ml. Supernatants from each time point were assayed in duplicate.

II.3.6 Immunofluorescence staining of ALI.

ALI were fixed with 10% formalin and paraffin embedded. Immunofluorescence staining was performed using standard techniques and serial sections were stained with hematoxylin and eosin (H&E). ALI sections were incubated with anti IL-13 (10 μg/ml) antibody followed by Alexa 546-conjugated secondary antibody. Nuclei were counterstained with Hoechst 33342. All images were obtained using a Leica AOBS™ SP2 confocal microscope and analyzed by Velocity software.

II.3.7 Statistical Analysis.

Comparisons between multiple groups were made by ANOVA; when significant differences were found further comparisons were made by Student's *t*-test.

II.4 Results

II.4.1 Airway epithelial cells synthesize and release IL-13 in response to mechanical injury.

Given the persistent epithelial damage in asthma, we hypothesized that the elevated IL-13 may reflect as a part of the normal AEC response to injury. mRNA expression of IL-13 increased after mechanical injury in 1HAEo$^-$ cells (Fig. II.1A). In cell lysates, IL-13 expression increased rapidly after injury and remained elevated for at least 24 hr (Fig. II.1B). As shown in Fig. II.1C, IL-13 is rapidly released following injury, with quantifiable levels of the cytokine detected in conditioned media (CM) as early as 30 min after injury. Expression of IL-13 by ALI after mechanical injury was also examined (Fig. II.1D). In non-injured ALI, IL-13 expression was restricted to the apical surface of columnar cells (Panel a). Mechanical injury induced expression of IL-13 in both basal and columnar cells at the wound edge 1 min (panel b) and 30 min (panel c) after injury.

II.4.2 IL-13 mediates airway epithelial repair in an in vitro model of epithelial repair.

AEC release of IL-13 in response to injury generates the question what role this cytokine has in epithelial repair. To address this question we used a recombinant soluble form of IL-13Rα2 (shIL-13Rα2.FC, R&D Systems, Minneapolis, MN, USA) to neutralize the IL-13 released by injured AEC. This component has previously been shown to attenuate the effects of IL-13 in fibroblasts (17). Different concentrations of shIL-13Rα2.FC were added to the monolayers of 1HAEo$^-$ cells immediately after injury.

Figure II.2A shows that addition of 10 μg/ml of shIL-13Rα2 significantly reduced epithelial repair 24 hr after mechanical injury (* $p<0.05$). This data demonstrates that the endogenous release of IL-13 is important in epithelial repair. Next, we tested whether exogenous IL-13 can also enhance epithelial repair. Injured monolayers of 1HAEo$^-$ cells were treated with different concentrations of IL-13 (1-100 ng/ml). As shown in Fig. II.2B, addition of IL-13 at 10, 30, and 100 ng/ml significantly stimulated epithelial repair (‡ $p<0.01$). This range of IL-13 was similar to the levels of endogenous IL-13 released by AEC during repair of monolayer wounds.

II.4.3 Airway epithelial cells release soluble EGFR ligands in response to mechanical injury.

A rapid, damage-induced phosphorylation of the EGFR in epithelial cells has already been shown in many systems including airway epithelial cells (3). Activation of EGFR after mechanical injury in the absence of exogenous ligand suggests that activation is occurring through the release of endogenous mediators. To test whether bronchial epithelial cells produced soluble EGFR ligand(s) after mechanical injury, CM were collected from injured and non-injured monolayers of 1HAEo$^-$ cells. These CM were added to intact 1HAEo$^-$ monolayers. Phosphorylation of EGFR as induced by CM was assessed. Our data confirmed that mechanically injured 1HAEo$^-$ cells release mediator(s) that phosphorylate and activate EGFR (Fig. II.3A). Concurrent treatment of confluent monolayers with CM and a neutralizing anti-EGF antibody (0.1 μg/ml) decreased CM mediated EGFR phosphorylation by 50% (Fig. II.3B). Parallel confluent 1HAEo$^-$ monolayers were treated with CM and neutralizing antibodies for EGF (0.1 μg/ml) and

HB-EGF (3 μg/ml). As shown in Fig. II.3C, phosphorylation of EGFR was further decreased (8%, 30% and 64% by 30 min, 2 hr and 6 hr CM respectively) by the addition of anti-HB-EGF.

II.4.4 Airway epithelial cells release EGF and HB-EGF in response to epithelial injury.

We directly examined release of EGFR ligands by AEC in response to mechanical injury. Multiple linear wounds were made on confluent monolayers of 1HAEo$^-$ cells and CM was collected at indicated times after wounding. As shown in Fig. II.4A epithelial injury leads to a rapid release of EGF from 1HAEo$^-$ cells (* $p<0.05$). No further release or accumulation of EGF was detected in CM beyond 2 hr. We examined the level of HB-EGF protein expression and release in total cell lysates and CM collected from injured monolayers of AEC. Levels of HB-EGF increased gradually after injury, with a maximal expression observed at 8 hr in SFM or at 4 hr when cells grown in the presence of 10% Fetal calf serum (FCS) (Fig. II.4B). Injured monolayers of 1HAEo$^-$ cells in SFM conditions secrete HB-EGF into the supernatant as early as 30 min after injury with maximum secretion between 2-8 hr after injury (5-fold compared to injured monolayers at T0) (* $p<0.05$ and ‡ $p<0.01$) (Fig. II.4C).

II.4.5 Release of HB-EGF by injured epithelium is necessary for epithelial repair.

To examine whether release of HB-EGF by injured epithelium is necessary for repair, injured monolayers of 1HAEo$^-$ cells were treated with a neutralizing anti-HB-EGF antibody, the diphtheria toxin analog, CRM197, and the metalloproteinase inhibitor,

GM6001. As proHB-EGF is bound by diphtheria toxin, this analog inhibits only HB-EGF induced EGFR activation (18). Addition of the neutralizing anti-HB-EGF antibody significantly reduced epithelial repair compared to medium alone monolayers (* $p<0.05$) and the monolayers stimulated with HB-EGF (‡ $p<0.001$). Moreover, addition of anti-HB-EGF to HB-EGF, abrogates the effect of HB-EGF (§ $p<0.01$) (Fig. II.5A). Activation of HB-EGF is dependent upon protease cleavage of the proHB-EGF form (19). As shown in Fig. II.5B, addition of GM6001 significantly reduced the basal and HB-EGF stimulated epithelial repair (* $p<0.05$ and ‡ $p<0.001$, respectively), supporting the hypothesis that cleavage of proHB-EGF is necessary for epithelial repair. Addition of HB-EGF to GM6001-treated monolayers significantly improved the rate of epithelial repair, indicating that the inhibition of repair was not due to direct effect of the protease inhibitor on receptor function or some non-specific toxic effect of the inhibitor on the cells. Addition of CRM197 similarly inhibited repair of the injured 1HAEo$^-$ monolayers compared to medium alone and HB-EGF treated monolayers (* $p<0.05$ and ‡ $p<0.001$, respectively) (Fig. II.5C).

II.4.6 IL-13 induces the production of HB-EGF, but not EGF, by AEC.

Our previous data demonstrated an essential role for IL-13 and HB-EGF in epithelial repair. It has been shown that IL-13 can produce EGFR ligands and transactivate EGFR (11, 12). Next, we asked whether IL-13 has a role in release of EGFR ligands in AEC. To address this question, we investigated whether IL-13 could induce expression of HB-EGF in bronchial epithelial cells. Confluent monolayers of 1HAEo$^-$ cells were treated with 10 ng/ml of IL-13 and expression of HB-EGF and EGF were

measured at various time points. As shown in Fig. II.6A and B, expression and release of HB-EGF significantly increased following IL-13 exposure (* $p<0.05$). However, IL-13 showed no effect on EGF release (Fig. II.6B).

II.4.7 IL-13 enhances EGFR phosphorylation and stimulates epithelial repair via HB-EGF.

Our previous data showed that IL-13 increases HB-EGF production and release (Fig. II.6A and B). HB-EGF is a known ligand for EGFR, therefore, IL-13 should be able to increase EGFR phosphorylation. Confluent monolayers of 1HAEo$^-$ cells were treated with 10 ng/ml of IL-13 and phosphorylation of EGFR was detected using anti-pEGFR (pY^{845}). IL-13 stimulated EGFR phosphorylation of 1HAEo$^-$ cells 1 hr after exposure. IL-13-induced EGFR phosphorylation was prevented when the cells were treated with both IL-13 and the anti-HB-EGF antibody (Fig. II.7A). To test whether the stimulatory effect of IL-13 on epithelial repair is mediated via EGFR and its ligand, HB-EGF, injured monolayers of 1HAEo$^-$ were treated with IL-13 with or without AG1478, anti-HB-EGF, and GM6001. As Fig. II.7B shows IL-13 significantly stimulated epithelial repair (* $p<0.05$) and addition of AG1478, anti-HB-EGF neutralizing antibody, or GM6001 to IL-13 treated monolayers suppressed this effect (‡ $p<0.01$). Prevention of EGFR phosphorylation and HB-EGF activity inhibited the IL-13 effects on wound repair. Altogether these data show that IL-13-stimulated EGFR phosphorylation and epithelial repair is mediated through HB-EGF.

II.4.8 Inhibition of EGFR tyrosine kinase activity enhances IL-13 production from AEC.

Our previous experiments showed that IL-13 is a mediator of normal epithelial repair (Fig. II.1, II.2). Over expression of IL-13, as seen in asthma, may result from persistent secretion in an effort to affect the incomplete epithelial repair. This incomplete repair may result from the lack of EGFR function. To test this hypothesis we examined IL-13 release from AEC when EGFR tyrosine kinase activity was inhibited by AG1478. Multiple linear wounds were created on confluent monolayers of AEC. Wounded monolayers were treated with tyrphostin AG1478 (1 μM) or kept in SFM. Corresponding wound areas were determined and CM were collected at indicated times after wounding. Percent of epithelial repair 24 hr after injury was significantly lower (15.4±1.5) in monolayers treated with AG1478 compared to non-treated monolayers (23.3±2.5) ($p<0.05$). As shown in Fig. II.8, AEC release significantly more IL-13 in the presence of AG1478 (* $p<0.05$). This effect of tyrphostin AG1478 on IL-13 release in response to mechanical injury could not be attributed to any nonspecific effect, as AG1478 when added to confluent monolayers without injury had no effect on IL-13 production.

III.5. Discussion

Airway remodeling, which includes goblet cell hyperplasia, mucus hypersecretion, subepithelial fibrosis, and epithelial damage, is a characteristic feature of chronic asthma. To the extent that the epithelial abnormality is the result of inappropriate or incomplete repair remains to be described. IL-13 is known as a Th2 cytokine produced by T helper type 2 cells and other cells recruited to the lung during allergic responses which has been described to play a key role in many aspects of airway remodeling (20-22). In the present study we hypothesized that IL-13 is a part of the normal response to injury. Overproduction of IL-13 in response to inadequate epithelial repair could act on both epithelium and sub-epithelial elements leading to airway remodeling. We found that mechanical epithelial injury causes increased production and release of IL-13 by both AEC monolayers and cells grown in ALI. We also showed that this increased production of IL-13 augments epithelial repair that is mediated by EGFR activation. A previous study by Pourazar and colleagues showed an over expression of IL-13 by bronchial epithelial cells in response to exposure to diesel exhaust using an *in vivo* model (2). However, our investigation, to our knowledge, is the first study to show that IL-13 is a repair mediator released by airway epithelial cells in response to injury. We also demonstrated a mechanism by which IL-13 facilitates the repair, HB-EGF secretion.

Epithelial repair consists of a complex cascade of events which starts immediately after injury and leads to effective and normal repopulation of the epithelium. Persistent epithelial damage might be as a result of incomplete repair where any component of this cascade does not work properly. Autocrine activation of EGFR, at least in part, plays an essential role in mediating the key events during epithelial wound healing (23-25).

Puddicombe *et al.* showed a rapid, damage-induced phosphorylation of the EGFR in AEC grown in monolayer, irrespective of the presence of exogenous ligand (3). Activation of EGFR after mechanical injury in the absence of exogenous ligand suggests that activation is occurring through the release of endogenous mediators. Bronchial epithelial cells produce several ligands for EGFR, including EGF, TGF-α, HB-EGF and amphiregulin (26). An induction in the expression and release of EGFR ligands by AEC in response to different stimuli such as cigarette smoke extract (4), compressive stress (1), and oxidative stress has been demonstrated (6).

In the present study we found that bronchial epithelial cells release EGF and HB-EGF in response to mechanical injury. Our data also showed an early release of EGF and a late release of HB-EGF by AEC after mechanical injury. We further examined the role of endogenous HB-EGF in epithelial repair. Addition of a neutralizing antibody for HB-EGF as well as CRM197 significantly reduced the rate of epithelial repair. Members of the EGF family are synthesized as membrane-anchored forms and are then processed by proteolytic cleavage to give bioactive soluble forms (19). Addition of GM6001, a broad-spectrum metalloproteinase inhibitor, attenuated epithelial wound closure in our model even when no exogenous HB-EGF was added. These data showed that proteolytic release of HB-EGF is essential for complete airway epithelial repair.

Unlike the majority of the studies which have focused on the role of a single mediator in epithelial repair, we studied the role of IL-13 and two EGFR ligands. Our data demonstrated a rapid increase in EGF and IL-13 secretion by AEC after injury followed by a later response by HB-EGF release. Up-regulation of multiple EGFR ligands has been observed in a few experimental models of epithelial stress. Chu *et al.*

showed a sustained up-regulation of the EGFR ligands HB-EGF, epiregulin, and amphiregulin, but not TGF-α after compressive stress of bronchial epithelial cells. Similarly, in healing skin wounds both HB-EGF and amphiregulin mRNA, have been shown to be up-regulated (27). Given the diverse binding specificities and signaling networks associated with EGFR ligands, the sequential expression of multiple ligands may serve to diversify the autocrine and paracrine responses to mechanical perturbation.

Many effects of IL-13 on airway epithelium are mediated through transactivation of EGFR (11, 12). A recent study has demonstrated that IL-13 and EGFR are complementary pathways in chronic goblet cell metaplasia (28). IL-13 has also been shown to induce proliferation of bronchial epithelial cells through production of TGF-α and activation of EGFR (13). Our data showed that IL-13 enhances epithelial repair and induces EGFR phosphorylation. We also demonstrated that IL-13 increases the expression and release of HB-EGF, but not EGF. Finally, we demonstrated that both IL-13-induced EGFR phosphorylation and epithelial repair are mediated through HB-EGF.

Impaired epithelial repair may contribute to airway remodeling as a result of prolonged presence and/or over-production of inflammatory mediators and growth factors. In the present study we examined whether inhibition of EGFR-mediated epithelial repair has any effect on IL-13 production by AEC. We showed that AEC markedly increase the amount of IL-13 secreted when EGFR activity is inhibited and epithelial repair is prevented. In the response to injury, normal expression of IL-13 contributes to epithelial repair. However, excessive or prolonged release of this cytokine in an attempt to affect repair would have additional adverse effects on both epithelial and sub-epithelial cells and structures. Puddicombe *et al.* found that disruption of EGFR-

mediated epithelial repair is paralleled by enhanced release of TGF-β2 by bronchial epithelial cells (3).

One limitation of our study was that we used monolayers of 1HAEo$^-$ cells in our injury-repair model. These cells show characteristics of non-differentiated basal human airway epithelial cells. However, we have confirmed production of IL-13 by ALIs, which mimic many aspects of fully differentiated airway epithelium *in vivo*. Another concern is that the present study is not a complete investigation of the EGFR activation following epithelial injury by multiple ligands but demonstrates the sequential involvement of at least two ligands for normal repair.

In conclusion, we investigated a basic mechanism of epithelial repair in an *in vitro* model of airway epithelial injury and repair. We utilized both AEC monolayer and well-differentiated epithelial cells (ALI) which are similar in structure and function to human airway epithelium *in vivo*. We found that AEC release in a sequential manner, EGF, IL-13 and, HB-EGF in response to mechanical injury. We found that IL-13 is a mediator of normal epithelial repair which acts via the EGFR pathway. We also demonstrated an essential role for HB-EGF in epithelial repair. The results of this study highlight an important interaction between EGFR and IL-13 in the airway epithelium. Defective repair, not solely the presence of elevated cytokines, may be a major contributor to the chronic airway changes.

Fig. II .1

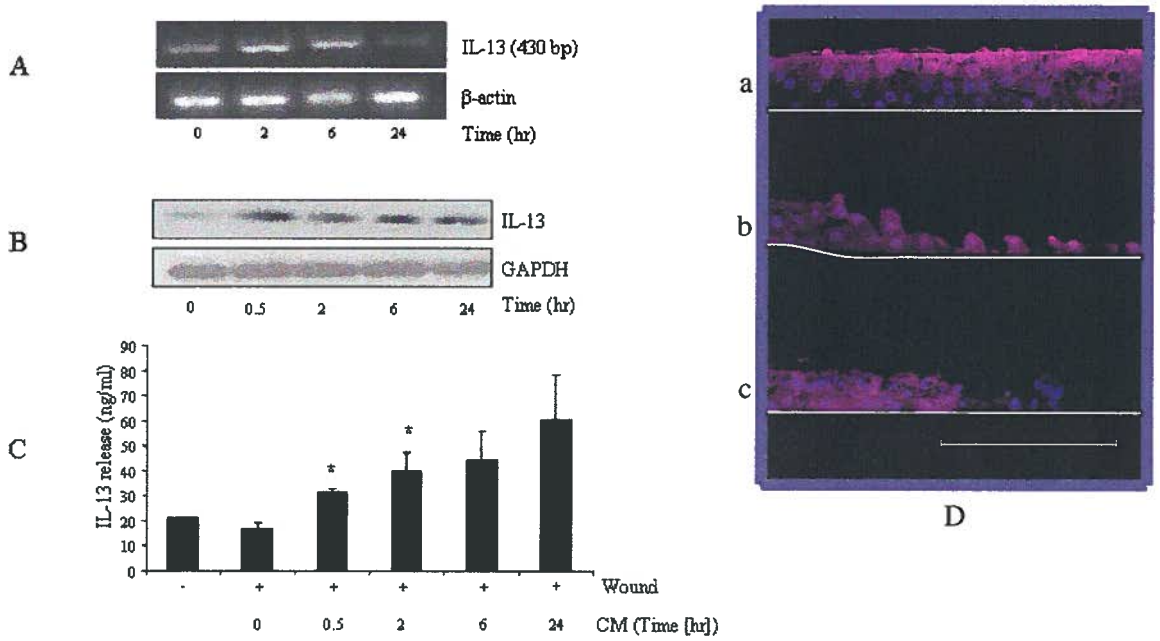


Figure II.1. Airway epithelial cells synthesize and release IL-13 in response to mechanical injury. Multiple linear wounds were made in confluent monolayers of 1HAEo$^-$ cells. Total RNA, protein lysates, and CM were collected at indicated times. mRNA expression (A), protein synthesis (B) and release (C) of IL-13 after mechanical injury were examined by RT-PCR, western blotting, and ELISA. Two linear wounds are created on ALI and followed for 1 min and 30 min and then fixed with 10% formalin. Sectioned ALI were stained for IL-13 using standard protocols. Representative images are noted in Panel D with (a) confluent ALI, (b) ALI 1 min and (c) ALI 30 min after injury. Scale bar = 40 μm.

Fig. II.2

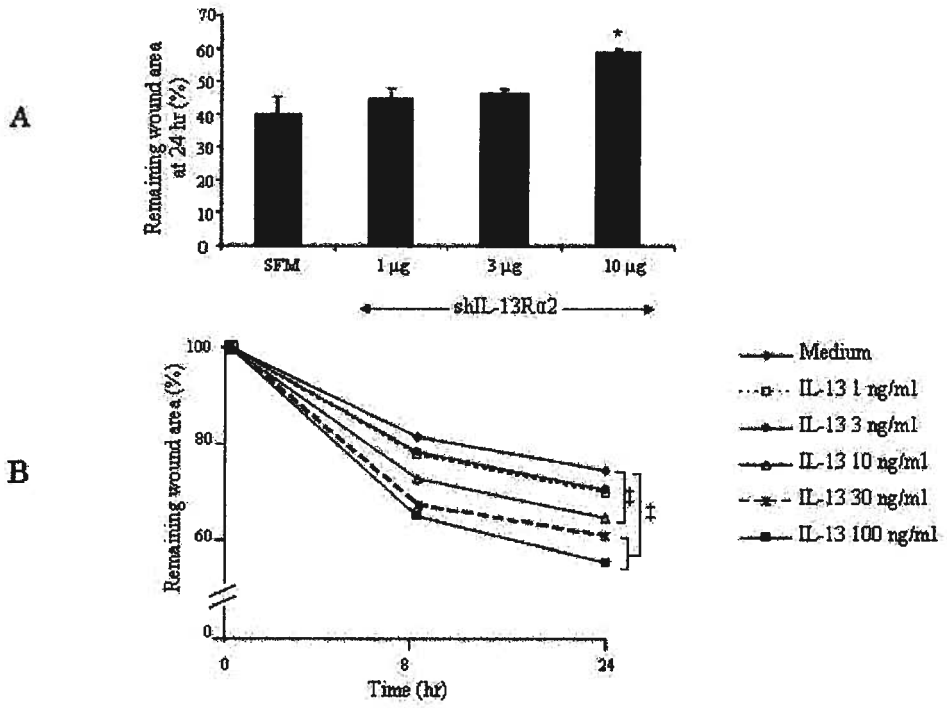


Figure II.2. IL-13 mediates airway epithelial repair. Injured monolayers of 1HAEo$^-$ cells were treated with different concentration of shIL-13Rα2.FC (1-10 μg/ml) (A) or IL-13 (1-100 ng/ml) (B) immediately after injury or kept in serum-free medium and corresponding wound areas were determined. Data are mean ± SEM for 8 wounds in each group.

Fig. II.3

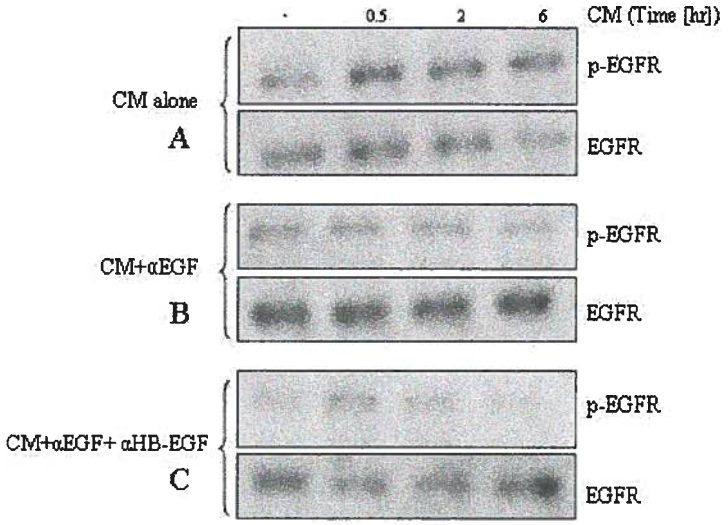
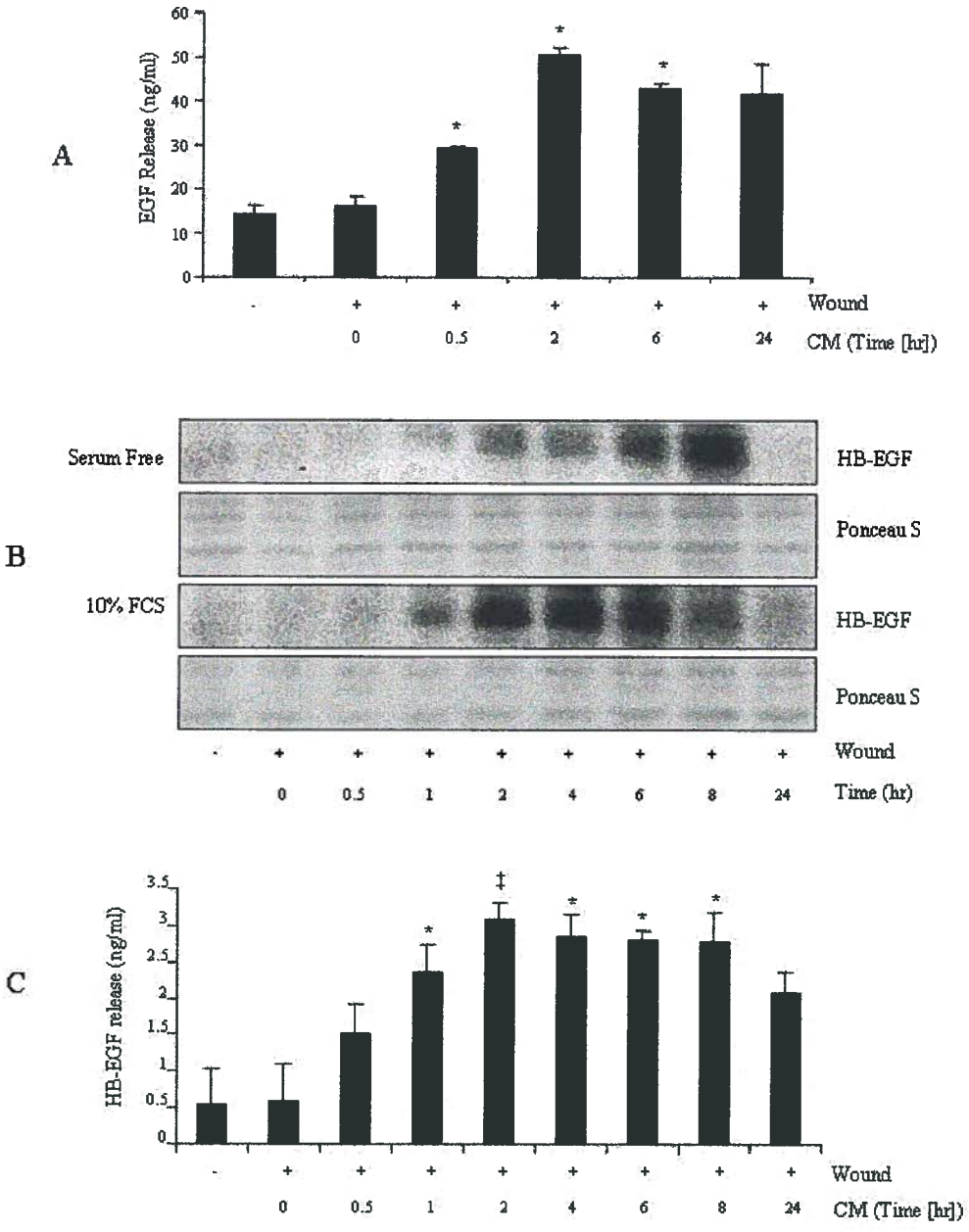


Figure II.3. Airway epithelial cells release soluble EGFR ligands in response to epithelial injury. Conditioned medium (CM) were collected from injured 1HAEo$^-$ monolayers at different time points. Confluent monolayers of 1HAEo$^-$ cells were treated with CM in the presence or absence of anti-EGF (0.1 μg/ml) or in combination with anti-HB-EGF (3 μg/ml). Protein lysates were collected from the treated monolayers after 1 hr of CM exposure and phosphorylation of EGFR was determined by western blot.

Fig. II.4


A
EGF Release (ng/ml)
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Wound
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Wound
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2
1.5
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0.5
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*
- + + + + + + + +
Wound
0 0.5 1 2 4 6 8 24
CM (Time [hr])

Figure II.4. Airway epithelial cells release EGF and HB-EGF in response to mechanical injury. Total protein lysates and CM were collected from injured monolayers of 1HAEo$^-$ cells at indicated times. Release of EGF into CM was measured by ELISA (A). Synthesis (B) and release (C) of HB-EGF after mechanical injury were examined by western blotting and ELISA. All membranes were stained with Ponceau S to confirm loading. Synthesis of HB-EGF after injury was studied in both SFM and in the presence of 10% FCS (B) while release was examined only in serum free conditions (C).

Fig. II.5

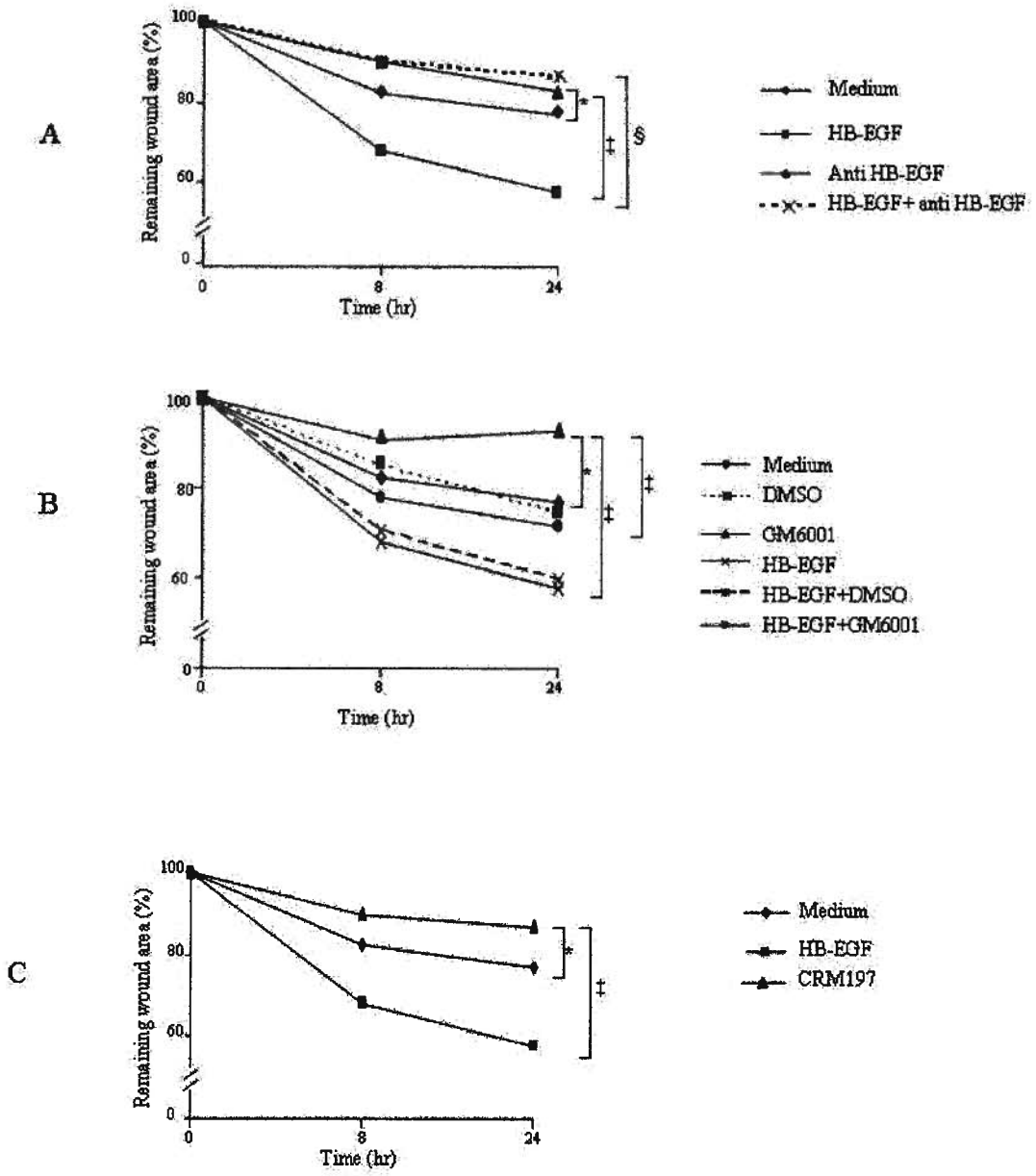

Figure II.5. Release of HB-EGF by injured epithelium is necessary for epithelial repair. After mechanical injury monolayers of 1HAEo$^-$ cells were exposed to a neutralizing anti-HB-EGF antibody (3 μg/ml) or GM6001 (50 μM) in the absence or presence of HB-EGF (20 ng/ml) (A and B, respectively), or 10 μg/ml of CRM197 (C). Corresponding wound areas were determined 0, 8, and 24 hr after wound creation using time-lapse videomicroscopy. Data are mean ± SEM for 8 wounds in each group. Error bars are omitted for clarity.

Fig. II.6

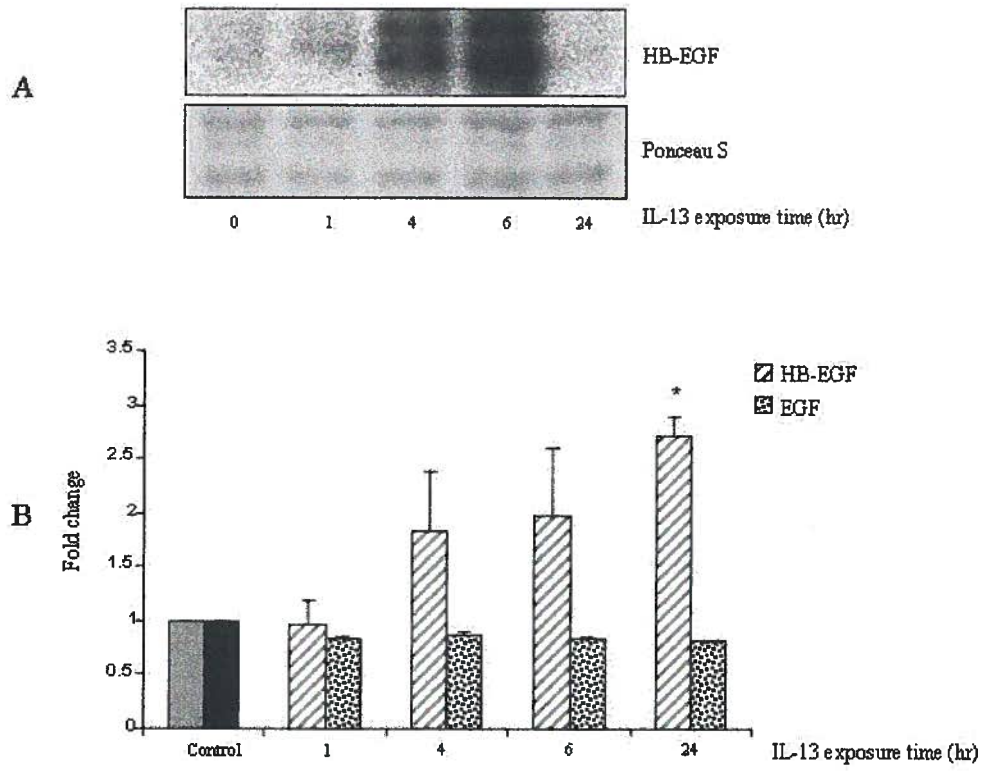

Figure II.6. IL-13 enhances production and release of HB-EGF in a culture model of airway epithelium. Confluent monolayers of 1HAEo$^-$ cells were treated with IL-13 (10 ng/ml) and total protein lysates and CM were collected. Synthesis (A) and release (B) of HB-EGF and release of EGF were examined by western blotting and ELISA. In each experiment monolayers with no treatment was considered as control. Data in panel B is expressed as fold-increase relative to the control monolayers with no treatment (gray and black bars). IL-13 induced synthesis (A) and release (B) of HB-EGF from 1HAEo$^-$ cells in a time-dependent manner. IL-13 had no effect on release of EGF by 1HAEo$^-$ cells (B).

Fig. II.7

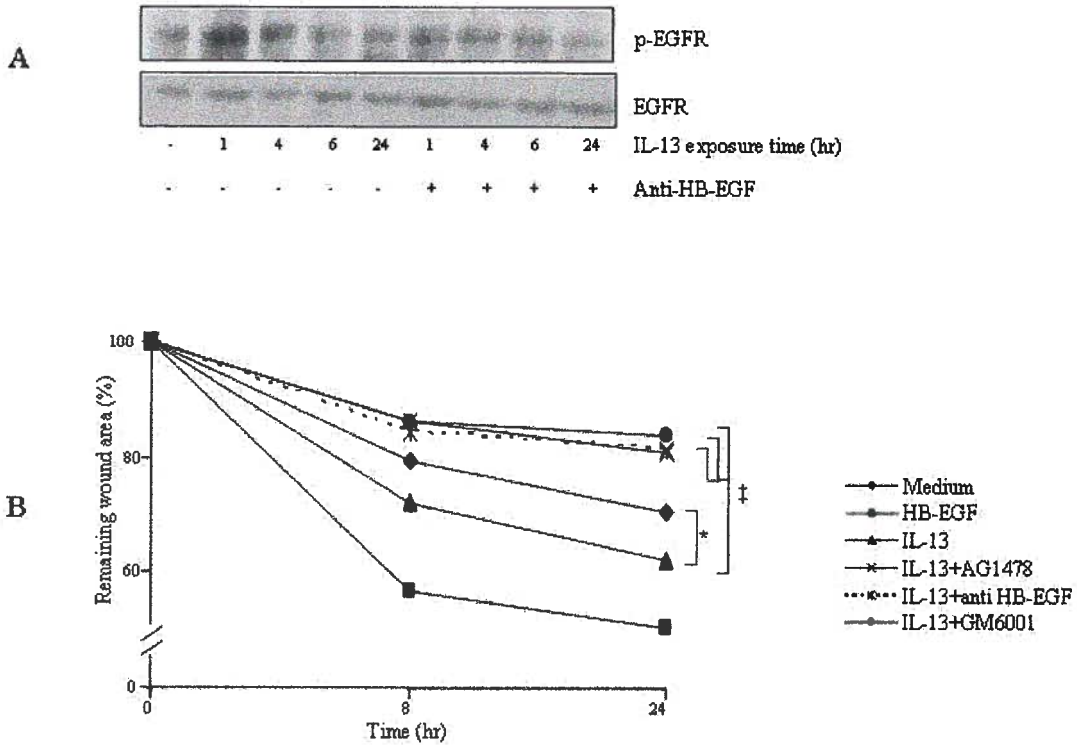

Figure II.7. IL-13 induces EGFR phosphorylation and enhances airway epithelial repair via HB-EGF. Confluent monolayers of 1HAEo$^-$ cells were treated with IL-13 (10 ng/ml) with or without concurrent treatment of anti-HB-EGF (3 μg/ml). Total protein lysates were collected and phosphorylation of EGFR was examined by western blotting (A). Injured monolayers of 1HAEo$^-$ cells were treated with IL-13 (10 ng/ml) with and without AG1478 (1 μM), anti-HB-EGF (3 μg/ml), and GM6001 (50 μM). Corresponding wound areas were determined at 0, 8, and 24 hr after injury. Data are mean ± SEM for 8 wounds in each group.

Fig. II.8

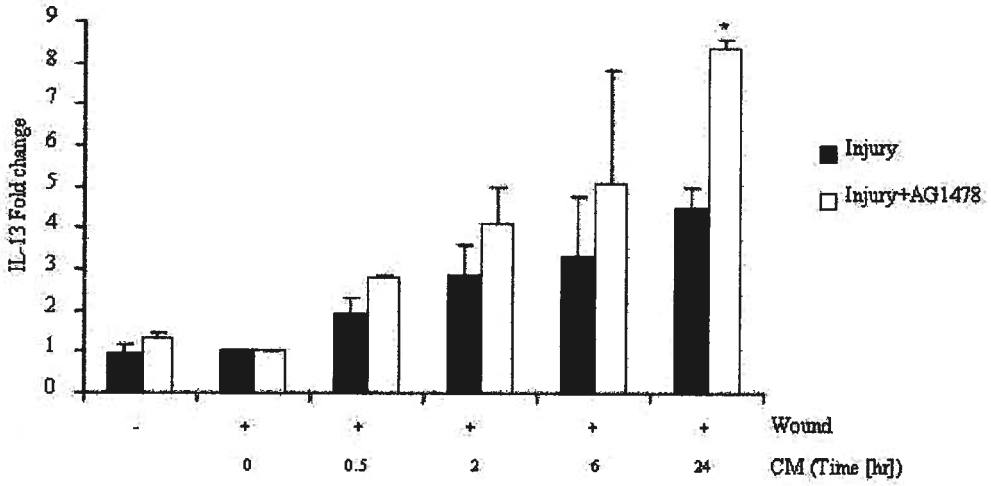

Figure II.8. Disruption of EGFR Tyrosine kinase activity enhanced IL-13 release from AEC. Wounded monolayers of 1HAEo$^-$ cells were treated with tyrphostin AG1478 (1 μM) or kept in SFM. Conditioned media (CM) were collected at indicated times and release of IL-13 was evaluated by ELISA. A significant increase in IL-13 release is induced in wounded monolayers when EGFR phosphorylation is inhibited by tyrphostin AG1478 (* $p<0.05$).

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Data presented in this section are an extension to chapter III. These are on going experiments and have not been published as a separate unit yet.

II.7 IL-13 SIGNALING THROUGH IL-13 RECEPTOR α2 MEDIATES AIRWAY EPITHELIAL WOUND REPAIR

IL-13 is a Th2 like cytokine which has been considered as a central mediator of airway remodeling in asthma. Our laboratory has recently demonstrated that IL-13 promotes airway epithelial repair. We also showed that this effect is mediated through the autocrine release of HB-EGF and activation of EGFR (1). The effects of IL-13 are mediated by a complex receptor system that includes IL-4 receptor α (IL-4Rα), IL-13 receptor α1 (IL-13Rα1) and IL-13 receptor α2 (IL-13Rα2). While IL-13Rα2 is thought to act only as a decoy receptor (2, 3), recent investigations have demonstrated that IL-13Rα2 acts as a signaling receptor as well (4). In the current investigation we studied the role of IL-13Rα1 and α2 in airway epithelial wound repair.

Confluent monolayers of human airway epithelial ($1HAE^{o-}$) cells were wounded with a sterile rubber tip and permitted to heal in the presence or absence of antibodies specific for neutralizing IL-13Rα1 or Rα2. In another set of experiments 80% confluent monolayers of $1HAE^{o-}$ cells were transfected with IL-13Rα1 or IL-13Rα2 siRNA (5 nM) for 48 hr before they were mechanically injured. In both set of experiments protein extracts and conditioned medium (CM) were collected at 0, 6 and 24 hr after injury. Wounded monolayers were also photographed using a Nikon light timelapse videomicroscope for wound closure kinetics.

Expression of IL-13Rα1 remained constant during the epithelial repair, however, expression of IL-13Rα2 increased after injury (Fig II.E.1). Epithelial injury leads to an

increase in the expression and release of HB-EGF from 1HAE^{o-} cells (1). Neutralization of IL-13Rα2 inhibited HB-EGF synthesis and release from injured monolayers, decreased EGFR phosphorylation and, prevented epithelial repair (Fig II.E.3). Inhibition of IL-13Rα1 altered neither HB-EGF and p-EGFR expressions nor epithelial repair. However, HB-EGF release from injured monolayers was significantly increased in this group (Fig II.E.2). IL-13Rα1 inhibition provides more IL-13 accessible to IL-13Rα2 signaling pathway which may result in excessive release of HB-EGF in this group.

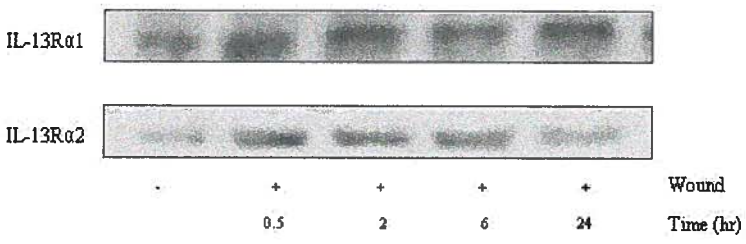

Figure II.E.1. Expression of IL-13Rα1 and Rα2 following injury. Total protein lysates were collected from injured monolayers of 1HAEo$^-$ cells at indicated times. Expression of IL-13Rα1 and IL-13Rα2 was examined by western blotting.

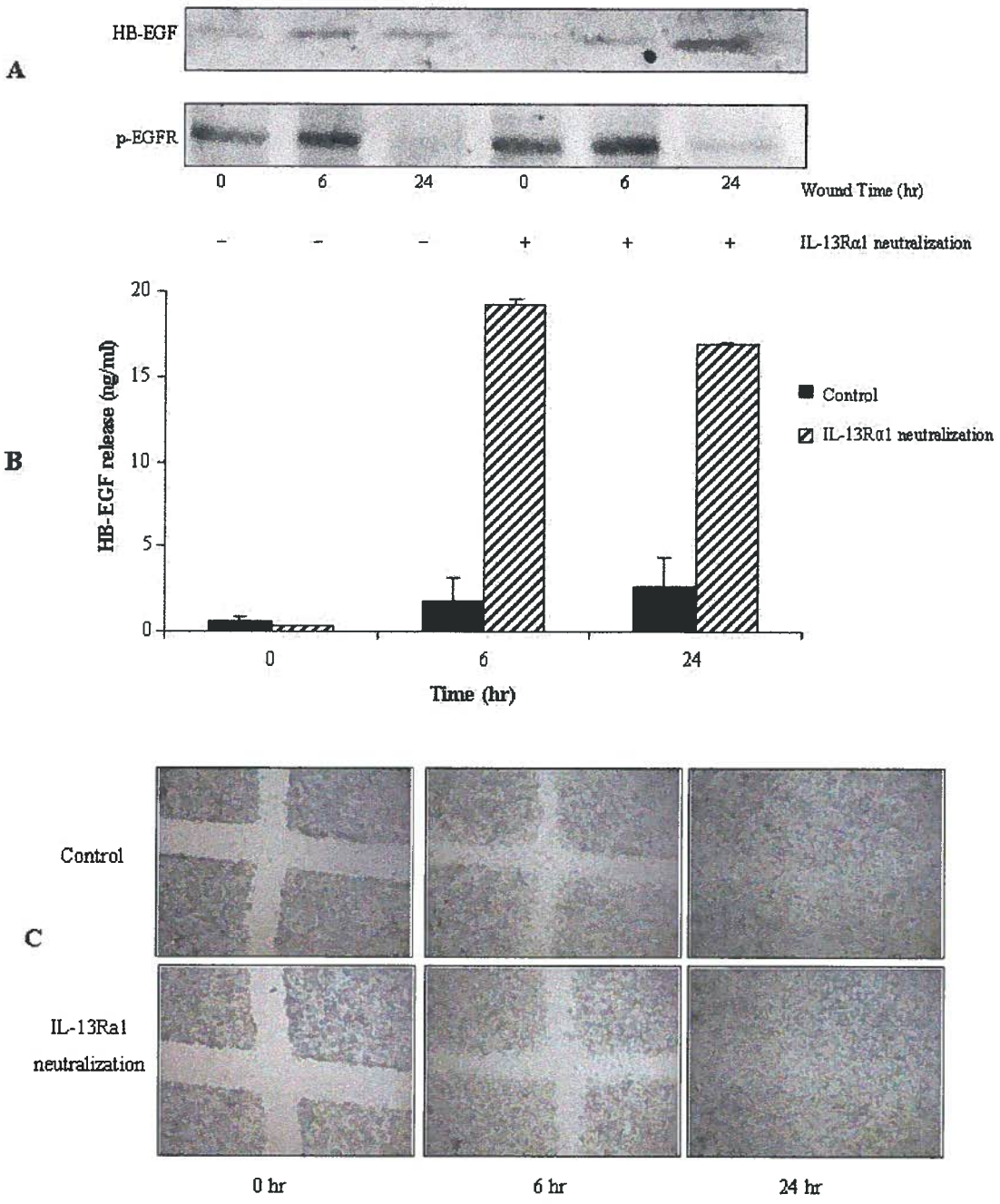

Figure II.E.2. IL-13Rα1 neutralization after mechanical injury does not change HB-EGF and p-EGFR expression and has no effect on epithelial repair. Total protein lysates and conditioned medium were collected from injured monolayers of 1HAEo$^-$ cells at indicated times. Expression of HB-EGF and p-EGFR was examined by western blotting (A) and release of HB-EGF was measured by ELISA (B). A cross-hatched wound was created on confluent monolayer of 1HAEo$^-$ cells. Monolayers were then treated with a neutralizing anti IL-13Rα1 antibody (20 μg/ml) or maintained in medium (control) (C).

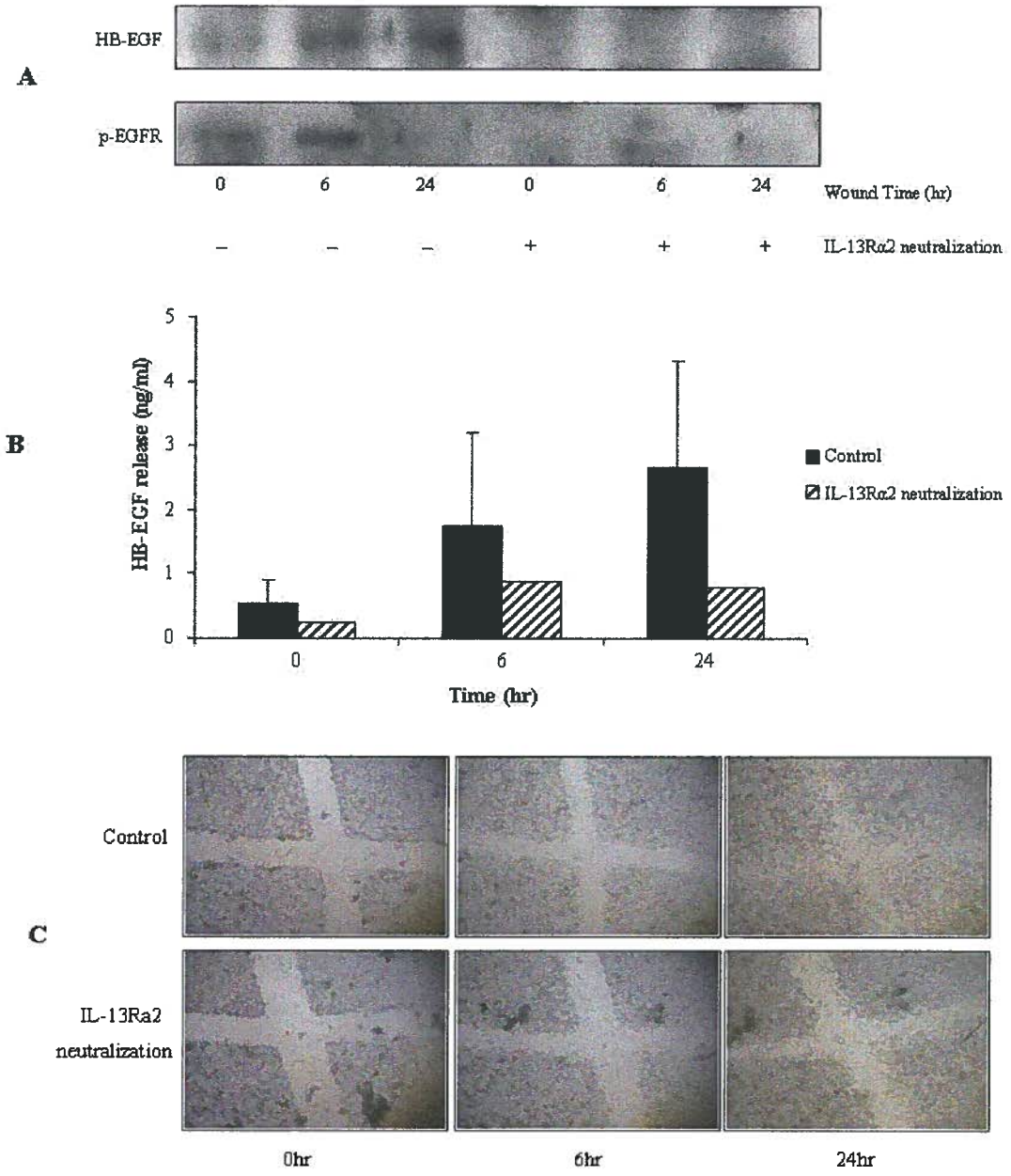

Figure II.E.3. IL-13Rα2 neutralization reduces HB-EGF and p-EGFR expression after mechanical injury and inhibits epithelial repair. Total protein lysates and CM were collected from injured monolayers of 1HAEo$^-$ cells at indicated times. Expression of HB-EGF and p-EGFR was examined by western blotting (A) and release of HB-EGF was measured by ELISA (B). A cross-hatched wound was created on confluent monolayer of 1HAEo$^-$ cells. Monolayers were then treated with a neutralizing anti IL-13Rα2 antibody (2 μg/ml) or maintained in medium (control) (C).

To confirm the role of IL-13Rα1 and Rα2 in HB-EGF synthesis in AEC, we knocked down the expression of IL-13Rα1 and Rα2 in 1HAEo$^-$ cells using siRNA. Fig. II.E.4 shows the protein expression of IL-13Rα1 and Rα2 with 5 nM of their specific siRNA.

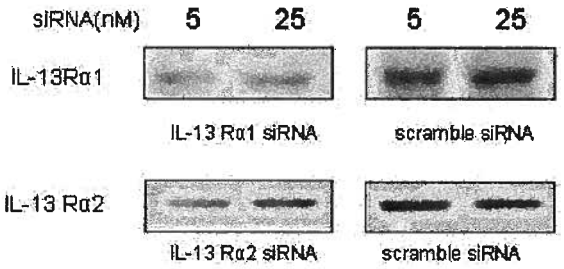

Figure II.E.4. IL-13Rα1 and Rα2 targeted siRNAs knock down the expression of IL-13Rα1 and IL-13Rα2. 1HAEo$^-$ cells grown to 80-90% confluency in 6-well culture plates and then transfected with 5 and 25 nM of IL-13Rα1, IL-13Rα2, or scramble siRNA in the presence of 24μl HiperFect. Total protein lysates were collected after 48 hr and expression of IL-13Rα1 and IL-13Rα2 were examined by western blotting.

As shown in Fig. II.E.5 non-transfected 1HAEo$^-$ cells showed an increase in HB-EGF synthesis 6 hr after injury. IL-13Rα1 knocked-down-cells express the same amount of HB-EGF after injury; however, the expression of HB-EGF was significantly reduced in IL-13Rα2 knocked-down-cells.

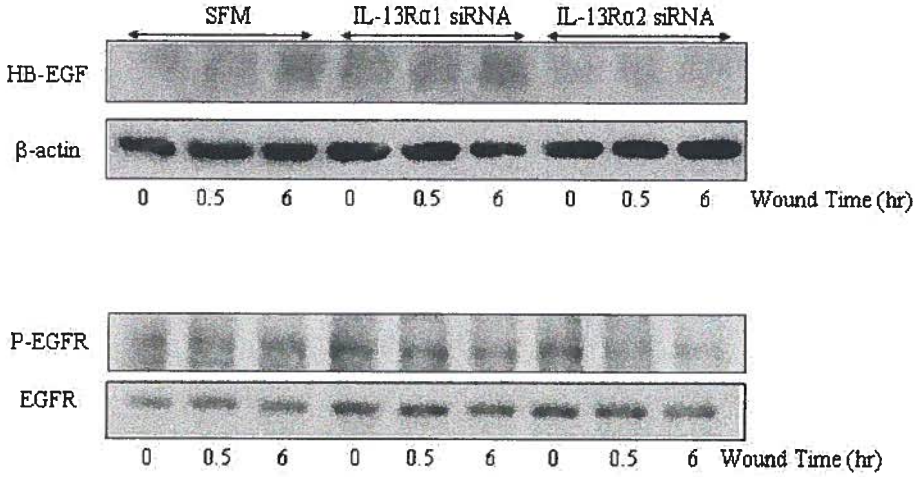

Figure II.E.5. HB-EGF expression and EGFR phosphorylation after mechanical injury are reduced in AEC when IL-13Rα2 is knocked down. Confluent monolayers of 1HAEo$^-$ cells were transfected with IL-13Rα1 and IL-13Rα2 siRNAs as described above. After 48 hr of transfection the medium was replaced by SFM and multiple linear injuries were created on monolayers using a rubber stylet. Monolayers with no scratch wounds were used as the control. Protein cell lysates were collected at different time points after injury. Expression of HB-EGF, p-EGFR, EGFR and β-actin were determined by western blotting.

Altogether our data suggest that IL-13Rα2 acts as a signaling receptor and is involved in airway epithelial repair. Further investigations are required to elucidate the cross-talk between IL-13Rα1 and IL-13Rα2 and also between IL-13 Rα2 and other signaling pathways.

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CHAPTER III. AIRWAY EPITHELIAL WOUND REPAIR: ROLE OF CARBOHYDRATE SIALYL LEWIS X (sLe^x)*

III.1 Summary

Epithelial repair is a complex cellular and molecular process, the details of which are still not clearly understood. Plasma membrane glycoconjugates can modulate cell function by altering the function of protein and lipids. Sialyl-Lewis x (sLe^x), a fucose containing tetrasaccharide, decorates membrane bound and secreted proteins and mediates cell-cell interaction. In the present study we investigated the role of sLe^x in airway epithelial repair. Using immunohistochemistry we showed an increased expression of sLe^x in areas of damaged bronchial epithelium when compared to intact regions. Confluent monolayers of airway epithelial cells were mechanically wounded and allowed to close. Wounded monolayers were photographed for wound closure kinetics, fixed for immunocytochemical studies, or subjected to RNA extraction. Examining the expression of different α1,3-fucosyltransferases (FucT), enzymes which mediate the final step in the synthesis of sLe^x, we found that FucT-IV was the common gene expressed in all cell lines and primary airway epithelial cells. We demonstrated an increased expression of sLe^x over time after mechanical injury. Blocking of sLe^x with an inhibitory antibody completely prevented epithelial repair. Our data suggest an essential functional role for sLe^x in epithelial repair. Further studies are necessary to explore the exact mechanism for sLe^x in mediating cell-cell interaction in bronchial epithelial cells to facilitate epithelial migration and repair.

* This work is published, as mentioned in LIST OF PUBLICATIONS, PRESENTATIONS, AND AWARDS and cited as Allahverdian et al. Airway epithelial wound repair: role of carbohydrate sialyl Lewis x. Am J Physiol Lung Cell Mol Physiol. 2006 Oct;291(4):L828-36.

III.2 Introduction

As the barrier to the external environment, the bronchial epithelium is continuously exposed to gaseous and particulate components of inhaled air and therefore is frequently injured. Inflammation is an initial response to tissue injury, which provides immune cells dedicated to debris removal and growth factors to promote tissue repair (1). In addition to this inflammatory response, wound healing involves migration and spreading of epithelial cells into the damaged region and proliferation of new epithelial cells (2, 3). The complete healing of wounds represents an important process by which the respiratory epithelial barrier integrity is maintained.

Several proteins essential for normal cell physiology, like membrane bound receptors for growth factors and cytokines, are glycosylated. Several lines of evidence support the theory that oligosaccharide moieties are crucial for the function of some of those proteins and that variation in their glycosylation pattern often leads to changes in their function (4-6). Oligosaccharides on cell surface proteins and lipids have functional roles in cell adhesion (7), migration (8), proliferation (9) and growth potential (10). The molecular events that initiate, mediate, and regulate different processes involved in epithelial repair have not been fully elucidated, but a number of studies have suggested that glycoconjugates attached to proteins within the plasma membrane of epithelial cells play a central role in these events. Our laboratory has determined the pattern of cell surface glycosylation in normal human airway epithelial cells (11). We have shown that glycosylation profiles in airway epithelium change overtime during repair of a wound created by mechanical injury (12). Our data also suggested that cell surface N-glycosylation has a functional role in airway epithelial cell adhesion and migration and

that N-glycosylation with terminal fucosylation plays an essential role in the complex process of repair by coordination of certain cell-cell functions (13). In asthma, detailed cellular and ultrastructural examination of bronchial biopsies and bronchoalveolar lavage fluid have provided evidence for epithelial damage, even in mild cases (14-17). This excessive epithelial damage can arise from an enhanced susceptibility to injury or an inadequate repair response, or a combination of both (1). Kauffmann *et al.* (18) has reported an under-representation of carbohydrate structures with terminal fucose in asthmatic patients with a correlation between this deficiency and the severity of the disease. These data suggest that defects in epithelial repair in asthma patients may be due, in part, to improper glycosylation of airway epithelial cells. Taken together, these studies indicate an involvement of cell surface carbohydrates especially those with terminal fucose in regulation of epithelial repair processes.

Lewis blood group antigens are biosynthetically and structurally related carbohydrate structures used as markers of cell differentiation and embryonic development (19). Expression of these antigens is not limited to erythrocytes and they can be found in different tissues and organs. It has been shown that these oligosaccharide structures are involved in cell-cell interaction. Sialyl Lewis x, (sLe^x), a fucose containing tetrasaccharide [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc] belonging to this family, has been recognized as a ligand for E-selectin and therefore has an important role in lymphocyte trafficking (7, 10, 20). This antigen has been detected in various tumors where it mediates binding of cancerous cells to endothelial selectins and thereby promoting tumor metastasis (21, 22). sLe^x is also found at the non-reducing termini of N-linked or O-linked oligosaccharides on glycoproteins as well as on glycosphingolipids.

Final step in synthesis of sLe^x is catalyzed by specific α1,3-fucosyltransferases (FucT). Six human α1,3-FucTs have been cloned and partially characterized: FucT-III, FucT-IV, FucT-V, FucT-VI, FucT-VII, and FucT-IX which show different pattern of expression among tissues.

Neither the normal processes leading to complete epithelial repair nor the abnormalities that permit chronic damage in disease states of the epithelium are fully understood. While previous studies in epithelial repair suggested a role for glycoconjugates, none of these studies specified an oligosaccharide structure(s) to be involved in repair. Our present study, to our knowledge, for the first time demonstrates a critical role for the tetrasaccharide sLe^x in airway epithelial repair. We found an over expression of sLe^x, *in vivo* and *in vitro* after injury. In a culture model of epithelial repair, we were able to demonstrate an inhibition of repair after blocking of sLe^x. These observations have important implications not only for understanding the epithelial injury-repair cycle but also for identifying novel therapies for conditions resulting from impaired epithelial repair, such as asthma.

III.3 Materials and Methods

III.3.1 Collection of airway specimens from normal human subjects.

Approval for the use of all human tissue was granted by University of British Columbia and Providence Health Care Ethics Review Board. Normal bronchial segments were collected from pathological specimens from adults undergoing lung resection in a regional chest hospital in Großhansdorf, Germany. Samples generally were of fourth, fifth or sixth generation central airways in transverse section, so that a complete circumference could be examined. Bronchial specimens were fixed in 4% paraformaldehyde (PFA) for 1 hr at room temperature. Samples were then shipped on ice to our laboratory, washed in Dulbecco's Phosphate-Buffered Saline (DPBS) and stored at 4°C. After paraffin-embedding airways were retrieved and tissue sections were prepared in transverse orientation from the paraffin blocks for preparation of 5 μm thick sections. No subject had a history of asthma.

III.3.2 Immunohistochemistry.

Immunostaining was performed using a mouse monoclonal anti-human sLe^x antibody (KM 93, Seikagaku America, Ijamsville, MD, USA) or an isotype matched non-specific antibody.

III.3.3 Quantification.

To study sLe^x immunoreactivity, the entire epithelium of one airway section was systematically assessed in each subject. Several sections were obtained from the same airway and more than one airway per donor sample was assessed. Several images were

taken from the entire airway section (usually 10-15 images based on the size of airway) in each subject. In this manner the entire circumference of the airway was documented in images. If a wound was detected in one of these images this section then became the representative airway section for that donor. Next, from the pool of images for the representative section, 3 numbers were randomly selected and if they contained damaged area they would be further assessed for sLe^x expression in the areas of damaged and intact epithelium. In this manner selection bias was minimized. Epithelial damage was characterized morphologically by the absence of differentiated ciliated and secretory cells (23). To study the immunoreactivity of sLe^x in areas of epithelial damage and normal epithelium, percentage of positively stained basal and columnar cells in three areas exhibiting epithelial damage and three areas of intact epithelium was determined in each subject. A total of 6 normal airways were assessed. Therefore, together 18 areas of intact epithelium were compared with 18 areas of damaged epithelium. Previous studies have shown that migratory epithelium presents within 40 μm from the wound edge (2). Therefore, to evaluate sLe^x immunoreactivity in a damaged area, basal and columnar cells within 40 μm from either wound edge were counted. Next, epithelium farther than 40 μm from the wound edge was considered as intact epithelium if both the pseudo-stratified layer was complete and 40 μm of this area was evaluated for sLe^x staining. The *ImagePro Plus* image analysis software (Media Cybernetics) was used to collate the airway images and to determine the positive cells using a point counting grid. Positive counts were confirmed by manual inspection by one author (S.A.).

III.3.4 Cell culture.

1HAEo$^-$ and 16HBE 14o$^-$ cells are SV40-transformed normal human airway epithelial cells that have been characterized previously (24, 25) and express multiple surface carbohydrate markers of normal primary basal airway epithelial cells (11). Primary normal human bronchial epithelial (NHBE) cells were collected from subjects undergoing lung resection. These cells were derived from different donors. Epithelial cell purity was determined by examining the typical morphological features of primary epithelial cells in culture and staining the cells with anti-cytokeratin 18 antibody (USBiological, Swampscott, Massachusetts, USA). Cells were subcultured and used between passages 3 and 5.

III.3.5 Monolayer wound repair assay.

We have established this method previously using 6-well culture dishes (13, 26-28). In two experiments NHBE cells grown in a monolayer were treated with an inhibitory anti-sLe^x antibody (40 ng/ml) (KM 93, Seikagaku America, Ijamsville, MD, USA) or an isotype-matched non-specific antibody (40 ng/ml) immediately after mechanical injury. In three experiments, 1HAEo$^-$ cells were treated concurrently with both Epidermal Growth Factor (EGF) (15ng/ml) and 4-Deoxy-fucose, a general inhibitor of fucosyltransferases (FuTi) (10^{-3} to 10^{-5} M) (Calbiochem, La Jolla, CA, USA). In three experiments, 1HAEo$^-$ cells were treated concurrently with both EGF (15ng/ml) and soluble sLe^x (10^{-4} to 10^{-7} M) (Calbiochem, La Jolla, CA, USA). In two experiments, 1HAEo$^-$ cells were treated with either FuTi (10^{-3} to 10^{-5} M) or soluble sLe^x (10^{-4} to 10^{-7} M) without the addition of EGF. In each experiment, one well was used as a negative

control with no treatment and one well was treated with 15 ng/ml of EGF, which has previously been demonstrated to be a potent accelerant in models of epithelial monolayer wound closure (26).

III.3.6 Immunocytochemistry.

NHBE cells were grown on four-well chamber slides until confluency. Three linear wounds were created using a rubber stylet. Monolayers were then fixed at 0, 2, 6, 12, 24, and 48 h after mechanical injury using Clark's solution (90% ethanol, 10% glacial acetic acid). Expression of sLe^x was detected using mouse anti-human sLe^x (KM 93, Seikagaku America, Ijamsville, MD, USA).

III.3.7 RNA isolation and Real-Time Polymerase Chain Reaction.

1HAEo$^-$, 16HBE 14o$^-$, and NHBE cells were grown to confluency and then RNA extracted. Expression of four subtypes of α1,3-FucTs was studied by real-time RT-PCR using primers specific for FucT-III , -IV, -VII, and –IX. RNA was extracted at specific time points after injury using the TRIzol reagent (GIBCO/BRL), according to the manufacturer's protocol. mRNA expression was quantified by real-time PCR using *LightCycler™* (Roche, Mannheim, Germany). The levels of target mRNAs were normalized to the level of β-actin mRNA in the same sample.

III.3.8 Flow cytometry analysis.

Flow cytometric analysis was performed on 1HAEo$^-$ and NHBE cells using anti-E, -L and, -P selectin mouse monoclonal antibodies (RDI, Flanders, NJ).

III.3.9 Statistical analysis.

Data were entered in and analyzed by means of SPSS 7.1 for Windows. Wound closure is expressed as a percentage of area at time 0. In previous videomicroscopy experiments with cell monolayers (28), intra-observer variability was <2%, and inter-observer variability was <4% for all measurements.

III.4 Results

III.4.1 Expression of sLe^x is higher in areas of epithelial damage compared to intact epithelium.

We initiated investigating the role of sLe^x in epithelial repair by studying the expression of sLe^x in damaged and intact areas of normal airway epithelium. Immunoreactivity of sLe^x was analyzed in sections of bronchial specimens obtained from normal subjects (n=6). In each subject three areas of epithelial damage and three areas of intact epithelium were identified as described in Materials and Methods. The percentage of positively stained basal and columnar cells was significantly higher in areas of damaged compared to intact epithelium ($p<0.002$, Mann-Whitney U-test) (Figs. III.1 and III.2).

III.4.2 Mechanical injury enhances the expression of sLe^x in a culture model of airway epithelium.

We examined the effect of mechanical injury on sLe^x expression in mechanically wounded monolayers of NHBE cells. Epithelial cells were characterized by staining the cells with anti-cytokeratin-18 antibody and examination for typical morphological features of airway epithelial cells in culture. Immunocytochemical staining of the wounded monolayer showed a time-dependant increase in sLe^x expression coordinate with wound closure and a decrease once the repair is complete (Fig. III.3). Increased expression of sLe^x after mechanical injury was associated with a change in the epithelial cell shape. The majority of the epithelial cells expressing sLe^x exhibit an elongated

morphology. These elongated cells appear to be migratory epithelial cells in phenotype. Evaluation of migration and migratory phenotype of these cells was beyond the scope of the present study.

III.4.3 Blocking of sLe^x with an anti-sLe^x inhibitory antibody prevents epithelial monolayer wound repair.

To determine whether sLe^x plays a role in bronchial epithelial repair, we studied kinetics of epithelial wound repair in the presence of a blocking anti-sLe^x antibody. A linear wound was made in confluent monolayer of NHBE cells using a rubber stylet. Cells were treated with an anti-sLe^x antibody (40 ng/ml) or an isotype-matched non-specific antibody (40 ng/ml) immediately after injury. In each experiment, one well was used as a negative control with no treatment. Corresponding wound areas were determined at 0, 2, 6, 12, and 24 h after wound creation using time-lapse videomicroscopy (Fig. III.4, Panel A). The remaining wound area 24 h after wounding was significantly higher in monolayers treated with anti-sLe^x antibody compared to non-treated monolayers and the ones treated with non-specific antibody ($p<0.05$) (Fig. III.4, Panel B). These data showed that blocking of sLe^x in our culture model of airway epithelial wound repair inhibited wound closure.

III.4.4 α1,3-fucosyltransferases exhibit a diverse pattern of expression in 1HAEo$^-$, 16HBE 14o$^-$, and NHBE cells.

Expression of four subtypes of α1,3-FucTs, the enzyme responsible for the fucosylation step in sLe^x antigen synthesis, was studied in 1HAEo$^-$, 16HBE 14o$^-$, and

NHBE cells using real-time RT-PCR (Table III.1). FucT-IX was not expressed in any of the cell lines nor primary cells examined. FucT-III and -VII showed a different pattern of expression among two cell lines and primary cells. We found that FucT-IV is the only gene transcribed in both primary cells and cell lines of airway epithelium.

III.4.5 A general fucosyltransferase inhibitor (FuTi) reduces epithelial repair in a culture model of epithelial cell monolayer wound repair in the presence and absence of exogenous EGF.

Our previous data provide some evidence on the role of sLe^x in epithelial repair. We further investigated the role of fucose containing oligosaccharides in epithelial repair by inhibiting synthesis of fucose containing oligosaccharides using FuTi. Initial wound area and perimeter for monolayers within each experimental series were equivalent and consistent. In control and EGF-only experiments pooled across experimental series, the remaining wound area after 24 h was 30±3.8% in control cultures and 3.7±1.5% in EGF-stimulated wounds ($p< 0.01$; n=10). Concurrent treatment of monolayers with EGF (15 ng/ml) and the FuTi inhibited wound repair in a dose-dependent manner when compared to EGF-alone. Monolayers treated with 10^{-3} compared to monolayers treated with 10^{-4} M and 10^{-5} M FuTi and monolayers treated with 10^{-4} M compared to 10^{-5} M FuTi had higher wound area 24 h after treatment ($p<0.05$). The remaining wound area at 24 h after treatment with 10^{-4} M FuTi + EGF (35±1.4%) and 10^{-3} M FuTi + EGF (43±2.6%) were significantly higher compared to EGF alone treated group (3.5±1.4%, $p< 0.01$) (Fig. III.5, Panel B). In the absence of exogenous EGF, FuTi in its highest doses (10^{-3} and 10^{-4} M) inhibited epithelial repair compared to control monolayers. The remaining wound area at

24 h after treatment with 10^{-4} M FuTi (34±1.0%) and 10^{-3} M FuTi (35±3.0%) were significantly higher compared to control group with no treatment (23±1.6%, $p< 0.05$) (Fig. III.5, Panel D).

III.4.6 Soluble sLe^x reduces epithelial repair in a culture model of epithelial cell monolayer wound repair only in the presence of exogenous EGF.

We further investigated the role of sLe^x in epithelial repair by blocking the potential receptors for sLe^x using soluble sLe^x. Initial wound area and perimeter for monolayers within each experimental series were equivalent and consistent. In control and EGF-only experiments pooled across experimental series, the remaining wound area after 24 h was 25±4.1% in control cultures and 1±0.3% in EGF-stimulated wounds ($p< 0.01$; n=10). Concurrent treatment of monolayers with soluble sLe^x and EGF inhibited wound repair in a concentration-dependent manner. Monolayers treated with 10^{-4} M soluble sLe^x compared to monolayers treated with 10^{-5}, 10^{-6}, and 10^{-7} M soluble sLe^x had higher wound area 24 h after treatment ($p<0.05$). The remaining wound area at 24 h after treatment with 10^{-4}, 10^{-5}, and 10^{-6} M soluble sLe^x + EGF was significantly higher compared to EGF alone treated group ($p<0.01$) (Fig. III.6, Panel B). In the absence of exogenous EGF, soluble sLe^x had no effect on epithelial repair (data are not shown). Altogether our data showed that co-treatment of the injured monolayers with EGF and FuTi or soluble sLe^x reverse the acceleration effect of EGF on epithelial repair. While the inhibitory effect of FuTi on epithelial repair remained in the absence of EGF, soluble sLe^x showed no effect without the concomitant stimulation of EGF. These data provide

further evidence on the role of fucose containing oligosaccharides and sLe^x in epithelial repair.

III.4.7 E-selectin is expressed by a subset of airway epithelial cells.

Our previous studies identified that fucose-containing ligands are essential for repairing airway epithelium. Over-expression of sLe^x may promote closure of wounds by increasing the selectin (CD62) receptor-ligand interaction in airway epithelial cells. Next we examined the expression of P-, E-, and L-selectin by 1HAEo$^-$ cells using cytofluorometric analysis. We found that E- but not P- and L-selectin was expressed by a subset of airway epithelial cells (data are not shown). We also examined the expression of E-selectin by NHBE and 1HAEo$^-$ cells before (N=5) and 24 h after mechanical injury (N=3) both in the presence of EGF (15ng/ml). There was no statistically significant difference in the percent of cells expressing E-selectin receptor before and after mechanical injury (Table III.2).

III.5 Discussion

In providing the physical barrier to the external environment, the bronchial epithelium is continuously exposed to injuries. The airway epithelium is therefore routinely challenged as part of normal function. Epithelial wound healing represents an important process by which the respiratory epithelial barrier restores the physical barrier and tissue integrity is maintained. Epithelial repair involves a series of ordered events including migration, spreading, proliferation and differentiation of epithelial cells. Cell surface glycoconjugates have crucial function in a variety of normal and disease states. It has been shown that glycans can modulate function of proteins and lipids they are attached to and are involved in cell-cell and cell-matrix interaction (4-7, 29). There is a growing interest in exploring the role of cell surface carbohydrates in epithelial repair. Using lectins in an *in vitro* model of wound repair, Adam *et al.* (30) recently demonstrated that *N*-acetylglucosamine (GlcNAc) which is recognized by the lectin wheat germ agglutinin (WGA) is required for epithelial repair. Our previous work demonstrated that cell surface N-glycosylation has a functional role in airway epithelial cell adhesion and migration. N-glycans with terminal fucosylation plays an essential role in the complex process of repair by coordination of cell-cell functions, including migration (13). In the present study, for the first time we examined the role of a specific fucose containing carbohydrate structure, sLe^x, in bronchial epithelial repair.

Sialyl Lewis x is a member of the Lewis blood group structures which are found at the non-reducing termini of N-linked or O-linked glycans on glycoproteins and glycolipids. sLe^x has been identified as a necessary component of selectin-ligand interaction which mediate cell-cell interaction and migration in several systems (7, 10,

20-22). To investigate the role of sLe^x in epithelial repair we examined the expression of this antigen on bronchial epithelium by immunostaining. There was increased expression of sLe^x in areas of damaged epithelium compared to intact regions. This finding suggests a possible contribution of sLe^x in epithelial repair. Gipson (31) showed previously that cell surface carbohydrates on epithelial cells that are spreading and/or migrating to cover a wound are different from cell surface carbohydrate structures found on normal epithelial cells. Our laboratory has shown that glycosylation profiles in airway epithelium change overtime during repair of a wound created by mechanical injury (12). It has been shown that injury of the respiratory epithelium enhances bacterium *Pseudomonas aeruginosa* adhesion to the epithelium and it has been speculated that changes of cell surface glycoconjugates related to wound repair, cell migration and/or spreading may favor P. *aeruginosa* adhesion (32).

The final step in synthesis of sLe^x is catalyzed by specific α1,3-FucT. The fucosyltransferase gene family encodes for a group of proteins that show a complex tissue- and cell type-specific expression pattern. FucT-IV and -VII are expressed in human leukocytes where they modify carbohydrate motifs that can act as E- and P-selectin ligands (33-35). In contrast FucT-III, -V and –VI are not expressed in leukocytes (36) and FucT-IX is abundantly expressed in brain, stomach, spleen, and peripheral blood leukocytes (37). Among six α1,3-FucTs responsible for synthesis of sLe^x, we examined the expression of FucT-III, -IV, -VII and -IX in two bronchial epithelial cell lines (1HAEo$^-$ and 16HBE 14o$^-$) and primary cells. FucT-V and -VI do not appear to have an essential biological role and not all humans have functional forms of these enzymes (38). We found that FucT-IV is the only gene expressed in all airway epithelial cells examined

(Table III.1), therefore FucT-IV is to be considered the main FucT in the study of airway epithelial repair. It has been shown that expression of FucT varies during development and malignant transformation (39-41). This may explain the diversity of FucT expression between transformed bronchial epithelial cell lines and primary cells studied. Our data showed a time-dependent increase in the expression of FucT-IV after mechanical injury coordinate with both sLe^x expression and wound closure (data are not shown). Several studies have pointed to the similarities between pathways and genes activated during development, malignant transformation and tissue healing. It has been shown by several studies that FucT-IV expression is significantly higher in tumors than in adjacent normal cells (40-42). Cailleau-Thomas et al. (39) examined the expression of FucT during human development. They found that FucT-IV and –IX are the only FucT strongly expressed during the first two months of embryogenesis.

In the present study, we demonstrated an increased expression of sLe^x during repair of a wounded *in vitro* monolayer. To our knowledge, there is no other report indicating expression of specific carbohydrate structure after injury. This finding confirms our *in vivo* observation of over expression of sLe^x in the area of epithelial damage. Over expression of sLe^x by the epithelial cells distant from the wound edge suggests involvement of a soluble factor(s) released by the injured epithelium. This soluble factor would initiate the repair process including migration of distant cells.

To confirm the role of fucose containing oligosaccharides and specifically sLe^x in epithelial repair we treated human airway epithelial cells in monolayer culture with a fucosyltransferase inhibitor or soluble sLe^x in the presence and absence of EGF, a potent accelerator of epithelial repair (22). Wounded monolayers were followed for closure by

use of time-lapse videomicroscopy. Our data demonstrated that preventing the synthesis of fucosylated glycans by FuTi inhibited epithelial repair, in the presence and absence of EGF. However, blocking of potential receptors for sLe^x by soluble sLe^x, inhibited epithelial repair only in the presence of EGF. There are several mechanisms by which sLe^x can participate in epithelial repair. First, sLe^x is a decorating motif for many membrane-bound and secreted proteins and can modulate the function of certain glycoproteins. Second, sLe^x has been shown to act as a common ligand for the selectin family of receptors (20, 43). Selectins are a family of three adhesion molecules (L-, E-, and P-selectin) initially described as receptors specialized for capturing leukocytes from the bloodstream on the blood vessel endothelium. It seems that interaction of selectins with their ligands mediate cell adhesion and migration in several cell systems, including leukocyte adhesion on the endothelium, and cancer cell metastasis through interaction with E-selectin presented on vascular endothelial cells (35, 44-47). Our finding that soluble sLe^x only inhibited accelerated repair with no effect on a non-accelerated repair suggests that interaction of sLe^x with its selectin receptor does not have a prominent role in epithelial repair and is not the sole mechanism utilizing sLe^x to affect repair. It also suggests that sLe^x binding to CD62E during repair requires pathways activated by EGF. FuTi on the other hand inhibited epithelial repair in either the presence or absence of EGF. This demonstrates that fucose containing structures have an essential role in epithelial repair. The universal fucosyltransferase inhibitor, FuTi, prevents the synthesis of fucose-containing structures and thus sLe^x on the surface of the repairing airway epithelial cells. To address the specific role of sLe^x in epithelial repair we inhibited sLe^x motifs with an inhibitory antibody, KM 93 which demonstrated an inhibitory effect on

epithelial repair. The exact structure carrying the sLe^x structure needed for repair remains to be identified.

Our data suggests that binding of sLe^x to its receptor, in part, contributes to epithelial repair after mechanical injury, so we investigated the expression of E-, P- and L-selectins on bronchial epithelial cells by flow cytometry. We showed that only E-selectin is expressed by a subset of airway epithelial cells. We also demonstrated that expression of E-selectin by bronchial epithelial cells does not change during repair in the presence of exogenous EGF. Constant expression of E-selectin by a subset of airway epithelial cells in response to injury suggests that this receptor does not play an essential role in epithelial repair whereas the regulation of the synthesis of the ligand, (sLe^x) rather than E-selectin expression itself is the essential link during repair to affect closure. We have previously demonstrated the role for N-linked fucosylation but now more specifically demonstrate the role for the fucose containing sLe^x (10).

In conclusion, our data demonstrates that the oligosaccharide sLe^x plays an essential role in airway epithelial repair. Our data may explain the previous observation of under-representation of fucose-containing carbohydrate structures in asthmatic patients reported by Kauffmann *et al.* (18). In that report severity of asthma and thus epithelial damage was inversely related to the amount of detected fucose-containing antigens. As such, these results and reports suggest that defects in epithelial repair in asthma patients may be due, in part, to improper glycosylation of airway epithelial cells. We demonstrated that FucT-IV is the main FucT expressed in bronchial epithelial cells. The expression of FucT-IV is increased upon mechanical injury. sLe^x has been identified as a tumor specific antigen which promotes tumor cell motility through interaction with

endothelial E-selectins. Another unexplored possibility is that sLe^x as a carbohydrate modification of another protein structure control cell motility. Our data showed an important role for the carbohydrate structure sLe^x in epithelial repair, however the interaction of sLe^x with E-selectin receptor only in part plays a role in epithelial repair. Further investigation is required to elucidate how sLe^x as a post-translational modification of cell protein(s) may alter protein binding or receptor activity in bronchial epithelial cell to affect migration and repair.

Fig. III.1

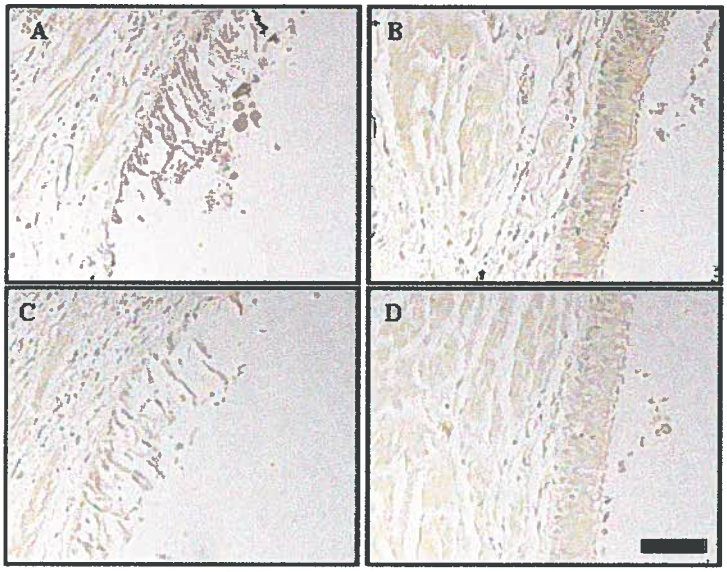

Figure III.1. Expression of sLe^x on airway epithelium in normal subjects. Bronchial segments obtained from pathological specimens of adults undergoing lung resection, were processed and sectioned as described in Materials and Methods. New fuschin was applied for visualization and positive sLe^x detection is noted by red staining. Panels A and B show immunoreactivity for sLe^x in areas characterized by epithelial damage (A) and intact epithelium (B). Panels (C) and (D) show the appearance of an isotype matched non-specific antibody staining in the areas of damaged and intact epithelium. Scale bar = 10μm.

Fig. III.2

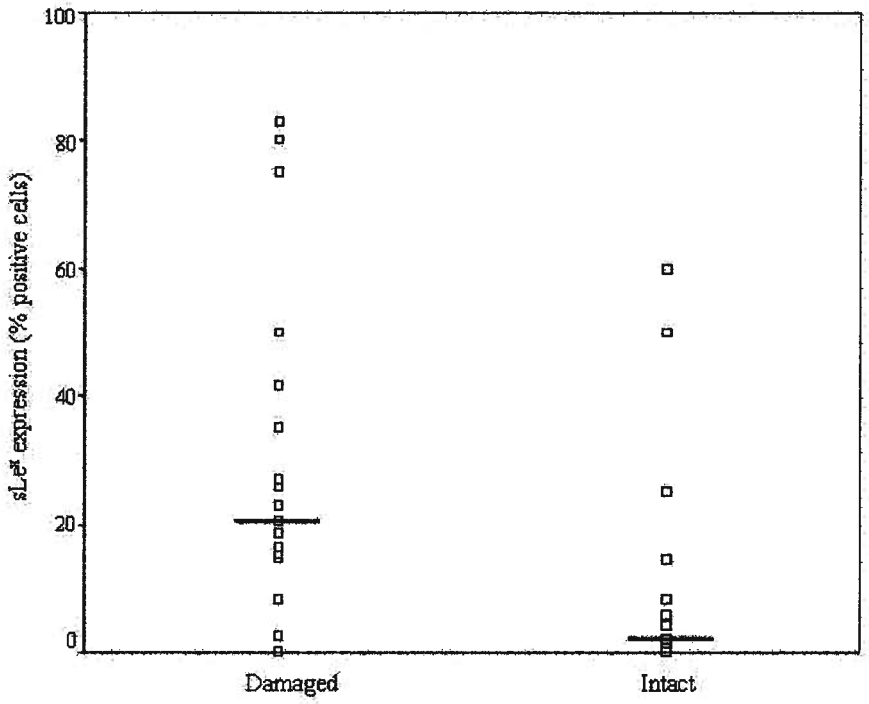

Figure III.2. Expression of sLe^x is higher in areas of epithelial damage compared to intact epithelium. Immunostaining of sLe^x in intact and damaged epithelial zones was assessed in each subject. Epithelial damage was characterized morphologically by the absence of ciliated and secretory cells. Quantification was performed with *ImagePro Plus* image analysis software. To evaluate sLe^x immunoreactivity in damaged areas, positive staining basal and columnar cells within 40 μm from either wound edge were counted. Next intact epithelium farther than 40 μm from the wound edge was considered as intact epithelium, 40 μm of this area was assessed for sLe^x staining. Percentage of positively stained basal and columnar cells in three areas exhibiting epithelial damage and three areas of intact epithelium were determined in each subject. The statistical significance was determined by Mann-Whitney U-test. The horizontal line represents the median. Expression of sLe^x is significantly higher in areas of epithelial damage compared to intact epithelium ($p<0.002$).

Fig. III.3

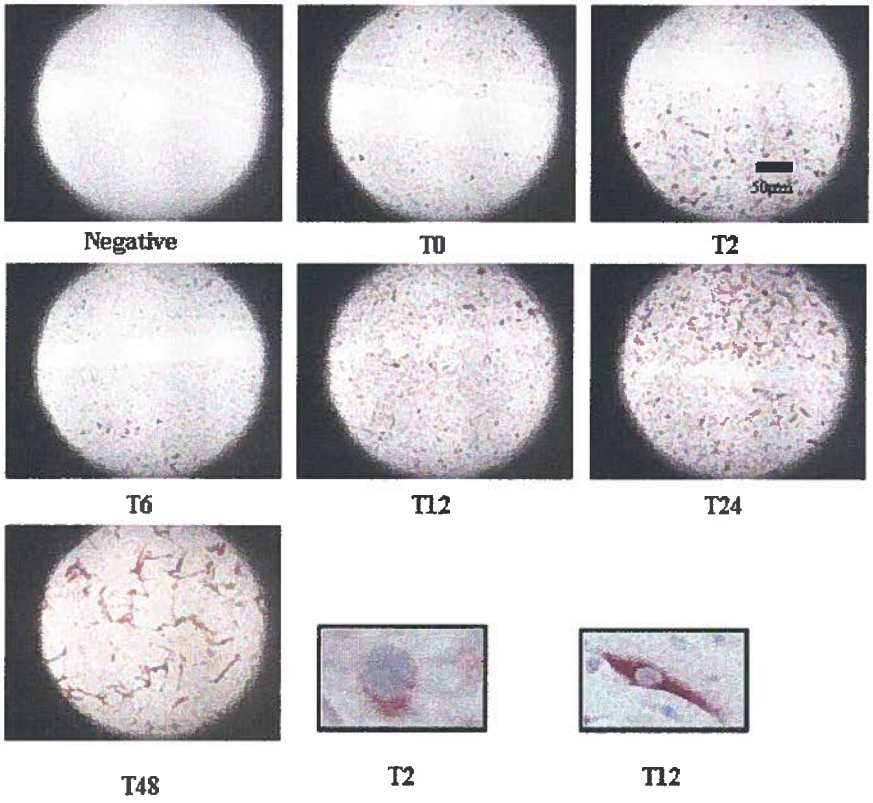

Figure III.3. Mechanical injury induces the expression of sLe^x in a culture model of epithelial repair. A linear wound was made in confluent monolayers of primary bronchial epithelial cells using a rubber stylet. Monolayers were fixed with Clark's solution at 0, 2, 6, 12, 24, and 48 h after the mechanical injury. Expression of sLe^x was detected using mouse α-human sLe^x and Vector Red for visualization. Detection of sLe^x increased with repair and noted by increased red stain. A correlation between increased expression of sLe^x and the cell-shape phenotype also changed over time after mechanical injury. Cells that had higher detection of sLe^x demonstrated an elongated shape characteristic of migratory cells (see enlarged inset from T12).

Fig. III.4

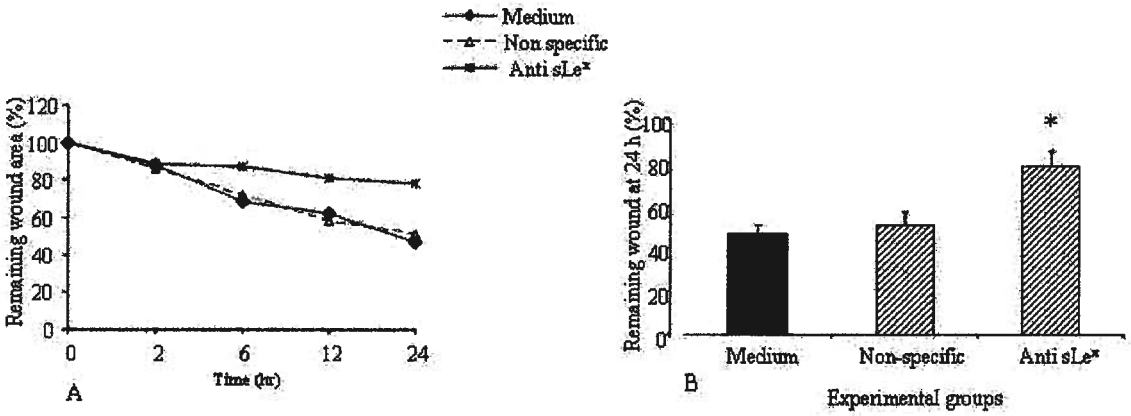

Figure III.4. Blocking of sLe^x with an anti-sLe^x inhibitory antibody prevents epithelial monolayer wound repair. A linear wound was made in confluent monolayers of primary bronchial epithelial cells as described and wounds were treated with an anti-sLe^x antibody or control antibody. In each experiment monolayers with no treatment considered as control group. Corresponding wound areas determined 2, 6, 12, and 24 h after wound creation using time-lapse videomicroscopy are presented in Panel A. Data are mean ± SEM, for 24 wounds measured in two independent experiments. The effect of anti-sLe^x antibody on wound repair is demonstrated in Panel B. Anti-sLe^x antibody significantly reduced wound repair compared to controls (* $p<0.05$). The statistical significance of the differences between groups was determined by one-way ANOVA.

Fig. III.5

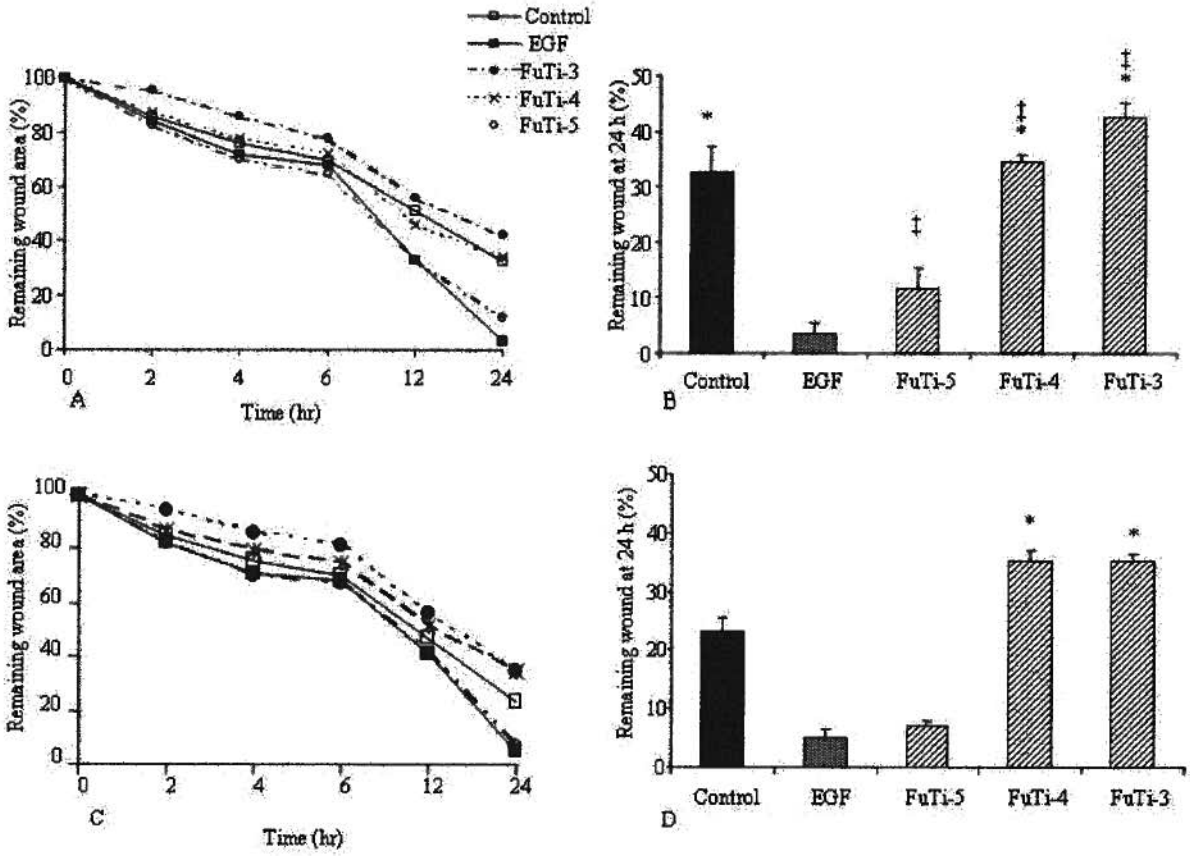

Figure III.5. Wound repair of 1HAEo⁻ cells is impaired in the presence of a fucosyltransferase inhibitor. Confluent monolayers of 1HAEo⁻ were serum starved for 24 h prior to the creation of a small wound as described in Materials and Methods. Cells were treated with 10^{-3}-10^{-5} M of a fucosyltransferase inhibitor [FuTi-3, -4, and -5] in the presence (Panels A and B) and absence (Panels C and D) of EGF (15 ng/ml) after mechanical injury. In each experiment, one well was used as a negative control and one well was treated with EGF (15 ng/ml). Corresponding wound areas determined 0, 2, 6, 12, and 24 h after wound creation using time-lapse videomicroscopy are demonstrated in Panels A and C with wound repair inhibition at 24 h demonstrated in Panels B and D. The remaining wound area after 24 h was significantly higher in control cultures compared to EGF-treated groups (* $p< 0.01$, n=10). In the presence of EGF, monolayers treated with the FuTi when compared to monolayers treated only with EGF demonstrated a dose-dependent inhibition of repair. At 24 h all FuTi treatments were significantly different (‡ $p<0.05$, Panel B) and the 10^{-3} and 10^{-4} M FuTi were also significantly different when compared to EGF alone (* $p< 0.01$, Panel B). In the absence of exogenous EGF, 10^{-3} and 10^{-4} M FuTi significantly inhibited epithelial repair compared to control group (* $p< 0.01$, Panel D). The statistical significance of the differences between groups was determined by one-way ANOVA.

Fig. III.6

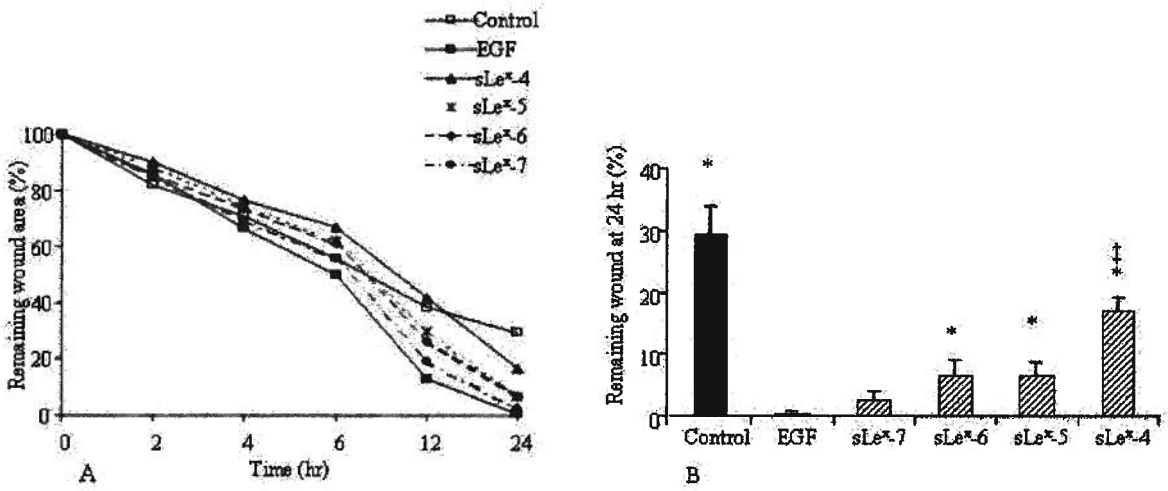

Figure III.6. Wound repair of 1HAEo$^-$ cells is reduced by soluble sLe^x only in the presence of exogenous EGF. Confluent monolayers of 1HAEo$^-$ were serum starved for 24 h prior to the creation of a small wound as described in Materials and Methods. Cells were treated with 10^{-4} to 10^{-7} M of soluble sLe^x [sLe^x-4, -5, -6, and -7] and EGF (15 ng/ml) (Panels A and B) after mechanical injury. In each experiment, one well was used as a negative control and one well was treated with EGF (15 ng/ml). Corresponding wound areas determined 0, 2, 6, 12, and 24 h after wound creation using time-lapse videomicroscopy and wound repair inhibition at 24 h demonstrated. The remaining wound area after 24 h was significantly higher in control cultures compared to EGF-treated groups (* $p< 0.01$, n=10). The remaining wound area at 24 h after treatment with 10^{-4}, 10^{-5}, and 10^{-6}M soluble sLe^x + EGF (15 ng/ml) was significantly higher compared to EGF alone (* $p<0.01$, Panel B). Monolayers treated with 10^{-4} M soluble sLe^x compared to monolayers treated with 10^{-5}, 10^{-6}, and 10^{-7} M soluble sLe^x have higher wound area 24 h after treatment (‡ $p<0.05$, Panel B). The statistical significance of the differences between groups was determined by one-way ANOVA.

Table III.1

	FucT-III	FucT-IV	FucT-VII	FucT-IX
1HAEo$^-$	–	+	+	–
16HBE 14o$^-$	+	+	+	–
Primary	+	+	–	–

Table III.1. α1,3-fucosyltransferases show a diverse pattern of expression in 1HAEo$^-$, 16HBE 14o$^-$, and NHBE cells. Expression of four subtypes of α1,3-FucTs (FucT III, IV, VII and IX) was studied in 1HAEo$^-$, 16HBE 14o$^-$, and NHBE cells using real-time RT-PCR. α1,3-FucTs showed a different pattern of expression among two cell lines and primary cells but FucT-IV was the only gene transcribed in both primary cells and cell lines of airway epithelium.

Table III.2

	Uninjured (T0)	After injury (T24)
1HAEo⁻	8.6±0.4%	9.2±0.5%
NHBE	7.8±0.7%	7.5±0.9%

Table III.2. Expression of E-selectin is not changed after mechanical injury in 1HAEo⁻ and NHBE cells. Expression of E-selectin by 1HAEo⁻ and NHBE cells was examined before (N=5) and 24 h after mechanical injury (N=3) in the presence of exogenous EGF (15ng/ml). There was no statistically significant difference in the percent of cells expressing E-selectin receptor before and after mechanical injury.

III.6 References

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CHAPTER IV. SIALYL LEWIS X MODIFICATION OF EPIDERMAL GROWTH FACTOR RECEPTOR REGULATES RECEPTOR FUNCTION DURING AIRWAY EPITHELIAL WOUND REPAIR

IV.1 Summary

Epidermal growth factor receptor (EGFR) is a major regulator of airway epithelial cell (AEC) function such as migration, proliferation and differentiation which has an essential role in epithelial repair. EGFR is a glycoprotein with 12 potential N-glycosylation sites in its extracellular domain. Glycosylation of EGFR has been shown to modulate its function. Previously our laboratory demonstrated an important role for carbohydrate sLe^x in airway epithelial repair. In the current investigation we examined whether sLe^x decoration of EGFR can modulate receptor function during AEC repair. We demonstrated a co-localization of the carbohydrate structure sLe^x with EGFR on primary normal human bronchial epithelial (NHBE) cells using both confocal microscopy and immunoprecipitation. The final step in the synthesis of sLe^x is catalyzed by specific α-1,3-fucosyltransferases (FucT). FucT-IV showed a temporal expression coordinate with epithelial repair. Down regulation of FucT-IV expression in NHBE by small interfering RNA (siRNA) suppressed sLe^x expression. Using a blocking antibody for sLe^x after mechanical injury we observed a reduction in EGFR phosphorylation following injury. In the same manner, knocking down of FucT-IV by siRNA significantly reduced phosphorylation of EGFR and prevented epithelial repair. Present data suggests that sLe^x has an important role in modulating EGFR activity to affect NHBE repair.

IV.2 Introduction

Bronchial epithelial wound healing represents an important process by which the respiratory epithelial barrier is restored and tissue integrity maintained. Epithelial repair is a complex cellular process which involves a series of ordered events including migration, spreading, proliferation and differentiation of epithelial cells. Although the details of molecular events involved in epithelial repair are still not clearly understood, a number of studies have suggested that plasma membrane glycoconjugates have important roles in regulation of repair processes. Several proteins essential for normal cell physiology including adhesion molecules, cell surface receptors, enzymes and, hormones are glycosylated (1, 2). More importantly, several lines of evidence support the theory that oligosaccharide moieties are crucial for the function of those proteins and that variation in their glycosylation pattern often leads to changes in their function (3-5). Our laboratory has previously determined an important role for cell surface carbohydrates in particular those with terminal fucose in the regulation of epithelial repair (6). Sialyl Lewis x (sLe^x) is a fucose containing tetrasaccharide, [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc], which has been recognized as a decorating motif for many membrane-bound and secreted proteins. The final step in the synthesis of sLe^x is catalyzed by specific α1,3-fucosyltransferases (FucT) which show different patterns of expression among tissues (7). Recently we have studied the role of sLe^x in airway epithelial repair. Our data demonstrate an important role for sLe^x in bronchial epithelial repair (8).

Epidermal growth factor receptor (EGFR) is a major regulator of epithelial cell functions such as migration, proliferation and differentiation and has been shown to mediate epithelial repair (9-11). EGFR is a transmembrane glycoprotein with 12 potential

sites for N-glycosylation in its extracellular domain (12-14). Glycosylation patterns of EGFR include fucose containing structures such as Le^x, Le^y, and blood group A and H antigens (15, 16). It has been shown that glycosylation is necessary for the ligand binding and tyrosine kinase activity of EGFR (14-17). Carbohydrate moieties of EGFR are critical for the direct interaction of the receptor with other structures (18). Moreover, glycosylation defines localization of EGFR to a specific domain of the plasma membrane which could facilitate association of the receptor with other molecules and its subsequent transactivation (19). Modification of receptor N-glycans can regulate this receptor trafficking and duration of cell surface residency (20).

Our previous study has clearly demonstrated an important role for sLe^x in airway epithelial repair however; it does not explain how sLe^x mediates the repair process. As a carbohydrate, sLe^x must either modify a protein or lipid to regulate its function or participate in binding to a specific receptor to effect the desired action. In the current investigation we hypothesized that sLe^x mediates epithelial repair through regulation of the function of EGFR, a key protein involved in epithelial migration and repair. We used a cell culture model of epithelial injury and repair in airway epithelial cell (AEC). Using confocal microscopy we found a co-localization of sLe^x with the EGFR after injury. Immunoprecipitation studies confirmed the association of sLe^x with EGFR in AEC. We demonstrated that while mechanical injury leads to phosphorylation of EGFR, blocking of sLe^x in the injured monolayers by an inhibitory antibody or suppression of sLe^x expression by RNA interference (RNAi) for FucT-IV, prevents EGFR phosphorylation and reduces epithelial repair. Further investigations are required to understand how sLe^x modification of EGFR affects receptor function.

IV.3 Materials and Methods

IV.3.1 Cell culture.

Primary normal human bronchial epithelial (NHBE) cells were collected from subjects undergoing lung resection. Approval for the use of human tissue was granted by University of British Columbia and Providence Health Care Ethics Review Board. These cells were derived from different donors. Epithelial cell purity was determined by examining the typical morphological features of primary epithelial cells in culture and by staining the cells with anti-cytokeratin 18 antibody (USBiological, Swampscott, Massachusetts). Cells were subcultured in bronchial epithelial growth medium (BEGM) and used between passages 3 and 5.

IV.3.2 Immunostaining.

NHBE cells were grown on collagen IV (Sigma) coated chamber slides until confluency. Three linear wounds were created using a rubber stylet. Monolayers were then fixed at 0, 1, 6, and 24 hr after mechanical injury using Clark's solution (90% ethanol, 10% glacial acetic acid). After blocking of non-specific sites with a universal serum block (Dako Cytomation), monolayers were incubated overnight at 4°C with mouse anti-human sLe^x (KM 93, Seikagaku America, Ijamsville, MD) and sheep anti-human EGFR antibody (RDI, Flanders, NJ,). Following several washes with TBS, monolayers were incubated with anti-mouse Alexa 568 and anti-sheep Alexa 488-conjugated secondary antibody. Nuclei were counterstained with Hoechst 33342

(Molecular Probes, Eugene, OR). All images were obtained using a Leica AOBS™ SP2 confocal microscope and analyzed by Volocity™ (Improvisions, Boston, MA).

IV.3.3 Immunoprecipitation.

Confluent monolayers of NHBE cells were subjected to multiple linear injuries (7X7 linear scratches in each well) using a rubber stylet. Monolayers were washed with phosphate buffered saline (PBS) followed by the addition of defined medium. Monolayers with no scratch wounds were used as the control. Protein cell lysates were collected at different time points after injury. For sLe^x immunoprecipitation 125 μg of total cell lysates were incubated with the anti-sLe^x antibody (1.6 μg) overnight at 4°C with gentle rocking and then added to 25 μl of protein G-Sepharose (Sigma). For EGFR immunoprecipitation 50 μg of total cell lysates were incubated with 0.6 μg of a mouse anti-EGFR antibody (BIOSOURCE, Camarillo, CA). Immunoblotting for sLe^x and EGFR were performed using the appropriate antibodies. In both conditions cell lysates were precleaned by 25 μl of protein G-Sepharose prior to incubation with primary antibody. As controls for immunoprecipitation in each experiment, total protein lysates were

precipitated with beads only. Resulting immunoblots from these samples was negative.

IV.3.4 Monolayer wound repair assay.

We have established this method previously (6, 8). Briefly, NHBE cells were grown in 6-well plates until confluency and then circular wounds (~2.0 mm^2) were made using a rubber stylet (4 wounds per well). Wounds were imaged at the beginning and 24

hr after wound creation using a Nikon Eclipse TE200 inverted scope equipped with a Nikon Coolpix E995.

Wound areas were determined using ImagePro Plus and the remaining wound areas calculated as a percentage of area at time 0.

IV.3.5 Preparation of protein extracts and immunoblotting.

Confluent monolayers of NHBE cells were subjected to multiple linear injuries as described above and protein cell lysates were collected at different time points after injury. Monolayers with no scratch wounds were used as the control. In other experiment NHBE cells grown in a monolayer were treated with an inhibitory anti-sLe^x antibody (KM-93) (40 ng/ml) immediately after mechanical injury. In other experiment monolayers of NHBE were transfected with FucT-IV siRNA or scrambled siRNA and then subjected to multiple linear wounds and protein cell lysates collected.

IV.3.6 Immunocytochemistry.

NHBE cells were grown on collagen IV (Sigma) coated chamber slides until confluency and transfected with FucT-IV siRNA or kept in defined medium. After 48 hr three linear wounds were created using a rubber stylet and monolayers were fixed 24 hr after mechanical injury using Clark's solution (90% ethanol, 10% glacial acetic acid). Expression of sLe^x was detected using mouse anti-human sLe^x.

IV.3.7 siRNA Preparation.

Synthetic RNAs were purchased from QIAGEN (Mississauga, ON, Canada). The sequence of the human FucT-IV siRNA was 5'-CAA AUU UAU UAC AAA UUU A-3'; 3'-UAA AUU UGU AAU AAA UUU-5'. Scramble oligo control siRNA duplex was also purchased from QIAGEN, and was used as controls. The transfection was performed using 80% confluent NHBE cells. FucT-IV siRNA and HiperFect were mixed and incubated for 10 min at room temprature and then added to the cells. After 48 hr of transfection, fresh medium was added and cells were incubated in fresh media for 8 hr before RNA extraction or any treatment. RT-PCR of FucT-IV was performed to determine the down-regulation of FucT-IV.

IV.3.8 RNA isolation and Reverse Transcriptase Polymerase Chain Reaction.

RNA was extracted from NHBE cells using the TRIzol reagent (GIBCO/BRL), according to the manufacturer's protocol. mRNA expression of FucT-IV and β-actin (internal control) was performed by conventional PCR using primers specific for FucT-IV and β-actin.

IV.3.9 Statistical Analysis.

Comparisons between multiple groups were made by ANOVA; when significant differences were found further comparisons were made by Student's *t*-test.

IV.4 Results

IV.4.1 sLe^x modifies EGFR in NHBE cells after mechanical injury.

EGFR is glycosylated with structures including Le^x, Le^y, and blood group A and H antigens (15, 16). Our laboratory has previously shown an over-expression of sLe^x in NHBE cells after mechanical injury (8). As a carbohydrate, sLe^x must decorate proteins and lipids to be able to be effective in cell function. We hypothesized that sLe^x modifies EGFR, an important mediator of epithelial repair, in NHBE cells during repair. Using confocal microscopy we found a co-localization of sLe^x with EGFR after mechanical injury in NHBE cells (Fig. IV.1A). Cells demonstrating this co-localization of sLe^x and EGFR are characterized by an elongated, migratory phenotype. To confirm sLe^x attachment to EGFR we immunoprecipitated total cell lysates from wounded and control monolayers of NHBE cells with anti-EGFR and anti-sLe^x antibodies. The immunoprecipitated proteins were immunoblotted for sLe^x and EGFR respectively (Fig. IV.1B, data from immunoprecipitation with anti-EGFR are not shown). These data indicated that EGFR is modified by sLe^x antigen in NHBE. Western blotting of sLe^x from total protein lysates isolated from non-injured and injured AEC monolayers demonstrate a clear band at 170 kDa, consistent with being EGFR (data are not shown).

IV.4.2 KM-93 alters EGFR activation following injury.

Figure IV.1 data showed that EGFR is modified by sLe^x after mechanical injury. To investigate the role of this modification in EGFR function during repair we studied the pattern of EGFR phosphorylation after injury in the presence and absence of KM-93, an

anti- sLe^x antibody previously shown to inhibit wound closure in AEC (8). Figure IV.2A shows the time course of tyrosine phosphorylation of EGFR after mechanical injury. EGFR phosphorylation peaked 30 minutes after injury (the earliest time tested), started to decline after 2 hr, but was still higher than control at 6 hr after wounding. Addition of KM-93 to injured monolayers altered the pattern of EGFR phosphorylation after injury. As shown in Fig IV.2B there is an attenuated and sustained EGFR phosphorylation in the presence of KM-93 without the expected reduction in total EGFR. As EGFR is activated it is internalized and undergoes degradation. This is not observed where KM-93 is added.

IV.4.3 Mechanical injury induces the expression of FucT-IV by NHBE cells.

Fucosyltransferases (FucT) are the enzymes that mediate the final step in synthesis of Lewis antigens. Previous data in our laboratory showed that FucT-IV is the common FucT gene transcribed in bronchial epithelial cell lines and primary cells (8). We studied the expression of FucT-IV in response to mechanical injury by real-time RT-PCR (Fig. IV.3). In NHBE cells mechanical injury significantly induced the expression of FucT-IV at 2, 6 and, 12 h after injury compared to unwounded groups coordinate with wound closure as demonstrated previously (8).

IV.4.4 NHBE cells express less sLe^x when FucT-IV is down regulated.

To confirm the association of FucT-IV with sLe^x expression in NHBE, we knocked down the expression of FucT-IV using siRNA. Fig. IV.4A shows the RT-PCR analysis of FucT-IV expression with a complete knock down of FucT-IV expression with 200 nm of siRNA. Fig. IV.4B shows that NHBE cells express less sLe^x when FucT-IV

expression is knocked down. This data demonstrates the association between the FucT-IV and sLe^x in human bronchial epithelial cells.

IV.4.5 Knockdown of FucT-IV expression attenuated wound induced EGFR activation and epithelial repair.

We have previously shown an over expression of sLe^x in NHBE cells after mechanical injury (8). Our present data demonstrates that sLe^x decorates EGFR (Fig. IV.1A) and that FucT-IV mediates sLe^x synthesis in NHBE cells (Fig. IV.4B). To investigate the role of sLe^x decoration of EGFR on receptor function during epithelial repair we compared the pattern of EGFR phosphorylation after mechanical injury in FucT-IV knocked-down NHBE cells and NHBE with normal expression of FucT-IV. As shown in Fig. IV.5A non-transfected NHBE cells and the monolayers transfected with scramble siRNA showed an increase in EGFR phosphorylation 30 min after injury. FucT-IV knocked-down-cells express the same amount of total EGFR; however, the expression of p-EGFR was markedly reduced. The same result observed in two other NHBE cells obtained from different individuals. Since EGFR phosphorylation after injury has been shown to mediate epithelial repair we measured the amount of epithelial repair in NHBE cells after FucT-IV knock down. Fig. IV.5B shows epithelial repair 24 hr after wounding was significantly reduced in monolayers transfected with FucT-IV siRNA compared to control monolayers ($p<0.05$). sLe^x modification of EGFR has an essential role in regulation of EGFR function to complete epithelial repair in our culture model of airway epithelial wound repair.

IV.5 Discussion

Sialyl Lewis x is a member of the Lewis blood group structures which are found at the non-reducing termini of N-linked or O-linked glycans on glycoproteins and glycolipids. sLe^x is also a decorating motif for many membrane-bound and secreted proteins and can modulate the function of certain glycoproteins. Previously our laboratory demonstrated an important role for sLe^x in airway epithelial repair (8). Contribution of sLe^x to repair may result from its role in modification of selectin ligands and therefore selectin-ligand interaction. Our data has shown that E-selectin is only expressed by a subset of airway epithelial cells and that expression does not change during repair (8). This in combination with an incomplete inhibition of repair by soluble sLe^x suggests that E-selectin does not play an essential role in epithelial repair. We hypothesized that sLe^x modifies another protein/lipid structure(s) which have an important role in airway epithelial repair.

EGFR is a major regulator of epithelial cell function such as cell growth, migration, and differentiation and has been shown to play crucial roles in epithelial repair (9-11). These observations make EGFR a plausible candidate for our study. First we studied whether sLe^x decorates EGFR in AEC. Our study showed that sLe^x modifies EGFR in AEC and that the association of sLe^x with the receptor increases after epithelial injury. Previous studies have identified the glycosylation patterns of EGFR which include fucose containing structures such as Le^x, Le^y, and blood group A and H antigens (15, 16). Recently, Wang *et al.* reported that sLe^x decorates EGFR in human hepatocarcinoma cell lines (21). Tumors have long been described to be similar in many features to repairing

tissues (22). In both cases cell proliferation, survival and in particular migration of the cells in response to growth factors, cytokines, and other stimuli is accompanied by a response. Sialyl Lewis antigens including sLe^x are known tumor associated antigens expressed on the surface of cancer cells (24, 25). It has been documented that EGFR carries most of these sialylated and fucosylated antigens in cancer tissues. Basu *et al.* found that while such carbohydrates are absent on receptors from normal human tissues and antigen-negative tumor cell types, they are intrinsic to the EGFR expressed by antigen-positive carcinoma lines (26). In the current investigation we identified that the sLe^x content associated with the EGFR increases after mechanical injury. This finding suggests that sLe^x may alter EGFR function in a manner that could affect cell survival, migration and/or proliferation, all of which are necessary for tissue repair.

The final step in the synthesis of Lewis antigens is catalyzed by specific α1,3-fucosyltransferases (FucT). Six human α1,3-FucTs have been cloned and partially characterized: FucT-III, FucT-IV, FucT-V, FucT-VI, FucT-VII, and FucT-IX which have demonstrated different patterns of expression among human tissues (7). Although FucT-VII has been considered as the main FucT in the synthesis of sLe^x (21, 27), we could not demonstrate its expression in AEC cell lines and primary cells both at either baseline or after mechanical injury (8). FucT-VII is expressed mainly in leukocytes and it is possible that in epithelial cells other FucTs are responsible for sLe^x synthesis. Our previous work has shown that FucT-IV is the only FucT gene expressed in all airway epithelial cells that we examined (8). In the present study we found a time-dependent increase in the expression of FucT-IV after mechanical injury. It has been demonstrated that FucT-IV over expression promotes cell proliferation (28). Several studies have demonstrated that

FucT-IV expression is significantly higher in tumors than in the adjacent normal cells (29-31) and during the first two months of embryogenesis (32). Using a siRNA targeting FucT-IV, sLe^x expression was significantly reduced. This finding demonstrates an association between FucT-IV and level of sLe^x expression in AEC. This association might not be the same in other primary cells or cell lines since a new study by Zhang *et al.* has found no significant difference in the level of sLe^x expression after FucT-IV knock down in A431 cells (33).

Next, we tested whether sLe^x modification of EGFR can modulate EGFR activation and function. Using a blocking antibody against sLe^x (KM-93) and knocking down the expression of sLe^x by FucT-IV siRNA both inhibited EGFR phosphorylation after mechanical injury and significantly reduced epithelial repair. In recent years, increased attention has been paid to the relationship between structural changes in surface glycans and membrane-bound receptor signaling. Earlier studies showed that certain lectins, which bind and block specific carbohydrate structures, are able to modify EGFR function (34, 35). Overexpression of *N*-acetylglucosaminyltransferase (GnT)-III introduces a bisecting *N*-acetylglucosamine (GlcNAc) into the *N*-glycans of EGFR in U373 MG glioma cells and generates decreased EGF binding and autophosphorylation of EGFR, as well as reduced cell proliferation upon EGF stimulation (36). Using a series of human ErbB3 mutants that lack each of the 10 N-glycosylation sites Takahashi *et al.* showed that a specific N-glycan in domain III of the ErbB family plays an essential role in receptor dimerization and activity (37). Wang *et al.* reported that in embryonic fibroblast cells derived from α1,6-fucosyltransferase (FucT-VIII) knockout mice that EGF-induced phosphorylation of EGFR and the subsequent EGFR-mediated JNK and

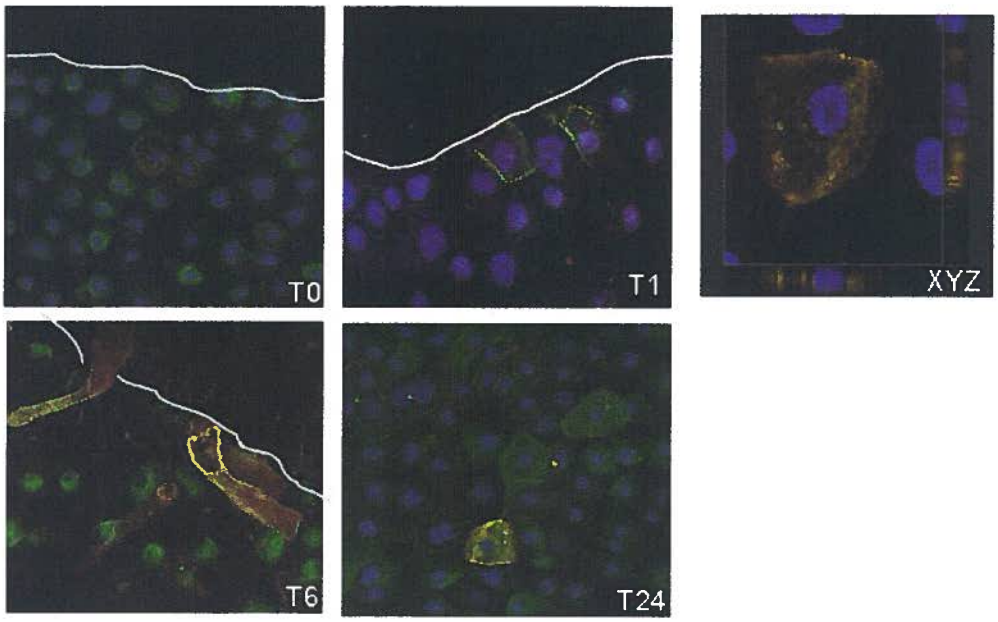
ERK activation were suppressed (38). Not only structural changes in the core portion of *N*-glycan, but alteration of the terminal residues on the outer chain of the glycans can also modify surface receptor signaling. An antibody against Le^Y, another antigen expressed on the EGFR, has been shown to inhibit EGFR-mediated signaling (39). Recently it has been documented that supression of FucT-I and FucT-IV expression reduces EGFR signaling and inhibits cell proliferation in A431 cells (33).

The literature on EGFR glycosylation suggests there are several ways by which carbohydratres can modify EGFR function. It has been noted that EGFR which express blood group A have a lower affinity for EGF, lower tyrosine kinase activity and lower turn over compared to the EGFR which does not express blood group A (40). Core fucosylation of EGFR does not affect EGFR presentation on the cell surface but it changes the binding affinity of EGF for its receptor (38). Consistent with this we found that knocking down the expression of sLe^x does not change the cell surface expression of the EGFR (data are not shown) leaving the possibility of altered ligand binding as the explanation for the marked reduction in detected phosphorylated EGFR and wound repair in FucT-IV siRNA treated AEC. Further investigations are required to demonstrate how exactly sLe^x decoration of EGFR alters receptor function.

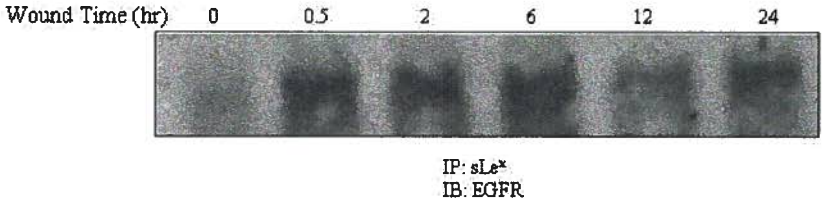
In summary we demonstrate that sLe^x modifies EGFR on AEC, this modification increases during epithelial wound repair and determines EGFR activation and function in repair. There is increasing evidence to support that normal epithelial repair is compromised in asthma (41). It has also been shown that an under-representation of fucose-containing carbohydrate structures in asthmatic patients is associated with the

disease. In this study the severity of asthma was inversely associated with the amount of fucose-containing antigens (42). As such, these results and reports suggest that defects in epithelial repair in asthmatic patients may be due, in part, to improper glycosylation of structures on airway epithelial cells. To target receptor glycosylation in disease processes may now represent a novel approach to both identify essential elements in a biological process or to intervene in a disease. Since EGFR expression has been found to be increased in several tumor types (43-45), targeting the post-translational addition of sugar residues to the EGFR would modify receptor function. With this approach it would be possible to block growth factor receptors more selectively or to regenerate a defective process such as wound repair in the AEC of asthmatics.

Fig. IV.1



A



B

Figure IV.1. sLe^x decorates EGFR in NHBE cells (A). Three linear wounds were created on confluent monolayers of primary NHBE cells grown on 4-well chamber slide using a rubber stylet. Monolayers were fixed with Clark's solution at different time points after mechanical injury. EGFR (green) and sLe^x (red) were detected using standard techniques. Using confocal microscopy we demonstrated co-localization of EGFR and sLe^x in NHBE cells and found that the signal intensity increases during wound closure and that there is a change in cellular location. Initially the signal is intra-cellular but it then moves to the cell-surface by 6 h after wound creation. Co-localization (yellow in this XYZ format) is demonstrated. White lines represent the wound edge.
Immunoprecipitation profiles of airway epithelial cell protein extracts (B). Multiple linear wounds were created on confluent monolayers of NHBE grown on 6-well plates as described previously. Equal amounts of protein were incubated with monoclonal anti-sLe^x antibody overnight and then incubated with protein A/G agarose beads for 1h at 4°C. Precipitated proteins were separated by 8% SDS PAGE and, transferred to nitrocellulose membranes and immunoblotted with anti-EGFR antibody. Both panels A and B demonstrate that sLe^x decorates EGFR in NHBE cells and that this association increases after injury.

Fig. IV.2

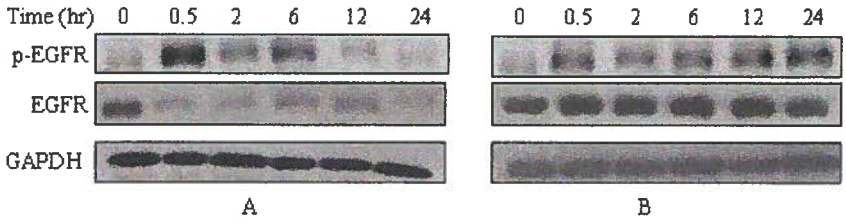


Figure IV.2. Mechanical injury stimulates phosphorylation of EGFR in a culture model of airway epithelium (A). NHBE were grown to confluency and multiple linear wounds were created as described. Protein lysates were collected at 0, 0.5, 2, 6, 12 and, 24 hr post-wounding. Expression of EGFR and p-EGFR were studied by western blotting. **An anti-sLe^x antibody alters EGFR activation following injury (B).** Injured monolayers of NHBE were treated with 40 ng/ml of an anti-sLe^x antibody and then permitted to attempt wound closure. Total protein lysates were collected at different time points and phosphorylation of EGFR was studied. Note that blocking of sLe^x on a wounded monolayer of NHBE alters the pattern of EGFR phosphorylation.

Fig. IV.3

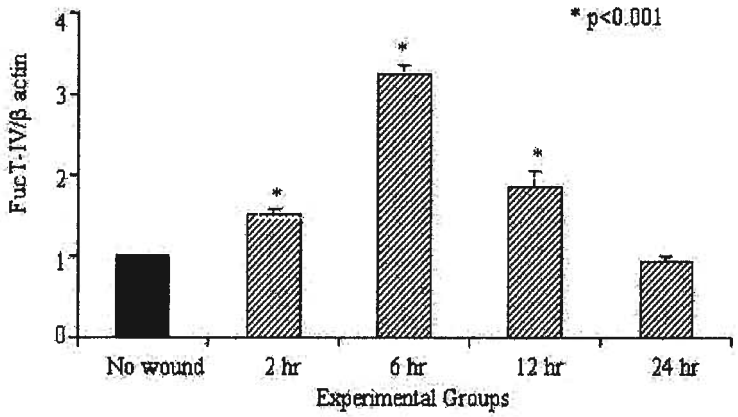


Figure IV.3. Mechanical injury induces the expression of FucT-IV by NHBE. Multiple linear wounds were created on confluent monolayers of NHBE using a rubber stylet. Control monolayers at each time point were confluent monolayers with no mechanical injury. Total RNA was extracted at different time points after mechanical injury. Reverse transcription of total cellular RNA and real-time PCR was carried out as described in Materials and Methods. In NHBE cells mechanical injury significantly induced the expression of FucT-IV at 2, 6 and, 12 h when compared to control (* $p<0.001$, $n=3$). The statistical significance of the differences between groups was determined by one-way ANOVA.

Fig. IV.4

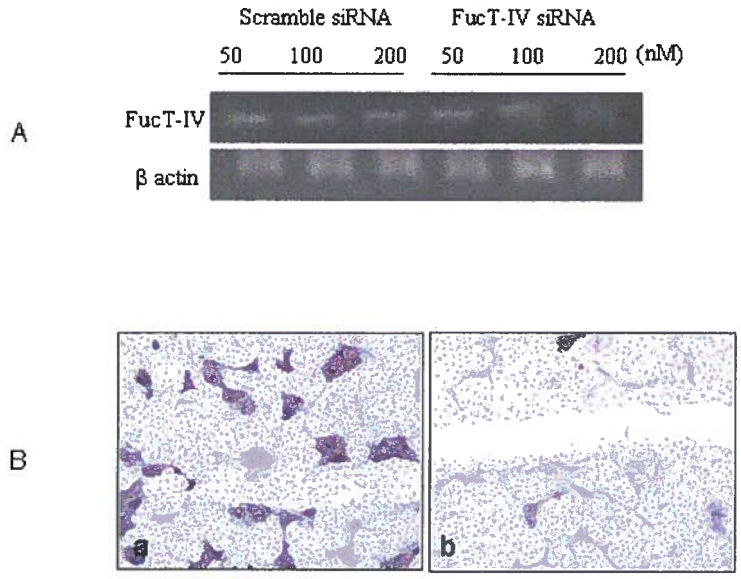
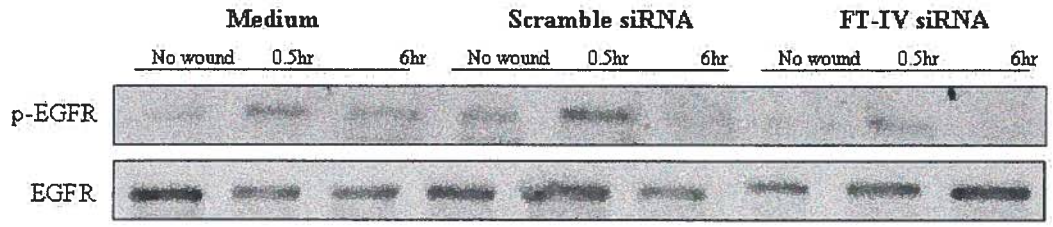
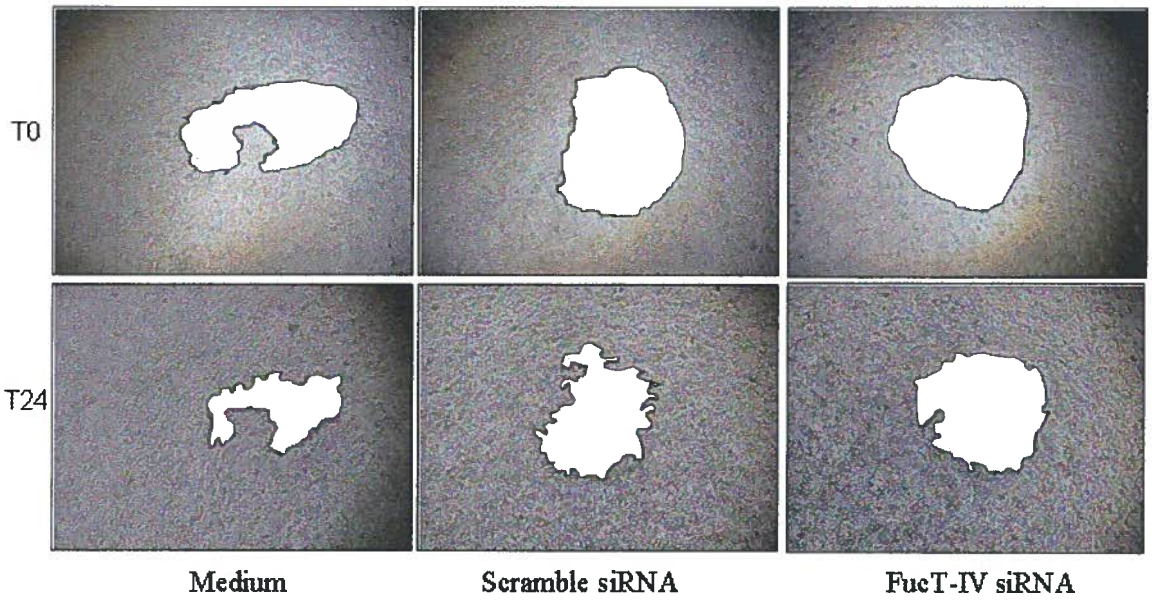


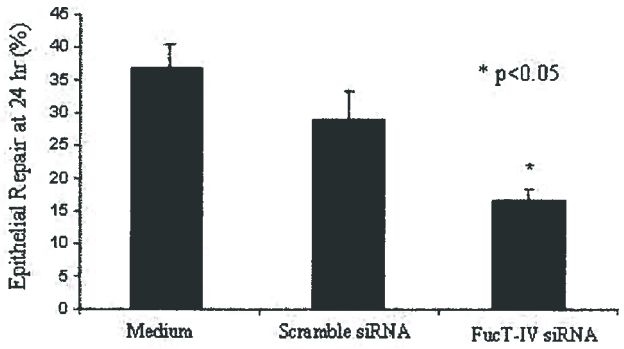
Figure IV.4. The effect of FucT-IV targeted siRNA on FucT-IV mRNA expression (A). NHBE grown to 80-90% confluency in 6-well culture plates and then transfected with 200 nM of FucT-IV siRNA in the presence of 12µl HiperFect. Total cellular RNA was extracted after 48 hr, reverse transcription of RNA and conventional PCR was carried out as described in Materials and Methods. Relative quantitation values of FucT-IV mRNA levels were normalized with respect to β-actin gene expression. **NHBE cells express less sLe^x when FucT-IV is down regulated (B).** Confluent monolayers of primary NHBE cells were kept in defined medium (a) or transfected with FucT-IV siRNA (b). After 48 hr medium was changed and fresh medium was added to both groups. Monolayers were kept in the fresh media for 8 hr and then mechanically injured using a rubber stylet. Injured monolayers were washed with PBS and fresh medium added. Monolayers were fixed with Clark's solution after 24 hr of repair and expression of sLe^x was examined as described before (8). (Magnification 4X)

Fig. IV.5


Medium
Scramble siRNA
FT-IV siRNA
No wound
0.5hr
6hr
No wound
0.5hr
6hr
No wound
0.5hr
6hr
p-EGFR
EGFR

A


T0
T24
Medium
Scramble siRNA
FucT-IV siRNA


Epithelial Repair at 24 hr (%)
45
40
35
30
25
20
15
10
5
0
* p<0.05
*
Medium
Scramble siRNA
FucT-IV siRNA

B

Figure IV.5. EGFR activation in response to mechanical injury is impaired in NHBE cells when FucT-IV is knocked down (A). Confluent monolayers of primary NHBE cells were transfected with FucT-IV siRNA or scrambled siRNA as described above and subjected to multiple linear injuries using a rubber stylet. Monolayers with no scratch wounds were used as the control. Protein cell lysates were collected at different time points after injury. Phosphorylation of EGFR was determined using an antibody that recognizes the Tyr-845 phosphorylated form of the receptor. **Airway epithelial repair is reduced in NHBE cells when FucT-IV is knocked down (B).** Confluent monolayers of primary NHBE cells were transfected with FucT-IV siRNA and mechanically wounded as described above. Injured monolayers were followed for 24 hr and photographed at different time points after injury using a Nikon Eclipse TE200 inverted scope equipped with a Nikon Coolpix E995. Corresponding wound areas were determined using ImagePro Plus and the epithelial repair was calculated as a percentage of area at time 0. (N=5)

IV.6 References

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CHAPTER V. CONCLUSION AND FUTURE DIRECTIONS

The objective of the work presented here is to determine the basic mechanism of airway epithelial repair, focusing on the EGFR and IL-13 pathways and the role for sLe^x and sLe^x decoration of EGFR in the repair. Bronchial epithelial repair is an important process by which the respiratory epithelium restores the physical barrier and tissue integrity. When any component of the repair cascade is defective this may result in persistent epithelial damage as seen in many disease states, including asthma (1-3). Impaired epithelial repair may also contribute to airway remodeling as a result of the prolonged presence and/or over-production of inflammatory mediators and growth factors. Understanding the basic mechanism of normal airway epithelial repair has important implications for identifying novel therapies for conditions resulting from impaired epithelial repair and/or a persistent repair phenotype, such as asthma.

Members of the EGF family have been shown to enhance airway epithelial repair when applied exogenously (4). However, the role for endogenous ligand(s), released by damaged or adjacent epithelium, in activation of EGFR and their role in epithelial repair had not previously been determined. IL-13 is known as a Th2 cytokine produced by T helper type 2 cells and other cells recruited to the lung during allergic responses and has been described to play a key role in pathogenesis of asthma and airway remodeling (5-7).

In Chapter II we identified that bronchial epithelial cells release EGF early and HB-EGF later in response to mechanical injury. Our data showed that proteolytic release of HB-EGF is essential for complete airway epithelial repair. Moreover, we found that IL-13 is a mediator of epithelial repair. This finding, to our knowledge, is the first study to show that IL-13 is involved in epithelial repair and coordinated via EGFR ligands. IL-

13 facilitates AEC repair by inducing HB-EGF production and secretion. Finally, in Chapter II we showed that inhibition of EGFR activity and epithelial repair resulted in a significant increase in the amount of IL-13 secreted by AEC (8). This finding suggests that the elevated expression of IL-13 may not be the primary cause for structural changes and airway remodeling in asthma but an attempt to affect repair when repair itself is impaired. This may shift the target of therapies from secondary (i.e. elevated levels of IL-13) to the primary defect of repair.

The effects of IL-13 are mediated by a complex receptor system that includes IL-IL-4Rα, IL-13Rα1 and IL-13Rα2. IL-13Rα2 was thought to act only as a decoy receptor (9, 10) until recently. However, new investigations have demonstrated that this receptor may also signal (11). Chapter II also concentrates on the role of IL-13Rα1 and α2 in airway epithelial wound repair. Our data demonstrates that the effects of IL-13 on HB-EGF synthesis and epithelial repair are mediated through IL-13Rα2. IL-13 may affect *repair* and *remodeling* via different receptor pathways. We found a novel role for IL-13Rα2 in epithelial repair. IL-13Rα1 by stat 6 activation appears to be the principal pathway that mediates mucus production, goblet cell metaplasia and other remodeling modifications (12). Identifying the relative contributions of IL-13Rα1 and IL-13Rα2 to repair and remodelling of the normal airway epithelium is the next step. For completeness this will require IL-13Rα1 and IL-13Rα2 deficient mice. Detailed investigations are then required to elucidate the pathways by which IL-13Rα2 affects HB-EGF production and epithelial repair. It is also crucial to determine the relative role and the cross-talk between IL-13Rα1 and IL-13Rα2 in disease states such as asthma.

Oligosaccharides on cell surface proteins and lipids have important roles in cell function such as adhesion (13), migration (14), and proliferation (15). Although several studies have shown important roles for glycoconjugates in epithelial repair (16-19), none of these studies identified a specific oligosaccharide structure to be involved. In Chapter III we demonstrate a critical role for the tetrasaccharide sLe^x in airway epithelial repair (20). sLe^x is a ligand of E-selectin and is important in lymphocyte homing (13, 21) and migration of cancer cells (22, 23). sLe^x is known as a tumor specific antigen. The general concept is that sLe^x promotes tumor cell motility and metastasis through interaction with endothelial E-selectins, however, another unexplored possibility is that sLe^x may present on specific receptors which control cell motility. In Chapter IV we demonstrate that sLe^x decorates EGFR on AEC. We demonstrated that while mechanical injury leads to phosphorylation of EGFR, blocking of sLe^x in the injured AEC monolayers by an inhibitory antibody or suppression of sLe^x expression by RNA interference (RNAi) for FucT-IV, prevents EGFR phosphorylation and markedly reduces epithelial repair. To understand the mechanism of sLe^x decoration and its effects on EGFR function require further investigations. It is possible that sLe^x alters localization of the EGFR on specific domain of the plasma membrane and affects receptor interaction with other structures. Another possibility is that sLe^x changes the receptor affinity to different ligands or to other members of the EGFR family and therefore alters pattern of receptor heterodimerization. Finally, sLe^x might affect EGFR trafficking and half-life on the plasma membrane.

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LIST OF PUBLICATIONS, PRESENTATIONS, AND AWARDS

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PRESENTATIONS

IL-13 signaling through the IL-13 receptor α2 mediates airway epithelial wound repair. **Allahverdian S**, Liu E. Yang J, Singhera GK, Dorscheid DR. American Thoracic Society Meeting, Toronto, Ontario, May 16-21, 2008. **(Poster)**

Alterations in Bronchial Immune Barrier in Response to Environmental Challenges Yang JS, Singhera GK, Hackett TL, Allahverdian S, Knight DA, van Eeden SF, Hegele R, Bai TR, and Dorscheid DR. American Thoracic Society Meeting, Toronto, Ontario, May 16-21, 2008. **(Poster)**

Improper EGFR Glycosylation Prevents Epidermal Growth Factor Receptor Activity and Epithelial Repair in Asthma. **Allahverdian S**, Dabiri D, Singhera GK, Dorscheid DR. American Thoracic Society Meeting, San Francisco, California, May 18-23, 2007. **(Poster)**

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HB-EGF Ectodomain Shedding and EGFR Activation Mediates Airway Epithelial Wound Repair. Harada N, **Allahverdian S**, Singhera GK, Knight DA, DR Dorscheid. 11th Congress of the Asian Pacific Society of Respirology, Kyoto, Japan, November 19-22, 2006. **(Poster)**

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Glycosylation of Epidermal Growth Factor Receptor Mediates Airway Epithelial Wound Repair. **Allahverdian S**, Dorscheid DR. American Thoracic Society 100th Anniversary. San Diego, California, May 20-25, 2005. **(Oral)**

β-catenin/TCF signaling augments vascular provisional matrix through targeting proteoglycan, versican, gene promoter. Rahmani M, Wong BW, **Allahverdian S**, Cheung C, Carthy JM, Keire P, Wight TN, McManus BM. Vascular Biology and Medicine Meeting Chicago, June 16-19, 2005. **(Oral)**

β-catenin/TCF signaling augments vascular provisional matrix through targeting proteoglycan, versican, gene promoter.Rahmani M, Wong BW, **Allahverdian S**, Cheung C, Carthy JM, McManus BM. National Research Forum for Young Investigators in Circulatory and Respiratory Health, Winnipeg, April 28- May 1, 2005. **(Oral - Finalist for Young Investigator Award)**

β-catenin/TCF signaling in vascular response to injury: role of proteoglycan versican and matrix metalloproteinase cross-talk on remodeling events. Rahmani M, Wong BW, Cheung C, **Allahverdian S**, Carthy JM, Walinski HP, McManus BM. Canadian Cardiovascular Congress, Montreal, Quebec, Oct 22-26, 2005. **(Oral)**

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Expression of Sialyl-Lewis X in Asthmatic and Normal Airway Epithelial Cells. **Allahverdian S**, Dorscheid DR. European Respiratory Society 13th Annual Congress, Vienna, Austria, September 27- October 1, 2003. **(Poster)**

Underweight, Overweight, Obesity and Relation to Dietary Intake in a Group of Adolescents: Tehran Lipid and Glucose Study. **Allahverdian S**, Mirmiran P, Mohammadi F, Sarbazi N, Azizi F. 17th International Congress of Nutrition, Vienna, Austria, 27-31 August, 2001. **(Poster)**

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Risk Profiles of Tehran Inhabitants: Interim Report from Tehran Lipid and Glucose Study (TLGS). Mirmiran P, Rahmani M, Madjid M, **Allahverdian S**, Emami H, Ghanbarian A, Hajipour R, Azizi F. 17th International Diabetes Federation Congress, Mexico City, Mexico, Nov 5-10, 2000. **(Oral)**

Coronary Artery Disease in Iranian Diabetic and Nondiabetic Patients Is Associated with the Ratio of ApolipoproteinA-I/B and Apolipoprotein B, But Not with Paraoxonase Enzyme Activity. Rahmani M, Raiszadeh F, **Allahverdian S**, Navab M, Azizi F. 17th

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Hyperinsulinemia is Associated With Increased Serum Concentrations of Apolipoproteins in Iranian Nondiabetic Subjects With Coronary Artery Disease. Rahmani M, Raiszadeh F, **Allahverdian S**, Azizi F. Oral presentation; 12th Congress of Iranian Heart Association in collaboration with Mayo Clinic and the Cleveland Clinic Foundation, Tehran, Iran, September 18-20, 2000. **(Oral)**

Association of Dietary Factors with Serum Concentration of Lipoproteins in Men: Tehran Lipid and Glucose Study (TLGS). **Allahverdian S**, Mirmiran P, Rahmani M, Madjid M, Emami H, Azizi F. The 11th Annual Congress of Iranian College of Internal Medicine, Tehran, Iran, May 2-5, 2000. **(Poster)**

Dietary Factors and Obesity in Adolescents: Tehran Lipid and Glucose Study (TLGS). **Allahverdian S**, Mirmiran P, Sheikholeslami M, Rahmani M, Azizi F. The 11th Annual Congress of Iranian College of Internal Medicine, Tehran, Iran, May 2-5, 2000. **(Oral)**

Estimation of Energy Recommended Dietary Allowances (RDA) for Adults: Tehran Lipid and Glucose Study (TLGS). Mirmiran P, **Allahverdian S**, Sheikholeslami M, Azizi F. The 11th Annual Congress of Iranian College of Internal Medicine, Tehran, Iran, May 2-5, 2000. **(Oral)**

Gender Differences in Dietary Intakes and Anthropometric Measurements in Adults: Tehran Lipid and Glucose Study (TLGS). Mohammadi F, **Allahverdian S**, Mirmiran P, Sarbazi N, Azizi F. The 11th Annual Congress of Iranian College of Internal Medicine, Tehran, Iran, May 2-5, 2000. **(Oral)**

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Apolipoprotein A-I/B Ratio As A Predictor of Coronary Artery Disease in NIDDM Patients Undergoing Coronary Angiography. Rahmani M, Kiaii SH, Motamedi MR, **Allahverdian S**, Navab M, Azizi F. The 5th International Congress of Endocrine Disorders, Tehran, Iran, September 5-9, 1999. **(Oral)**

The Prevalence of Dyslipoproteinemia in Tehran Urban District-13 Population: Interim Report of Tehran Lipid and Glucose Study (TLGS). Madjid M, Rahmani M, Moeini S, **Allahverdian S**, Emami H, Ghanbili J, Hajipour R, Mehrabi Y, Azizi F. The 5th International Congress of Endocrine Disorders, Tehran, Iran, September 5-9, 1999. **(Oral)**

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Iodine Intake, Urinary Excretion and Thyroid Function Tests in Rasht and Sari in 1998. Rahmani M, **Allahverdian S**, Sheikholeslami M, Hedayati M, Azizi F. 10th Annual Congress of Iranian College of Internal Medicine, Tehran, Iran, May 2-5, 1999. **(Oral)**

Effect of Dried Garlic Supplementation on Blood Lipids of Mild and Moderate Hypercholesterolemic Patients. A Randomised Placebo-Controlled, Double-Blind Parallel Clinical Trial. Rahmani M, Khaleghnejad Tabari A, Khososi Niaki MR, **Allahverdian S**, Sheikholeslami M. 39th Annual World Congress of the International College of Angiology, Istanbul, Turkey, June 29- July 4, 1997. **(Oral)**

Efficacy of Short-term (six week) Step One Diet with and without Garlic Powder Tablet in Primary Mild to Moderate Hypercholesterolemic Patients. A Randomised Placebo-Controlled, Double-Blind Parallel Clinical Trial. Sheikholeslami M, Rahmani M, **Allahverdian S**. 4th Iranian Society of Nutrition Annual Congress, Tehran University School of Hygiene, Tehran, Iran, October 26-29, 1996. **(Poster)**

Study of Lipids, Lipoproteins and Major CAD Risk Factors Analysed by Multiple Discriminant in MI and Control Subjects of Third Median of Mazandaran. Rahmani M, Keyhani SH, **Allahverdian S**. 9th Iranian Annual Student Congress, Isfahan University School of Medicine, Isfahan, Iran, October 6-9, 1995. **(Oral)**

AWARDS

Cordula and Gunter Paetzold Fellowship, University of British Columbia (2006-2007)

Cordula and Gunter Paetzold Fellowship, University of British Columbia (2005-2006)

Best poster presentation, Experimental Medicine Research Day, University of British Columbia (2005)

University Graduate Fellowship Award, University of British Columbia (2004-2005)

Albert B and Mary Steiner Summer Research Award, University of British Columbia (2004)

Best poster presentation, Experimental Medicine Research Day, University of British Columbia (2004)

University Graduate Fellowship Award, University of British Columbia (2003-2004)

The third rank award of Public Health and Nutrition Research in 7th National Razi Research Festival, Tehran, Iran (2002)