ROLE OF EPITHELIUM-SPECIFIC ETS TRANSCRIPTION FACTOR-1 IN AIRWAY EPITHELIAL REGENERATION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Laboratory Medicine and Pathobiology
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Abstract

Human epithelium-specific ETS transcription factor-1 (ESE-1), which is also known as E74-like factor-3 (Elf3) in mice, is strongly expressed in lung during fetal development and in certain lung cancers. The primary goal of the work presented in this thesis was to investigate whether ESE-1 is involved in regeneration of the injured lung epithelium by administering naphthalene to both wild-type (Elf3 +/+ ) and Elf3-deficient (Elf3 -/- ) mice. However, optimal conditions for proper utilization of the naphthalene-induced lung injury model must first be established. Therefore, dose-response studies were initially conducted by administering three different doses of naphthalene to both male and female mice, as described in chapter 2. Although it is shown that the extent of naphthalene-induced Clara cell injury is dose-dependent in both male and female mice, female mice are more sensitive to naphthalene-induced injury than male mice independent of the dose. Furthermore, it is also demonstrated that these gender-dependent differences in naphthalene injury can subsequently influence downstream lung repair kinetics. In light of these findings, lung regeneration was examined in both sexes of both Elf3 +/+ and Elf3 -/- mice. As reported in chapter 3, the kinetics of bronchiolar epithelial cell proliferation and differentiation is delayed considerably in Elf3 -/- mice following naphthalene injury. Moreover, expression of transforming growth factor-beta type II receptor, which is a well-known transcriptional target gene of ESE-1 and is involved in the induction of epithelial cell differentiation, is significantly
lower in the bronchiolar airway epithelium of Elf3 -/- mice as compared to Elf3 +/+ mice under steady-state conditions and during repair of naphthalene-induced damage. Collectively, these findings occur to a similar extent in both sexes of both Elf3 +/+ and Elf3 -/- mice, and suggest that ESE-1 plays an important role in regulating the kinetics of airway epithelial regeneration after acute lung injury.
Acknowledgments

Firstly, I am extremely grateful to my supervisor, Dr. Jim Hu, for all the support and guidance he has given to me throughout the course of my graduate studies. I am also very appreciative to the members of my thesis advisory committee, Drs. Herman Yeger and Thomas Waddell, for providing me with their valuable insights, assistance, and expert advice during my PhD program. I would also like to acknowledge Dr. Ernest Cutz (staff pathologist, Hospital for Sick Children) for his proficient help with histological interpretation on numerous occasions. Also, special thanks to Dr. Rahul Kushwah for his beneficial collaborations over the recent years, and to Jie Pan and Jing Wu for their required technical assistance with few experiments. A huge thank you to all of the agencies that have provided me with funding throughout the entire course of my PhD program, including Ontario Graduate Scholarships, The Hospital for Sick Children (Restracomp Studentship), and the Canadian Institutes of Health Research (Canada Graduate Scholarships Doctoral Award).

Last but certainly not least, I am forever indebted to my parents, Russell and Barbara Oliver, for their unconditional love and support. For without them, this work never would have been accomplished. Most importantly, this thesis is dedicated to my grandmother, Anne Hanutin, and to the memory of my late grandfather, Isadore Hanutin.
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List of Abbreviations

AAT: alpha-1 antitrypsin
Ang-1: angiopoietin-1
Ang II: angiotensin II
ANOVA: analysis of variance
AP-1: activator protein 1
BADJ: bronchioalveolar duct junction
BASC: bronchioalveolar stem cell
β-TrCP: beta-transducin repeat-containing protein
CBP: CREB-binding protein
CC10/CCSP: Clara cell 10-kDa secretory protein
CF: cystic fibrosis
CFTR: CF transmembrane conductance regulator
CGRP: calcitonin gene-related peptide
COL2A1: type II collagen
COPD: chronic obstructive pulmonary disease
COX-2: cyclooxygenase-2
CREB: cAMP-response element-binding
Crif1: CR6-interacting factor 1
Crif1 -/-: Crif1-deficient
CYP2F2: cytochrome P450 isoenzyme 2F2
DAB: 3,3'-diaminobenzidine tetrahydrochloride
DC: dendritic cell
Ehf: ETS homologous factor
Elf3: E74-like transcription factor-3
Elf3 +/+: wild-type
Elf3 -/-: Elf3-deficient
Elf5: E74-like transcription factor-5
ErbB-2: erythroblastic leukemia viral oncogene homolog 2
Ert: ETS-related transcription factor
ESE-1: epithelium-specific ETS transcription factor-1
ESE-2: epithelium-specific ETS transcription factor-2
ESE-3: epithelium-specific ETS transcription factor-3
Esx: epithelial-restricted with serine box
ETS: E26 transformation-specific
FoxJ1: forkhead box J1
G3PDH: glyceraldehyde-3-phosphate dehydrogenase
Gfi1: growth factor independent-1
GRP: gastrin-releasing peptide
GRPR: GRP receptor
H&E: hematoxylin and eosin
HSC: hospital for sick children
IF: immunofluorescence
IL-1β: interleukin-1beta
IL-6: interleukin-6
IL-12: interleukin-12
iPS: inducible pluripotent stem
K4: keratin 4
LI: labeling index
LPS: lipopolysaccharide
LYZ: lysozyme
MI: mitotic index
MIP-3α: macrophage inflammatory protein-3alpha
NEB: neuroepithelial body
NF-κB: nuclear factor-kappaB
NO2: nitrogen dioxide
NOS2: inducible nitric-oxide synthase
O2: oxygen
O3: ozone
OVA: ovalbumin
Pak1: p21-activated kinase-1
PDEF: prostate-derived ETS transcription factor
PGP9.5: protein gene product 9.5
PH-3: phosphohistone-3
PNEC: pulmonary neuroendocrine cell
RPE: retinal pigment epithelium
SAR: serine- and aspartic acid-rich
Sca-1: stem cell antigen-1
Scgb1a1: secretoglobin 1a1
SCID: severely compromised immunodeficient
SE: standard error
SftpC: surfactant protein C
SO2: sulfur dioxide
Sp1: specificity protein 1
SPRR1A: small proline-rich protein 1A
SPRR1B: small proline-rich protein 1B
SPRR2A: small proline-rich protein 2A

SPRR3: small proline-rich protein 3

TGF-β RII: transforming growth factor-beta type II receptor

TGM-3: transglutaminase-3

TIMP3: tissue inhibitor of metalloproteinase 3

TNF-α: tumor necrosis factor-alpha

vCE: variant CC10/CCSP-expressing

WAP: whey acidic protein
Dissemination of Thesis Content

Publications


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Chapter 1
Introduction

1.1 The E26 transformation-specific transcription factor family

The E26 transformation-specific (ETS) family of transcription factors is characterized by a highly conserved 84 amino acid DNA binding domain, known as the ETS domain (Wasylyk et al., 1993). Because the first member of the ETS family, the v-ets oncogene, was originally discovered as part of a fusion protein with gag and myb expressed by the E26 avian erythroblastosis transforming retrovirus and its DNA binding domain is E26 transformation-specific, this 84 amino acid DNA binding domain was named the ETS domain (Wasylyk et al., 1993). The ETS domain is usually located within the carboxyl-terminal region of the protein as a winged helix-turn-helix structural motif and mediates binding to sites of purine-rich DNA, commonly containing a core consensus sequence of GGAA/T, within the promoter and enhancer regions of target genes (Oikawa and Yamada, 2003). Many ETS transcription factors also contain a pointed domain, which is located within the amino-terminal region as a helix-loop-helix structural motif and is involved in protein-protein interactions, and an A/T hook domain, which mediates binding to the minor groove of AT-rich DNA sequences (Oikawa and Yamada, 2003). Additionally, there are various other domains involved in regulating ETS transcription factor activity, such as activation, auto-inhibitory, and repression domains (Oikawa and Yamada, 2003). Approximately 30 members of the ETS transcription factor family have been identified in mammals, and have been shown to play crucial roles in the regulation of many physiological and pathological processes, such as cellular proliferation and differentiation (Oikawa and Yamada, 2003), embryonic development (Jedlicka and Gutierrez-Hartmann, 2008; Kageyama et al., 2006), oncogenesis/tumorigenesis (Jedlicka and Gutierrez-Hartmann, 2008; Lincoln and Bove, 2005; Turner et al., 2007; Turner and Watson, 2008), apoptosis (Oikawa and Yamada, 2003), hematopoiesis (Gupta et al., 2009; Lacorazza and Nimer, 2003), angiogenesis (Dejana et al., 2007; Lelievre et al., 2001; Randi et al., 2009), and inflammation (Oettgen, 2006).
1.1.1 Epithelium-specific ETS transcription factor-1

While many ETS family members are expressed in non-epithelial cells, such as hematopoietic and endothelial cells, epithelium-specific ETS transcription factor-1 (ESE-1) belongs to the ESE subfamily of ETS transcription factors, which are believed to be expressed exclusively in epithelial-rich tissues, such as stomach, small intestine, colon, pancreas, trachea, lung, kidney, salivary gland, prostate gland, mammary gland, uterus, and skin (Oettgen et al., 1997a; Tymms et al., 1997). Since its initial discovery and characterization, ESE-1 has been designated many other names, such as E74-like transcription factor-3 (Elf3), ETS-related transcription factor (Ert), epithelial-restricted with serine box (Esx), and Jen. Elf3 is the murine homolog for the human ESE-1 gene and is 89% identical to its human homolog at the amino acid level (Tymms et al., 1997). Northern blot analysis of ESE-1 mRNA expression in human tissues has previously shown the highest levels of expression to occur in the small intestine, colon, and uterus (Oettgen et al., 1997a; Tymms et al., 1997). Very little to no expression of ESE-1 was detected in many epithelial-poor tissues, such as spleen, thymus, brain, heart, and skeletal muscle (Oettgen et al., 1997a; Tymms et al., 1997). Similar patterns of expression for Elf3 have also been demonstrated in mouse tissues (Tymms et al., 1997). Expression of ESE-1 has also been detected in prostate, colon, breast, and cervical cancer-derived cell lines (Feldman et al., 2003). Other members of the ESE subfamily of ETS factors also include ESE-2 (also known as Elf5), ESE-3 (also known as Ehf), and prostate-derived ETS transcription factor (PDEF). In vitro transient transfection studies using multiple ETS-responsive reporter gene constructs have demonstrated that ESE factors can function both as transcriptional activators and repressors (Feldman et al., 2003).

The following sections will focus mainly on the various roles of ESE-1 in different pathophysiological processes occurring within epithelial-rich tissues. In particular, the involvement of ESE-1 in regulating many of these processes is based on findings obtained from in vivo studies utilizing mice with a null mutation of Elf3 as well as in vitro studies utilizing various primary cells and cell lines of epithelial origin. In contrast to prior belief, ESE-1 is not expressed exclusively in epithelial cells and numerous studies have shown an induction of ESE-1 in cells of non-epithelial origin in response to inflammatory stimuli. Therefore, various roles of ESE-1 in relevant inflammatory diseases will also be discussed. Since there is substantial evidence for the involvement of ESE-1 in regulating a diverse range of pathological processes...
overall, there is a high likelihood for this transcription factor to be a candidate susceptibility gene for various disorders. Lastly, the putative target genes regulated by ESE-1 are listed in Table 1-1, and will be discussed in further detail in the following sections with regard to how expression of each target gene is modulated by ESE-1 and in which cell type it has been shown to occur.
Table 1-1: List of putative target genes regulated by ESE-1

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Cell type in which ESE-1 regulates target gene</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transforming growth factor-beta type II receptor (TGF-β RII)↑</td>
<td>Mouse intestinal epithelium</td>
<td>(Ng et al., 2002)</td>
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<td></td>
<td>Mouse bronchiolar airway epithelium</td>
<td>(Oliver et al., 2011)</td>
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<tr>
<td></td>
<td>Mouse embryonal carcinoma cell line (F9)</td>
<td>(Kim et al., 2002)</td>
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<td></td>
<td>Human embryonic kidney cell line (HEK 293T)</td>
<td>(Kopp et al., 2004)</td>
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<td></td>
<td>Human hepatoblastoma cell line (HepG2)</td>
<td>(Choi et al., 1998)</td>
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<td>Human gastric cancer cell line (SNU-620)</td>
<td>(Choi et al., 1998)</td>
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<td></td>
<td>Human colon cancer cell line (RKO)</td>
<td>(Lee et al., 2003)</td>
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<td></td>
<td>Human breast cancer cell lines (SK-BR3, Hs578t)</td>
<td>(Chang et al., 2000)</td>
</tr>
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<td></td>
<td>Human cervical cancer cell line (HeLa 229)</td>
<td>(Kim et al., 2002)</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-3alpha</td>
<td>Human colonic epithelial cell line (Caco-2)</td>
<td>(Kwon et al., 2003)</td>
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<tr>
<td>(MIP-3α)↑</td>
<td></td>
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<tr>
<td>Small proline-rich protein 1B (SPRR1B)↑</td>
<td>Primary human tracheobronchial epithelial cells</td>
<td>(Reddy et al., 2003)</td>
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<td></td>
<td>Human bronchial epithelial cell line (BEAS-2B)</td>
<td>(Reddy et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Human lung adenocarcinoma epithelial cell line</td>
<td>(Reddy et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>(NCI-H441)</td>
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<tr>
<td>Epithelium-specific ETS transcription factor-3 (ESE-3)↑</td>
<td>Human bronchial epithelial cell line (BEAS-2B)</td>
<td>(Wu et al., 2008)</td>
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<td>Human lung carcinoma cell line (A549)</td>
<td>(Wu et al., 2008)</td>
</tr>
<tr>
<td>Interleukin-6 (IL-6)↑</td>
<td>Primary mouse airway epithelial cells</td>
<td>(Kushwah et al., 2011)</td>
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<td></td>
<td>Primary mouse bone marrow-derived dendritic cells</td>
<td>(Kushwah et al., 2011)</td>
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<td></td>
<td>Human bronchial epithelial cell line (BEAS-2B)</td>
<td>(Kushwah et al., 2011)</td>
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<td>Interleukin-12 (IL-12)↑</td>
<td>Primary mouse bone marrow-derived dendritic cells</td>
<td>(Kushwah et al., 2011)</td>
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<td>Lysozyme (LYZ)↑</td>
<td>Primary human bronchial airway epithelial cells</td>
<td>(Lei et al., 2007)</td>
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<td>Human lung carcinoma cell line (A549)</td>
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<td>Human cervical cancer cell line (HeLa)</td>
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<td>Whey acidic protein (WAP)↑</td>
<td>Mouse mammary epithelial cell line (HC-11)</td>
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<td>Erythroblastic leukemia viral oncogene homolog 2 (ErbB-2)↑</td>
<td>Simian kidney fibroblast cell line (COS)</td>
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<td></td>
<td>Human cervical cancer cell line (HeLa)</td>
<td>(Eckel et al., 2003)</td>
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<tr>
<td>Protein/Marker</td>
<td>Expression Sources</td>
<td>References</td>
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<td>Small proline-rich protein 2A (SPRR2A)</td>
<td>Primary human foreskin keratinocytes, Human keratinocyte cell line (HaCaT), Simian kidney fibroblast cell line (COS-1), Mouse embryonic fibroblast cell line (NIH 3T3), Human esophageal squamous carcinoma cell lines (TE-11, TE-12), Human cervical cancer cell line (HeLa)</td>
<td>(Oettgen et al., 1997a) (Cabral et al., 2003) (Cabral et al., 2003) (Cabral et al., 2003) (Brembeck et al., 2000)</td>
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<td>Transglutaminase-3 (TGM-3)</td>
<td>Normal human epidermal keratinocytes (NHEK), Human keratinocyte cell line (HaCaT), Human cervical cancer cell line (HeLa), Human neuroblastoma cell line (SK-N-AS)</td>
<td>(Andreoli et al., 1997) (Andreoli et al., 1997) (Andreoli et al., 1997) (Andreoli et al., 1997)</td>
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<td>Prostaglandin</td>
<td>Normal human epidermal keratinocytes (NHEK), Human keratinocyte cell line (HaCaT), Human cervical cancer cell line (HeLa), Human neuroblastoma cell line (SK-N-AS)</td>
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<td>Keratin 4 (K4)</td>
<td>Human esophageal squamous carcinoma cell lines (TE-11, TE-12), Human cervical cancer cell line (HeLa)</td>
<td>(Brembeck et al., 2000)</td>
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<td>Small proline-rich protein 1A (SPRR1A)</td>
<td>Primary human neonatal foreskin epidermal keratinocytes</td>
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<tr>
<td>Small proline-rich protein 3 (SPRR3)</td>
<td>Primary human neonatal foreskin epidermal keratinocytes</td>
<td>(Fischer et al., 1999)</td>
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<td>K12 keratin</td>
<td>Mouse corneal epithelium, Human corneal epithelial cell line (HCE)</td>
<td>(Yoshida et al., 2000) (Yoshida et al., 2000)</td>
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<td>Tissue inhibitor of metalloproteinase 3 (TIMP3)</td>
<td>Rat retinal pigment epithelium, Human retinal pigment epithelium cell lines (D407, hTERT-RPE1)</td>
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<td>Angiopoietin-1 (Ang-1)</td>
<td>Primary human synovial fibroblasts, Mouse embryonic fibroblast cell line (NIH 3T3), Human embryonic kidney cell line (HEK 293), Human breast cancer cell line (MCF-7)</td>
<td>(Brown et al., 2004) (Brown et al., 2004) (Brown et al., 2004) (Brown et al., 2004)</td>
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<td>Type II collagen (COL2A1)</td>
<td>Primary human chondrocytes, Human chondrocyte cell lines (T/C-28a2, C-28/12)</td>
<td>(Peng et al., 2008) (Peng et al., 2008)</td>
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<td>Inducible nitric-oxide synthase (NOS2)</td>
<td>Primary human umbilical vein endothelial cells (HUVECs), Primary human aortic smooth muscle cells (HASMCs), Primary rat aortic smooth muscle cells (RASMCs), Human acute monocytic leukemia cell line (THP-1), Mouse leukaemic monocyte macrophage cell line (RAW 264.7)</td>
<td>(Rudders et al., 2001) (Rudders et al., 2001) (Rudders et al., 2001) (Rudders et al., 2001)</td>
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<tr>
<td>Gene</td>
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<td>-----------------</td>
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<td>-----------------</td>
</tr>
<tr>
<td>Cyclooxygenase-2 (COX-2)↑</td>
<td>Human acute monocytic leukemia cell line (THP-1)</td>
<td>(Grall et al., 2005)</td>
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</tr>
<tr>
<td></td>
<td>Human chondrocyte cell line (T/C-28a2)</td>
<td>(Grall et al., 2005)</td>
</tr>
</tbody>
</table>

Note: ↑Expression of target gene is up-regulated by ESE-1; ↓Expression of target gene is down-regulated by ESE-1.
1.1.1.1 ESE-1 in early embryonic development

Mice with a null mutation of Elf3 have previously been generated through targeted gene disruption and approximately 30% of the resultant homozygous mutant mice die *in utero* at around embryonic day 11.5 (Ng et al., 2002), suggesting that Elf3 may potentially play an important role in early embryonic development. Indeed, others have also reported an involvement of Elf3 and other ETS transcription factors in regulating mouse preimplantation embryonic development (Kageyama et al., 2006). Interestingly, evidence of abnormalities in the endometrium of the uterus characterized by disorganization of the columnar epithelium and decreased number of uterine glands has been observed in surviving Elf3-deficient (Elf3 -/-) mice (Ng et al., 2002), which may in part also be related to the partial embryonic fatality of the Elf3 -/- offspring *in utero*. Future studies aimed at elucidating the exact role of ESE-1 in early embryonic development could potentially provide new insight for this epithelial-specific transcription factor in regulating various differentiation pathways. For instance, experiments focused on examining embryonic stem cell differentiation to endoderm and then subsequently to various other epithelial cell lineages (Albert and Peters, 2009; Zorn and Wells, 2007) in Elf3 -/- mice as well as utilizing the inducible pluripotent stem (iPS) cell strategy (Takahashi and Yamanaka, 2006; Wernig et al., 2007) of genetically reprogramming somatic cells (e.g. fibroblasts) isolated from Elf3 -/- mice into pluripotent embryonic stem cell-like cells or iPS cells can potentially reveal a direct involvement of ESE-1 in regulating cellular differentiation pathways during embryonic development.

1.1.1.2 ESE-1 in regulating epithelial cell differentiation during intestinal development

Many of the Elf3 -/- progeny that survive to birth have been observed to have diminished weight gain and eventually develop a 'wasting syndrome' that is characterized by a malnourished physical appearance, watery diarrhea, and lethargy (Ng et al., 2002). Most importantly, Elf3 -/- mice also exhibit a distinct phenotype in the small intestine during fetal/neonatal development, which includes severe morphological alterations in tissue architecture manifested by poor villus formation along with improper morphogenesis of the microvilli and defective terminal differentiation of absorptive enterocytes and mucus-secreting goblet cells (Ng et al., 2002). Moreover, it has been shown that the enterocytes within the small intestinal epithelium of Elf3 -/-
mice express reduced protein levels of transforming growth factor-beta type II receptor (TGF-β RII), which is a potent inhibitor of cell proliferation and an inducer of epithelial cell differentiation (Ng et al., 2002). It was subsequently demonstrated that ectopic expression of the human TGF-β RII transgene specifically in the intestinal epithelium of Elf3 -/- mice could rescue the previously characterized intestinal defects of Elf3 -/- mice (Flentjar et al., 2007). Thus, this phenotypic rescue had provided strong in vivo evidence that Elf3 is the critical upstream regulator of TGF-β RII gene expression in the mouse small intestinal epithelium (Flentjar et al., 2007). Indeed, many in vitro studies have also established that the TGF-β RII gene is a definite target of Elf3 and that Elf3 transactivates the TGF-β RII gene promoter by binding to two adjacent ETS binding sites (Agarkar et al., 2009; Agarkar et al., 2010; Chang et al., 2000; Choi et al., 1998; Kim et al., 2002; Kopp et al., 2004; Lee et al., 2003). Interestingly, human gastric cancer cell lines do not express ESE-1 mRNA and in turn show undetectable levels of TGF-β RII mRNA (Park et al., 2001). Further evidence of ESE-1 mediating TGF-β RII gene expression has also been provided from experiments with human colon cancer cell lines (Lee et al., 2003). In human colonic epithelial cells, ESE-1 has also been shown to regulate gene expression of the proinflammatory cytokine, macrophage inflammatory protein-3alpha (MIP-3α) (Kwon et al., 2003).

Another group has previously reported that CR6-interacting factor 1 (Crif1) plays an essential role in Elf3-mediated intestinal development by functioning as a transcriptional co-activator of Elf3 during terminal differentiation of the intestinal epithelium (Kwon et al., 2009). The intestinal epithelium-specific Crif1-deficient (Crif1 -/-) mice, which were used in this study, died soon after birth and displayed severe alterations in tissue architecture of the developing small intestine, including poor microvillus formation and abnormal differentiation of absorptive enterocytes, and these phenotypes were largely similar to those previously observed in Elf3 -/- mice (Kwon et al., 2009). It was also shown that Crif1 interacted with Elf3 through its ETS DNA binding domain and enhanced the transcriptional activity of Elf3 by regulating its DNA binding activity; whereas, knockdown of Crif1 by RNA interference conversely attenuated the transcriptional activity of Elf3 (Kwon et al., 2009). In addition, the expression level of TGF-β RII, a critical target gene of Elf3, was also dramatically reduced in the Crif1 -/- mice (Kwon et al., 2009), thus suggesting that both Elf3 and Crif1 cooperate in regulating transcription of the TGF-β RII gene. However, subsequent in vitro experiments aimed at examining the detailed
molecular interactions between Elf3 and its potential binding partners at the TGF-β RII gene promoter are required in order to know the exact molecular mechanism of how TGF-β RII gene transcription is regulated. In addition, even though we now have a clearer understanding of the molecular mechanisms of Elf3-mediated intestinal development in mice, further studies are needed in order to examine a potential association of human ESE-1 with related gastrointestinal diseases, such as inflammatory bowel disease and colorectal cancer.

1.1.1.3 ESE-1 in lung cancer and development

Expression of ESE-1 has been detected in some human lung cancers, such as large-cell carcinoma and adenocarcinoma, and in lung cancer-derived cell lines, such as A549 (Tymms et al., 1997). Over-expression of the squamous differentiation marker, small proline-rich protein 1B (SPRR1B), in the bronchial airway epithelium is a marker for early metaplastic changes induced by various toxicants/carcinogens (Reddy et al., 2003). Interestingly, it has been shown that induction of SPRR1B gene expression in bronchial epithelial cells is mediated in part by protein-protein interactions between ESE-1 and other transcription factors, such as specificity protein 1 (Sp1) and activator protein 1 (AP-1), at the proximal and distal promoter regions, respectively (Reddy et al., 2003). With regards to lung development, very high levels of Elf3 expression have previously been detected within the developing fetal mouse lung (Tymms et al., 1997). However, further experiments are definitely required in order to obtain a better understanding of the exact role played by this transcription factor in both lung cancer and development.

1.1.1.4 ESE-1 in airway inflammation

It has previously been shown that ESE-1 expression is up-regulated in human bronchial airway epithelial cell lines after treatment with the proinflammatory cytokines, interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) (Wu et al., 2008). Furthermore, this cytokine-induced expression of ESE-1 is mediated by activation of the transcription factor, nuclear factor-kappaB (NF-κB), and after thorough characterization of the ESE-1 gene promoter, the NF-κB binding sequences that are required for this up-regulation of ESE-1 expression were identified (Wu et al., 2008). Indeed, others have also previously shown the presence of TATA and CCAAT boxes as well as potential binding sites for various ETS factors and NF-κB within the promoter region of
the ESE-1 gene (Oettgen et al., 1999). In addition, it has been demonstrated that ESE-1 up-regulates the expression of another member of the ESE subfamily of ETS transcription factors, ESE-3, and downregulates its own induction by cytokines, IL-1β and TNF-α (Wu et al., 2008). Lastly, reduced protein levels of the proinflammatory cytokine, interleukin-6 (IL-6), were found in the bronchoalveolar lavage fluid, lung tissue extract, and serum of Elf3 -/- mice as compared to their wild-type littermates after intranasal instillation of lipopolysaccharide (LPS) (Wu et al., 2008), suggesting a possible role for Elf3 in the regulation of IL-6 expression within the setting of airway inflammation. Interestingly, in cultured primary airway epithelial cells, ESE-1 has also been reported to transactivate the promoter of the human lysozyme gene, which is an essential component of innate immune defense in lung epithelia (Lei et al., 2007).

Since Elf3 -/- mice had shown impairment in IL-6 production upon exposure to LPS and IL-6 is a key cytokine involved in T_H17 differentiation, a potential role of Elf3 in regulating pulmonary inflammation was recently examined using an airway inflammation model that is known to be dependent on T_H17 response (Kushwah et al., 2011). Upon epicutaneous sensitization with the antigen, ovalbumin (OVA), followed by subsequent intranasal airway challenge with OVA, it was found that Elf3 -/- mice mount an impaired T_H17 response (Kushwah et al., 2011). Surprisingly, higher T_H2 antibody titers along with a more severe extent of airway inflammation was observed in Elf3 -/- mice as compared to their wild-type littermates (Kushwah et al., 2011). Since these findings were likely due to an exaggerated T_H2 response in Elf3 -/- mice, a possible involvement of Elf3 in T_H2 driven allergic airway inflammation was also investigated. Using a model of intraperitoneal sensitization with OVA followed by airway OVA challenge, it was found that Elf3 -/- mice did indeed mount an exaggerated T_H2 response (Kushwah et al., 2011). Further analysis revealed that although Elf3 -/- T cells were normal, Elf3 -/- dendritic cells (DCs) underwent hypermaturation and were impaired in the production of the T_H1 inducing cytokine, IL-12, and the T_H17 inducing cytokine, IL-6, which accounted for these exaggerated T_H2 and impaired T_H17 responses (Kushwah et al., 2011). In addition, although the regulation of genes encoding for T_H2 polarizing cytokines was normal in Elf3 -/- airway epithelial cells, IL-6 production was markedly reduced (Kushwah et al., 2011). Taken together, these findings identify a key role for Elf3 in regulating allergic airway inflammation by controlling DC driven T cell differentiation in mice. Thus, human ESE-1 may be an important factor involved in regulating the development of T_H2 and T_H17 dependent diseases, such as allergy and asthma. However, the
extent of contribution from epithelial cells in these pathological processes is not completely clear yet and requires further investigation. Further studies aimed at examining a potential connection between polymorphisms within the ESE-1 gene and susceptibility to developing various airway inflammatory diseases, such as asthma and cystic fibrosis (CF), are also needed in order to delineate these possibilities.

1.1.1.5 ESE-1 in mammary gland development

*In situ* expression analysis in human mammary gland has shown that ESE-1 is expressed specifically in the epithelial cells of the ductules and lobular structures (Thomas et al., 2000). Also, ESE-1 has been reported to positively regulate transcription of the whey acidic protein (WAP) gene in mammary epithelial cells, independently of lactogenic hormone treatment (Thomas et al., 2000). WAP is one of the major milk proteins produced by mammary epithelial cells during pregnancy and lactation. Interestingly, Elf3 mRNA levels increase within the mammary gland epithelium during murine pregnancy and early lactation (Neve et al., 1998). This suggests that ESE-1 may function to control processes related to cellular proliferation and differentiation as the mammary gland undergoes extensive epithelial cell proliferation along with subsequent differentiation and milk protein synthesis during pregnancy and lactation. In addition, the fact that murine Elf3 is also induced during involution of the mammary gland epithelium after weaning suggests that ESE-1 may also play a role in regulation of apoptotic pathways as mammary alveolar structures collapse and the secretory epithelial cells are removed during the apoptotic and remodeling phases of glandular involution (Neve et al., 1998). Further studies aimed at investigating the specific function of ESE-1 in both mammary gland development and involution are required in order to identify the exact role of this transcription factor in regulating these important physiological processes.

1.1.1.6 ESE-1 in breast cancer

ESE-1 is located at human chromosome 1q32.1 in a region known to be amplified in 50% of early breast cancers (Oettgen et al., 1997b) and ESE-1 mRNA is over-expressed at an early stage of human breast cancer development, known as ductal carcinoma *in situ* (Chang et al., 1997). In addition, the presence of both fully spliced and partially unspliced forms of ESE-1 mRNA has
been detected in human breast cancer cell lines and breast cancer tissues (Kaplan et al., 2004). Moreover, higher levels of ESE-1 mRNA and protein expression were detected in breast cancer cells than in normal breast epithelial cells, and an over-expression of ESE-1 has been observed in primary breast tumor specimens as compared to normal mammary tissues (He et al., 2007). ESE-1 expression is also up-regulated in a subset of breast tumors and breast cancer-derived cell lines that express high levels of the Her2/Neu proto-oncogene, which is also known as erythroblastic leukemia viral oncogene homolog 2 (ErbB-2) (Chang et al., 1997). Transient reporter assays using the ESE-1 promoter have shown that ESE-1 transcription is regulated by ErbB-2 receptor signaling in epithelial breast cancer cells, where expression of ErbB-2 up-regulates ESE-1 promoter activity while inhibition of ErbB-2 or its downstream signaling pathways decrease both ESE-1 promoter activity and endogenous ESE-1 protein levels (Neve et al., 2002). Thus, these findings identified the ESE-1 promoter as a potential transcriptional target of ErbB-2, and indicate ESE-1 expression as a downstream mediator of ErbB-2 signaling and ErbB-2-induced gene expression in the context of breast tumorigenesis (Neve et al., 2002). It has also been shown that ESE-1 is able to activate several malignancy-associated gene promoters, including the Her2/Neu (ErbB-2) promoter, and that ESE-1 expression is required for cellular survival of both nontransformed MCF-12A and transformed T47D human mammary epithelial cells in colony formation assays (Eckel et al., 2003). Therefore, a positive feedback loop with both ErbB-2 and ESE-1 is believed to occur based on the observations of increased ESE-1 expression in response to Her2/Neu receptor activation and ESE-1 subsequently binding and activating the Her2/Neu gene promoter. Moreover, it has also been reported that ESE-1 and ErbB-2 can cooperate to confer an invasive phenotype in human mammary epithelial cells (Coppe et al., 2010). In contrast, over-expression of ESE-1 in human breast cancer cells has been shown to induce endogenous TGF-β RII expression resulting in restitution of the TGF-β signaling pathway along with a significant reduction in resistance to the growth inhibitory effects of TGF-β (Chang et al., 2000).

Interestingly, others have demonstrated that ESE-1 expression alone can induce a transformed and in vitro metastatic phenotype along with functional and morphological epithelial to mesenchymal transition in otherwise normal MCF-12A human breast epithelial cells (Schedin et al., 2004). Furthermore, specific knockdown of endogenous ESE-1 in the human breast carcinoma ZR-75-1 and MCF-7 cell lines decreased cellular proliferation, colony formation, and
anchorage independent growth (Walker et al., 2010). Collectively, these findings suggest that ESE-1 plays a key role in maintaining the transformed phenotype by controlling cell proliferation in human breast cancer cells, thus providing a potentially novel target for future breast cancer therapy. Although it was previously presumed that many ETS factors transform mammary epithelial cells via their nuclear transcriptional functions, it has been shown that ESE-1 protein is localized within the cytoplasm in human breast cancer cells and that transformation of MCF-12A cells by stable expression of ESE-1 is mediated by the serine- and aspartic acid-rich (SAR) domain of ESE-1, which acts in the cytoplasm via an unknown nonnuclear and nontranscriptional mechanism (Prescott et al., 2004). In contrast, it has been demonstrated that the nuclear localization of ESE-1 protein induces apoptosis in nontransformed mammary epithelial cells via a transcription-dependent mechanism (Prescott et al., 2004). Others have reported that the signaling kinase, p21-activated kinase-1 (Pak1), interacts with and selectively phosphorylates ESE-1 at serine 207, which is located within the SAR domain (Manavathi et al., 2007). It was further demonstrated that ESE-1 is a labile protein, which readily undergoes ubiquitin-dependent proteolysis by interacting with the F-box-binding protein, beta-transducin repeat-containing protein (β-TrCP), and that the stability and transforming potential of ESE-1 can be enhanced via this phosphorylation-dependent regulation by Pak1 (Manavathi et al., 2007). Taken together, these studies have provided valuable knowledge towards understanding the molecular mechanisms of ESE-1-mediated transformation; however, the exact cytoplasmic- and Pak1-dependent mechanism by which ESE-1 initiates transformation in mammary epithelial cells still remains to be determined.

1.1.1.7 ESE-1 in terminal differentiation of the epidermal skin epithelium

An induction of ESE-1 expression has been reported to occur during terminal differentiation of the skin epidermis and in a primary human keratinocyte differentiation system (Oettgen et al., 1997a). Moreover, transient reporter assays have shown that ESE-1 activates expression of genes critical for terminal differentiation of epidermal keratinocytes, such as small proline-rich protein 2A (SPRR2A) (Oettgen et al., 1997a), transglutaminase-3 (TGM-3) (Andreoli et al., 1997), and profilaggrin (Andreoli et al., 1997). It has also been demonstrated that Skn-1a, a specific isoform of the transcription factor Skn-1, interacts and functionally cooperates with ESE-1 in transcriptional activation of the SPRR2A promoter in a human keratinocyte cell line (Cabral et
al., 2003). The human keratin 4 gene plays an important role in early differentiation of the esophageal squamous epithelium, and ESE-1 was also reported to suppress basal keratin 4 promoter activity and simultaneously activate the late differentiation linked SPRR2A promoter in both esophageal and cervical cancer epithelial cell lines (Brembeck et al., 2000). Interestingly, both ESE-1 and AP-1 have been shown to cooperatively regulate the expression of another human keratinocyte terminal differentiation marker, SPRR1A, at the proximal promoter region of the gene (Sark et al., 1998). In addition, the promoter region of the human SPRR3 gene contains multiple regulatory elements involved in keratinocyte differentiation-specific expression, including a high-affinity ETS binding site bound by ESE-1 (Fischer et al., 1999). Collectively, the findings obtained from the aforementioned studies suggest that ESE-1 plays an important role in regulating terminal differentiation of the epidermal epithelium; however, further studies are required to understand the exact role of ESE-1 in this important physiological process.

1.1.1.8 ESE-1 in the corneal and retinal epithelium of the eye

ESE-1 has been shown to be up-regulated upon differentiation in the embryonic/postnatal and adult mouse corneal epithelium and in immortalized human corneal epithelial cells, and this up-regulation correlated with increased expression of K12 keratin, which is a marker for differentiated corneal epithelial cells (Yoshida et al., 2000). High levels of Elf3 expression have also been reported to occur within the retinal pigment epithelium (RPE) of the retina in rats (Jobling et al., 2002). ESE-1 has also been shown to be expressed in the human RPE cell lines, D407 and hTERT-RPE1 (Jobling et al., 2002). Moreover, ESE-1 was shown to up-regulate the promoter of an important ocular gene, tissue inhibitor of metalloproteinase 3 (TIMP3) (Jobling et al., 2002). Thus, the specific expression of ESE-1 in the RPE may reflect an important role for this transcription factor in retinal function. Furthermore, the regulation of TIMP3 expression by ESE-1 may have implications for degenerative retinal diseases, such as age-related macular degeneration. Future experiments aimed at discovering the precise role of ESE-1 within both the corneal epithelium and RPE of the eye are required in order to clarify these possibilities.
1.1.1.9 ESE-1 in non-epithelial cells in the setting of inflammation

While ESE-1 is believed to be expressed exclusively in epithelial cells, this may only be the case under basal conditions as numerous studies have shown that expression of ESE-1 can be induced by the proinflammatory cytokines, IL-1β and TNF-α, and LPS in non-epithelial cells, such as synovial fibroblasts (Brown et al., 2004; Grall et al., 2003), chondrocytes (Grall et al., 2003; Peng et al., 2008), osteoblasts (Grall et al., 2003), monocytes/macrophages (Grall et al., 2003; Grall et al., 2005; Rudders et al., 2001), vascular smooth muscle cells (Rudders et al., 2001; Wang et al., 2004), and endothelial cells (Rudders et al., 2001; Wang et al., 2004; Zhan et al., 2010). More specifically, it has been shown that this induction relies on the translocation of the NF-κB family members p50 and p65 to the nucleus and subsequent transactivation of the ESE-1 promoter by a high-affinity NF-κB binding site (Grall et al., 2003). Collectively, these findings suggest that ESE-1 may play a role in mediating some of the effects of proinflammatory stimuli in various cells at sites of inflammation.

1.1.1.9.1 ESE-1 in rheumatoid arthritis and osteoarthritis

Intriguingly, expression of ESE-1 has been observed in cells of the synovial lining layer and in some mononuclear and endothelial cells in inflamed synovial tissues from patients with rheumatoid arthritis and osteoarthritis (Grall et al., 2003). It has also been demonstrated that ESE-1 can specifically bind and transactivate the promoter of the gene encoding for angiopoietin-1 (Ang-1), which is an important angiogenic growth factor that promotes the chemotaxis of endothelial cells and facilitates the maturation of new blood vessels during the process of angiogenesis in various inflammatory responses (Brown et al., 2004). Moreover, ESE-1 and Ang-1 are induced in synovial fibroblasts in response to inflammatory cytokines, with ESE-1 induction slightly preceding that of Ang-1, and both ESE-1 and Ang-1 exhibit a similar and strong expression pattern in the synovium of patients with rheumatoid arthritis (Brown et al., 2004). Taken together, these findings imply that ESE-1 may also function as a transcriptional mediator of angiogenesis in the setting of inflammation. Interestingly, an induction of ESE-1 by IL-1β was found to occur in human chondrocytes with ESE-1 functioning as a potent transcriptional suppressor of promoter activity for the type II collagen (COL2A1) gene and accounting for the sustained, NF-κB-dependent inhibition of COL2A1 expression by IL-1β.
Moreover, intracellular staining for ESE-1 was observed in chondrocytes from patients with osteoarthritis, but not in normal cartilage, suggesting a fundamental role for ESE-1 in cartilage degeneration and suppression of repair (Peng et al., 2008).

**1.1.1.9.2 ESE-1 in vascular inflammation**

Substantial evidence for the involvement of ESE-1 in vascular inflammation has also been provided from many different studies. For instance, ESE-1 has been shown to interact with the p50 subunit of NF-κB during regulation of transcription of the inducible nitric-oxide synthase (NOS2) gene (Rudders et al., 2001). Furthermore, strong expression of ESE-1 has been observed within the vascular smooth muscle and endothelium in a mouse model of endotoxemia, which is associated with acute vascular inflammation (Rudders et al., 2001). Others have reported that ESE-1 binds to and activates the promoter of the cyclooxygenase-2 (COX-2) gene in monocytes/macrophages in response to LPS (Grall et al., 2005). Since COX-2 is a key enzyme involved in the production of prostaglandins that are major inflammatory agents, neutralization of COX-2 is a potentially important goal for designing many anti-inflammatory drugs and as an activator of COX-2 induction, ESE-1 is a potential target for such therapeutics as well (Grall et al., 2005). Other proteins, including the transcriptional co-activators p300 and cAMP-response element-binding (CREB)-binding protein (CBP), have been shown to interact with ESE-1 and enhance its transcriptional activity in vascular endothelial cells (Wang et al., 2004). In contrast, the Ku proteins, Ku70 and Ku86, which are involved in repair of DNA damage, have been reported to bind to the DNA-binding domain of ESE-1 and negatively regulate its transcriptional activity (Wang et al., 2004). In vivo evidence for an involvement of ESE-1 in vascular inflammation has also been provided in an angiotensin II (Ang II)-induced model of vascular inflammation and remodeling, where Elf3 -/- mice have been observed to exhibit increased inflammatory cell infiltration into the aortic vessel wall, intimal medial thickening, and perivascular fibrosis of the aorta, as well as elevated systolic blood pressure as compared to wild-type littermate control animals (Zhan et al., 2010). Interestingly, reduced expression of NOS2 was also observed in Elf3 -/- mice infused with Ang II, suggesting that maintenance of NOS2 expression may be a mechanism by which ESE-1 offers vascular protection (Zhan et al., 2010). Collectively, these findings provide valuable insight regarding the involvement of ESE-1 in vascular dysfunction in the context of inflammation; however, further studies are required to
confirm the putative vascular protective effects of ESE-1-mediated nitric oxide release in the Ang II-dependent model of vascular inflammation.

1.2 Airway diseases

Defective airway epithelial repair following persistent injury has been proposed as an early event in the initiation of various airway diseases, and chronic defects in reparative capacity and cellular composition may further contribute to disease progression and exacerbation (Snyder et al., 2009a). Furthermore, increasing experimental evidence derived from both human studies as well as studies conducted with animal models suggests that chronic airway inflammation and subsequent epithelial injury inhibits normal airway epithelial repair and renewal, leading to aberrant remodeling of the entire epithelial mesenchymal trophic unit (Snyder et al., 2009a). Thus, understanding the cellular and molecular mechanisms of airway epithelial regeneration and repair is essential towards the development of novel therapeutic strategies for treating various airway diseases, such as chronic obstructive pulmonary disease (COPD), asthma, CF, pulmonary fibrosis, and lung cancer.

1.2.1 Chronic obstructive pulmonary disease

COPD is a category of chronic lung diseases characterized by the pathological limitation of airflow in the airway that is not fully reversible (Aoshiba and Nagai, 2004). Individuals with COPD commonly experience shortness of breath as well as increased coughing associated with increased sputum production and are prone to developing serious conditions, such as recurring chest infections, respiratory failure, pulmonary hypertension, and heart failure (Chung and Adcock, 2008). While cigarette smoking is the principal cause of COPD, chronic exposures to pollutants can also contribute to the development and/or exacerbation of COPD (Cosio and Guerassimov, 1999). COPD is common among seniors in Canada, and the most common forms of COPD are chronic bronchitis and emphysema (Cosio Piqueras and Cosio, 2001).
1.2.1.1 Chronic bronchitis

Chronic bronchitis is characterized by a persistent cough with sputum production (Banning, 2006). Evidence of goblet cell hyperplasia and hypertrophy is observed in the airways resulting in increased mucus secretion and airway obstruction (Randell, 2006). Excessive inflammatory cell infiltration can also be detected within the airway walls resulting in fibrosis and remodeling that subsequently thickens the walls of the airways and leads to narrowing of the airway lumen (Aoshiba and Nagai, 2004).

1.2.1.2 Emphysema

Emphysema is characterized by enlarged alveolar sacs causing a reduction in gas exchange surface area as well as a loss in lung elasticity, which burdens the thoracic cavity and clinically manifests as difficulty in breathing (Banning, 2006). This disease is commonly seen in smokers; however, in rare cases, individuals with a hereditary disease, such as the congenital genetic disorder, alpha-1 antitrypsin (AAT) deficiency, are also predisposed to developing COPD. Although lung epithelial cells and alveolar macrophages can also make AAT, it is primarily synthesized in the liver and is secreted into the blood where it circulates and diffuses into the lung parenchyma. The major function of AAT is to protect tissues against neutrophil elastase and pulmonary emphysema associated with AAT deficiency can lead to the unrestrained proteolytic activity of neutrophil elastase on lung connective tissue, thus resulting in alveolar destruction (Lomas and Parfrey, 2004).

1.2.2 Asthma

Asthma is a chronic disease that affects both children and adults, and is characterized by chronic inflammation and narrowing of the airways resulting in reversible airflow obstruction, bronchospasm, and variable and recurring clinical symptoms, such as coughing, shortness of breath, wheezing, and chest tightness (Cabana and Coffman, 2011). Asthma may be caused by a combination of genetic and environmental factors, and various stimuli, such as allergens, viral infections, and even exercise, can induce “episodes” or attacks (Martinez, 2007). Some of the major hallmarks of asthmatic lung disease include: airway hyper-responsiveness, extensive
inflammatory cell infiltration in the lung, goblet cell metaplasia, and airway remodeling (Aoshiba and Nagai, 2004).

1.2.3 Cystic fibrosis

CF is an autosomal recessive monogenic disorder that affects up to 1 in 3,000 people amongst the Caucasian population and is characterized by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes for an epithelial chloride channel (Riordan et al., 1989). The predominant location for CFTR expression in the airways is the tracheal and bronchial submucosal gland serous epithelial cells (Koehler et al., 2001). In the airways, a defective CFTR protein results in changes in the salt and water composition of the periciliary fluid causing isotonic dehydration and an impaired ability to clear infections along with excessive inflammation. Clinical manifestations of CF involve incompetent electrolyte transport in several epithelia, including that of the airways, pancreas, gastrointestinal ducts, and sweat glands (Fuller and Benos, 1992). However, the major cause of morbidity and mortality in CF patients is lung disease (Davis, 2006). The lungs of CF infants are almost normal at birth except for widening of the mouths of the submucosal glands, which is indicative of mucus impact (Sturgess and Imrie, 1982). However, as infants age, there is gradual colonization of the airways by various bacterial species, such as Haemophilus influenzae, Staphylococcus aureaus, and eventually Pseudomonas aeruginosa (Saiman, 2004), which initially clear with rigorous antibiotic therapy. Later on, however, there is permanent establishment of bacterial colonies, which are nearly impossible to be cleared from the airways, and this is believed to be caused by slower capture and clearance of bacteria due to extremely thick and viscous mucus production (Davis, 2006). Although the mechanism of CFTR mutations leading to CF airway disease is not clear, it has been shown that the airway surface fluid volume in CF lungs is reduced and this reduction is believed to impair airway mucociliary function, which is the first line of lung defense (Pilewski and Frizzell, 1999). In addition, CF airway epithelia have exaggerated responses to proinflammatory stimuli (Machen, 2006). It is likely that this combination of inefficient airway mucociliary clearance and exaggerated inflammatory responses contributes to the excessive neutrophil infiltration in the CF lung. Thus, the chronic bacterial infection results in persistent and chronic inflammation leading to destruction of the airway epithelium and a resultant decline in lung function in CF patients (Tomashefski et al., 1985).
1.2.4 Pulmonary fibrosis

Pulmonary fibrosis is the formation or development of excess fibrous connective tissue (i.e. fibrosis) in the lungs and is also known as “scarring of the lung.” This abnormal replacement of functional lung parenchyma with fibrotic scar tissue causes an irreversible decrease in oxygen diffusion capacity (Hardie et al., 2010). This disease can be manifested by many different symptoms, such as shortness of breath with exertion, chronic dry hacking cough, fatigue and weakness, chest discomfort, and loss of appetite together with rapid weight loss. Pulmonary fibrosis may also be a secondary effect of other interstitial lung diseases or can appear without any known cause, in which case it is known as idiopathic pulmonary fibrosis (Coward et al., 2010). Although the etiology of this relatively rare disease is poorly understood, it is generally believed that chronic epithelial injury and impaired repair may lead to disrupted communication between the airway epithelium and neighboring stroma, thus resulting in mesenchymal cell hyperproliferation and fibrogenesis (Pardo and Selman, 2002).

1.2.5 Lung cancer

Lung cancer is a disease characterized by uncontrolled cell growth in tissues of the lung with the most common symptoms being shortness of breath, coughing up blood, and weight loss. The most common cause of lung cancer in smokers is long-term exposure to tobacco smoke as well as a combination of genetic and other environmental factors, such as radon gas, asbestos, and air pollution including secondhand smoke in non-smokers. Lung cancer is classified according to histological type and the two most prevalent types of lung carcinoma are small-cell lung carcinoma and non-small-cell lung carcinoma (Gazdar and Linnoila, 1988).

1.2.5.1 Small-cell lung carcinoma

Small-cell lung carcinoma, also known as “oat cell” carcinoma, is less common than other types of lung cancer and is strongly associated with smoking. Most cases arise in the large airways, such as the primary and secondary bronchi, and the small cells contain dense neurosecretory granules (i.e. vesicles containing neuroendocrine hormones). This type of lung cancer is often
metastatic at presentation and carries a worse prognosis than non-small-cell lung carcinomas (Pierce et al., 1996).

1.2.5.2 Non-small-cell lung carcinoma

Non-small-cell lung carcinomas are the most prevalent and are grouped together because their prognosis and management are similar (Ihde and Minna, 1991). They are further grouped into three main subtypes: squamous cell lung carcinoma, adenocarcinoma, and large-cell lung carcinoma.

1.2.5.2.1 Squamous cell lung carcinoma

Squamous cell lung carcinoma usually starts near a central bronchus and a hollow cavity associated with necrosis is commonly found at the center of the tumor (Ihde and Minna, 1991). It is closely correlated with a history of tobacco smoking and is often preceded for many years by squamous cell metaplasia or dysplasia in the respiratory epithelium of the bronchi (Carter, 1985; Linnoila, 1990).

1.2.5.2.2 Adenocarcinoma

Adenocarcinoma usually originates in peripheral lung tissue and can be associated with gland and/or duct formation and/or production of significant amounts of mucus (Ihde and Minna, 1991). Although most cases of adenocarcinoma are associated with smoking, it is the most common form of lung cancer among never-smokers.

1.2.5.2.3 Large-cell lung carcinoma

Large-cell lung carcinoma is a “diagnosis of exclusion” in that the tumor cells lack light microscopic characteristics that would otherwise classify the neoplasm as a small-cell carcinoma, squamous cell carcinoma, adenocarcinoma, or any other more specific histological type of lung cancer. More specifically, large-cell lung carcinoma is primarily differentiated from small-cell lung carcinoma by the larger size of the anaplastic cells, a higher cytoplasmic-to-nuclear size
ratio, and a lack of “salt and pepper” chromatin (Nieburgs, 1982). The risk of developing large-cell lung carcinoma increases with a previous history of tobacco smoking.

1.2.6 Gender differences in airway disease

Substantial epidemiological evidence suggests that gender affects the incidence, susceptibility, and severity of several pulmonary diseases (Carey et al., 2007). For instance, it has been observed that among non-smokers, COPD affects more females than males (Ben-Zaken Cohen et al., 2007; Sin et al., 2007). In addition, although a reliable animal model for studying the pathogenesis of COPD has not yet been well established, female mice exposed to cigarette smoke have been shown to develop emphysematous-like changes in alveolar structure more rapidly than male mice (March et al., 2006). Among the general population, there is a greater prevalence of asthma in women than in men during the time of puberty to the 5th/6th decade of life (Carey et al., 2007). Intriguingly, lung function in females with CF deteriorates 26% more rapidly than that in their male counterparts (Corey et al., 1997) and female CF patients have a worse prognosis than do male CF patients as women with CF, on average, die at a significantly younger age (28 years vs. 37 years) in Canada (Corey, 1996; Corey and Farewell, 1996). Gender differences also appear to exist in a rodent model of pulmonary fibrosis as a previous study demonstrated that female rats have higher mortality rates and more severe fibrosis in the lungs than male rats in response to bleomycin treatment (Gharaee-Kermani et al., 2005). However, ovariectomized female rats displayed less pulmonary fibrosis than did sham-operated controls and hormone replacement therapy with estradiol restored the fibrotic response, suggesting that the exaggerated response of female rats to lung injury may be mediated by sex hormones (Gharaee-Kermani et al., 2005). Interestingly, studies have also shown that sex hormones, such as estrogen, can regulate alveolar formation, loss, and regeneration in the lungs of female mice (Massaro et al., 2007; Massaro and Massaro, 2004). In addition, both estrogen receptor-alpha and estrogen receptor-beta are expressed in the airways, and upon binding to its receptors, estrogen has the ability to induce lung cell proliferation and differentiation (Ben-Zaken Cohen et al., 2007). Moreover, increased estrogen levels are known risk factors for the development of lung cancer in women (Ben-Zaken Cohen et al., 2007). Although the incidence of lung cancer and the mortality rate as a result of it has appeared to reach a plateau in men, it continues to rise in women (Carey et al., 2007). Among lifetime non-smokers, lung cancer is much more common in
women than in men worldwide (Siegfried, 2001) and odds ratios for different histological types of lung cancer are consistently higher for women than for men with equivalent tobacco exposures (Zang and Wynder, 1996). In addition, adenocarcinoma of the lung is more common in women than in men regardless of smoking status (Ben-Zaken Cohen et al., 2007; Malhotra et al., 2006; Patel, 2005; Patel et al., 2004; Siegfried, 2001). Furthermore, DNA repair capacity has been shown to be 10%-15% lower in female lung cancer patients than in their male counterparts (Wei et al., 2000) and a previous study found a higher frequency of a specific mutation (i.e. G to T transversion) within the p53 gene in lung tumors of women than in those of men (Kure et al., 1996).

1.3 The mammalian lung

1.3.1 Structure and cellular composition of the adult lung

The mammalian lung is a structurally unique organ made up of conducting airways and a gas-exchange system. The conducting airways include the trachea, bronchi, and bronchioles, all of which serve to lead inspired air to the gas-exchanging regions of the lungs. The gas-exchange region is comprised of both the alveolar airspaces and adjacent blood vessels, which are also known as the pulmonary capillaries, and it is at this blood-air barrier where gas-exchange takes place. In the adult mouse, the proximal and cartilaginous conducting airways include the trachea and primary bronchi, all of which contain a pseudostratified columnar epithelium consisting of two main cell types: ciliated cells and Clara-like secretory cells. Small submucosal glands are also found in the upper airways as well as numerous basal cells, few goblet cells, and some pulmonary neuroendocrine cells (PNECs). In the more distal conducting airways, such as the small bronchi and bronchioles, there is a simple columnar/cuboidal epithelium predominately composed of Clara cells and some ciliated cells. More PNECs are also found here but no basal cells. More distally, the junction between the terminal bronchioles and the alveolar ducts can be found, and is known as the bronchioalveolar duct junction (BADJ). The gas-exchange region of the lung is mainly comprised of two epithelial cell types lining the alveolar airspaces, known as type I and type II alveolar epithelial cells, along with vascular endothelial cells lining the pulmonary capillaries. Both the structural organization and the main cell types found along the
proximodistal axis of the adult mouse lung are illustrated in Figure 1-1, and will be described further in the following sections.
Figure 1-1: A schematic illustrating the structural organization and main cell types found along the proximodistal axis of the adult mouse lung.

Note that not all of the cartilage elements associated with the trachea and main bronchi are shown, and that submucosal glands are present only in the upper part of the trachea in the mouse. The pseudostratified epithelium of the proximal (i.e. tracheobronchial) airways contains: ciliated cells, which express the transcription factor, forkhead box J1 (FoxJ1, shown in green), Clara-like secretory cells, which express secretoglobin 1a1 (Scgb1a1, shown in orange), and basal cells, which express the transcription factor, p63 (shown in black). The smaller bronchi and bronchioles also contain ciliated cells and Clara cells, as well as many more PNECs than the trachea, as shown by expression of calcitonin gene-related peptide (CGRP, shown in red). The bronchioles open into the alveoli through bronchioalveolar ducts. This junction region, known as the BADJ, is usually associated with a blood vessel (bv). The alveoli contain type II epithelial cells, which express surfactant protein C (SftpC, shown in green), and thin, flattened type I epithelial cells, which are closely apposed by pulmonary capillaries. Reproduced with permission from Rawlins and Hogan, 2006.
1.3.1.1 Basal cells

Basal cells are relatively unspecialized and undifferentiated epithelial cells scattered along the upper airways of mice, and are distinguishable by expression of cytokeratins 5 and 14 as well as the transcription factor, p63 (Rock et al., 2010). They do not appear in the large airways (i.e. trachea) until around birth and after the differentiation of ciliated and secretory cells (Rawlins and Hogan, 2006). Airway basal cells function as resident stem cells capable of both self-renewal and differentiation along the ciliated and secretory epithelial cell lineages during postnatal growth in juvenile mice and to maintain the airway epithelium during both normal steady-state conditions and repair after injury in adult mice (Hong et al., 2004b; Rock et al., 2009). Since they have abundant desmosomal and hemidesmosomal attachments, basal cells are also thought to play a major structural role within the respiratory epithelium (Evans et al., 2001). Indeed, studies have suggested that basal cells do not only contribute to epithelial cell renewal during repair after injury but may also form an epithelial barrier by flattening along the lumen of the airways to establish contact with each other, thus serving as a defense mechanism for protection against further insult to the basement membrane of the respiratory epithelium (Erjefalt et al., 1997).

1.3.1.2 Goblet cells

Goblet cells are glandular columnar epithelial cells whose sole function is to produce and secrete mucins, which can dissolve in water to form mucus. Although goblet cells are relatively abundant in human airways, they are sparse in the airways of rodents under normal pathogen-free conditions but can be induced by specific cytokine stimuli in the setting of infection and/or injury (Liu et al., 2006). Under these pathological conditions, goblet cells can contribute directly to airway obstruction through the hyperproduction of mucus. Interestingly, a member of the ESE subfamily of ETS transcription factors, PDEF, is expressed in goblet cells of the intestinal and respiratory tracts, and transgenic expression of PDEF in the respiratory epithelium of mice has been shown to cause goblet cell hyperplasia (Park et al., 2007). It has also been demonstrated that transgenic over-expression of PDEF in a subset of airway Clara cells results in their conversion to mucin-producing goblet cells (Chen et al., 2009). Furthermore, this pulmonary goblet cell differentiation was shown to be reversible and to occur in the absence of cell proliferation (Chen et al., 2009). In addition, loss-of-function experiments had also demonstrated that PDEF is required for mucus cell metaplasia in mouse models of obstructive lung disease,
including exposure to dust mite antigen and transgenic over-expression of the proinflammatory cytokine, interleukin-13 (Chen et al., 2009).

1.3.1.3 Ciliated cells

Ciliated cells are columnar airway epithelial cells that have an abundance of motile cilia on their surface. Both ciliated and secretory airway epithelial cells, including goblet cells, Clara cells, and submucosal gland serous cells, are responsible for driving the process of mucociliary clearance by which inhaled microorganisms and particulates are cleared from the airways (Rock and Hogan, 2011). Importantly, ciliated cells express the transcription factor, FoxJ1, which is required for their differentiation and the formation of motile cilia during late-stage ciliogenesis (You et al., 2004). More specifically, FoxJ1 directs apical membrane localization and docking of basal bodies as well as subsequent induction of proteins, such as β-Tubulin IV, that are required for axoneme assembly and growth during the postcentriologenesis stage of ciliogenesis (i.e. after basal body formation) in airway epithelial cells (You et al., 2004). Using transgenic lineage-labeling techniques, it has been demonstrated in adult mice that ciliated airway epithelial cells are a terminally differentiated cell population in the steady-state with an average half-life of 6 months in the trachea and 17 months in the lung (Rawlins and Hogan, 2008).

1.3.1.4 Pulmonary neuroendocrine cells

PNECs are bottle- or flask-shaped airway epithelial cells with cytoplasmic dense core neurosecretory granules, which store amine and peptide neuroendocrine hormones. These cells can be organized as solitary cells or as innervated clusters of cells, known as neuroepithelial bodies (NEBs), which are often located at airway branch points. Although it is not common to find an abundance of them in the lung during adulthood, PNECs are much more numerous during development (Linnoila, 2006). Interestingly, PNECs are the first differentiated and specialized epithelial cell type to appear in the lung during embryonic development (Linnoila, 2006). Moreover, during the early stages of lung organogenesis, PNECs/NEBs play a pivotal role in regulating cellular growth and maturation/differentiation within the airway epithelium through the paracrine secretion of various neuropeptides, such as CGRP and gastrin-releasing peptide (GRP), and bioactive amines, such as serotonin (Linnoila, 2006). Indeed, many of these
substances exhibit growth factor-like properties and may exert direct mitogenic effects on neighboring airway epithelial cells. Later on, during the transition from the intrauterine environment to breathing air (i.e. late fetal to early neonatal period), PNECs/NEBs are believed to function as oxygen-sensing airway chemoreceptors that are sensitive to hypoxia and are critical in the regulation of respiration (Youngson et al., 1993). Furthermore, it has been shown that this chemoreceptor function is mediated by an oxygen sensor enzyme complex (i.e. NADPH oxidase) that is coupled to an oxygen-sensitive potassium channel within the plasma membrane of PNECs/NEBs (Fu et al., 2000; Wang et al., 1996). Importantly, PNECs are the proposed cell of origin for small-cell lung carcinoma during adulthood (Park et al., 2011).

1.3.1.5 Clara cells

Clara cells are non-ciliated secretory epithelial cells located in both the proximal (i.e. tracheobronchial) and distal (i.e. bronchiolar) conducting airways of mice (Rawlins and Hogan, 2006). Clara cells are also described as dome-shaped cells that have an abundance of uterodomes, which are apical protrusions filled with secretory granules (Rock and Hogan, 2011). One of the main functions of Clara cells is to protect the respiratory epithelium by synthesizing and secreting much of the material lining the airway lumen, including glycosaminoglycans, various proteins (e.g. lysozymes), and conjugation of the secretory component of IgA antibodies. Clara cells are also responsible for synthesizing and secreting Clara cell 10-kDa secretory protein (CC10/CCSP), which is one of the most abundant secretory proteins found in the airways and is believed to play an important role in regulating local inflammatory responses (Harrod et al., 1998; Hayashida et al., 2000). CC10/CCSP is also important for normal Clara cell secretory function and maintenance of normal airway lining fluid composition (Stripp et al., 2002). Additionally, Clara cells are rich in smooth endoplasmic reticulum, which is where many of the xenobiotic-metabolizing enzymes are found. Thus, Clara cells are also heavily involved in both metabolic bioactivation and detoxification of various xenobiotics. Importantly, Clara cells are also believed to function as local stem/progenitor cells of the conducting airway epithelium that can divide and differentiate to produce ciliated cells as well as more Clara cells, i.e. for self-renewal (see Figure 1-2). More specifically, it has been shown in mice that during postnatal growth, adult homeostasis, or repair of the airway epithelium, the majority of Clara cells in the
bronchioles can both self-renew and generate ciliated cells, whereas in the trachea, Clara cells can give rise to ciliated cells but do not self-renew extensively (Rawlins et al., 2009).
Figure 1-2: A graphic representation of the theoretical stem cell hierarchy in the distal bronchiolar airway epithelium.

In the bronchiolar airways, PNECs have been shown to function as a self-renewing progenitor population; however, variant CC10/CCSP-expressing (vCE) stem cells located within the NEB microenvironment can undergo self-renewal as well as contribute to the regeneration of both Clara and ciliated epithelial cells. In addition, vCE cells located in close proximity to the BADJ are also known as bronchioalveolar stem cells (BASCs), and have the potential to give rise to both Clara cells of the bronchiolar epithelium and type II cells of the alveolar epithelium. Importantly, Clara cells themselves can undergo self-renewal as well as contribute towards the generation of ciliated cells. Lastly, type II alveolar epithelial cells can undergo self-renewal as well as contribute towards the generation of type I alveolar epithelial cells. Reproduced with permission of the American Thoracic Society. Copyright (c) 2011 American Thoracic Society. Reproduced from Randell, 2006.
1.3.1.6 Type I alveolar epithelial cells

Type I alveolar epithelial cells are squamous thin-walled cells that provide a gas-exchange surface (Rock and Hogan, 2011). Since type I cells are known to express various ion channels and pores, they are also believed to regulate airway fluid homeostasis (Rock and Hogan, 2011). While these cells account for most of the alveolar surface area, they are approximately half as numerous as type II cells (Williams, 2003). This is due to differences in cell size as type I cells are large, thin cells stretched across a large surface area and type II cells are much smaller cells (Williams, 2003). Type I cells are also unable to proliferate and are susceptible to various toxic insults (Uhal, 1997).

1.3.1.7 Type II alveolar epithelial cells

Type II alveolar epithelial cells are cuboidal in morphology and contain numerous secretory vesicles (i.e. lamellar bodies) filled with surfactant material, which aids in decreasing alveolar surface tension during breathing (Whitsett et al., 2010). Type II cells have long been thought to be the local stem cells of the alveolar epithelium giving rise to both type I and type II cells during both lung development and repair after injury (see Figure 1-2) (Adamson and Bowden, 1975; Evans et al., 1975).

1.4 Lung injury models

Many different laboratories have utilized various in vivo lung injury models to examine the cellular and molecular mechanisms of airway epithelial injury and repair in experimental animals. Different epithelial cell types in distinct regions of the airways are sensitive to various agents that have been commonly used to generate many of these lung injury models, which are summarized in Table 1-2 and will be described further in the following sections.
Table 1-2: Various agents or pollutants commonly used to generate lung injury models

<table>
<thead>
<tr>
<th>Pollutants</th>
<th>Injury methods</th>
<th>Injury mechanism or targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂, O₃, NO₂</td>
<td>Inhalation</td>
<td>Hyperoxic</td>
<td>Menalenbelt et al., 1992b; Smith, 1985</td>
</tr>
<tr>
<td>SO₂</td>
<td>Inhalation</td>
<td>Conducting airway</td>
<td>Aramusson et al., 1973; Langley-Evans et al., 1996</td>
</tr>
<tr>
<td>Poliocondol</td>
<td>Intratracheal instillation</td>
<td>Surface of airway and alveoli</td>
<td>Borthwick et al., 2001; Suzuki et al., 2000</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Intraperitoneal injection</td>
<td>Clara cells</td>
<td>Reynolds et al., 2000b; West et al., 2001</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>Intratracheal instillation</td>
<td>Alveolar Type I cells, alveolar Type II cells</td>
<td>Bagby et al., 1985</td>
</tr>
<tr>
<td>Elastase</td>
<td>Intratracheal instillation</td>
<td>Alveolar walls</td>
<td>Dahaybo et al., 1991</td>
</tr>
<tr>
<td>Radiation</td>
<td>Exposure</td>
<td>Exposed regions</td>
<td>Thron et al., 2002</td>
</tr>
</tbody>
</table>

Reproduced with permission from Liu et al., 2006.
1.4.1 Naphthalene-induced lung injury model

Many research groups have used acute treatment with naphthalene in mice as an experimental model to study the pathophysiological process of airway epithelial injury and repair (Kim et al., 2005; Linnoila et al., 2007; Park et al., 2006; Rawlins et al., 2007; Stripp et al., 1995; Van Winkle et al., 1995). Naphthalene is a volatile, polycyclic aromatic hydrocarbon that can form a flammable vapor and is commonly found in many different sources, such as tars, petroleum, automobile emissions, pesticides, antiseptics, tobacco smoke, and even in the ambient air (Van Winkle et al., 2004; Van Winkle et al., 2001). Naphthalene has also been used in the manufacture of various plastics, dyes, synthetic tanning agents, and solvents. Conversion of naphthalene to the cytotoxic and reactive metabolite, 1R, 2S-naphthalene oxide, is mediated by cytochrome P450 isoenzyme 2F2 (CYP2F2), which is abundantly expressed in Clara cells, thus making this cell type the primary target for injury in both the proximal and distal airways of mice (Buckpitt et al., 2002; Buckpitt et al., 1992; Buckpitt et al., 1995; Lin et al., 2009; Plopper et al., 1992a; Plopper et al., 1992b). Furthermore, conversion of naphthalene into 1R, 2S-naphthalene oxide within airway Clara cells can subsequently lead to the generation of numerous other cytotoxic metabolites, including various epoxides and quinones (Cho et al., 1995). Indeed, many of these electrophilic intermediates derived from naphthalene metabolism are extremely reactive and can undergo covalent binding to vital cellular macromolecules, such as proteins, lipids, and nucleic acids, thus resulting in oxidative stress and subsequent depletion of intracellular glutathione (Phimister et al., 2005a; Phimister et al., 2005b). In fact, glutathione conjugation is a very important pathway for detoxification of various naphthalene metabolites as studies have shown that elevated glutathione re-synthesis in the airways and maintenance of the intracellular glutathione pool can confer protection to Clara cells from naphthalene injury in mice made tolerant by repeated exposures (West et al., 2000; West et al., 2002).

Many of the critical early events associated with the initiation of naphthalene cytotoxicity and subsequent Clara cell injury have been characterized previously and include: cellular swelling; rearrangement of cytoskeletal filaments; formation of cytoplasmic vacuoles (i.e. vacuolization); formation of apical plasma membrane blebs that contain an abundance of swollen and damaged smooth endoplasmic reticulum; and the appearance of swollen mitochondria with granular matrices, all of which are followed by loss of cell membrane integrity and increased membrane...
permeability (Van Winkle et al., 1999). By 1-2 days after naphthalene exposure, injured Clara cells undergo necrosis and exfoliate into the airway lumen, resulting in denudation of the basement membrane. Cell proliferation can subsequently occur as early as 2-3 days after naphthalene injury and by 3-4 weeks, the airway epithelium is completely regenerated (Stripp et al., 1995; Van Winkle et al., 1995).

A popular method of delivering naphthalene is by intraperitoneal injection whereby it eventually reaches the lungs through the blood stream. Clara cell toxicity can occur within hours following naphthalene injection. In addition to administration by intraperitoneal injection, naphthalene can also be administered by inhalation. Importantly, the spatial pattern of injury throughout the airways after naphthalene exposure in mice has been shown to be highly dependent on the route of administration (West et al., 2001). For example, exposure to naphthalene by inhalation results in Clara cell injury that is limited to the proximal airways at low concentrations, whereas at high concentrations, injury occurs in the more distal conducting airways as well as in the proximal airways (West et al., 2001). In contrast, exposure to naphthalene by intraperitoneal injection results in injury that is limited to the distal airways at low doses, whereas at higher doses, Clara cell injury occurs in the more proximal conducting airways as well as in the distal airways (West et al., 2001). Nonetheless, both inhalation and injection of naphthalene cause dose-dependent Clara cell toxicity and injury in mice (West et al., 2001). Other important biological factors, such as sex, age, and strain, can also affect potential differences in the pulmonary injurious response to naphthalene exposure in mice (Fanucchi et al., 1997; Lawson et al., 2002; Van Winkle et al., 2002).

1.4.2 Bleomycin-induced lung injury model

Bleomycins are a family of compounds with antibiotic and antitumor activity having the major side effect of causing pulmonary toxicity, which results in injury to the alveolar regions of the lungs upon intratracheal instillation (Hay et al., 1991). The cellular toxicity of bleomycin is based on its bithiazole component, which can partially intercalate into the DNA helix and cause oxidative DNA damage (Liu et al., 2006). However, bleomycin-induced lipid peroxidation may also account for the alveolar damage and subsequent pulmonary inflammation (Liu et al., 2006). Bleomycin-induced lung injury is characterized by pulmonary fibrosis due to the increased
production of collagen and other extracellular matrix components in the lung (Bigby et al., 1985; Hay et al., 1991; Izbicki et al., 2002). Type I alveolar epithelial cells are particularly sensitive to bleomycin and are the first cells to be injured, whereas type II alveolar epithelial cells have a more variable sensitivity (Liu et al., 2006). Select populations of type II cells are thought to be important in the repair of bleomycin injury (Aso et al., 1976; Daly et al., 1998). Furthermore, bleomycin-resistant type II alveolar epithelial cells have been shown to undergo metaplasia in the presence of the drug (Izbicki et al., 2002).

1.4.3 Polidocanol-induced lung injury model

Polidocanol is a surface-active detergent clinically used to enhance absorption of small proteins/peptides and can also be experimentally used to induce injury in the proximal airways of mice (Liu et al., 2006). Other surface-active agents, such as polyoxyethylene 9 lauryl ether (Laureth-9), sodium glycocholate, and Triton X-100, have also been shown to induce lung injury after intratracheal instillation (Suzuki et al., 2000). Direct instillation of polidocanol to the mouse trachea can cause widespread denudation of the airway epithelium by 24 hours after treatment (Borthwick et al., 2001).

1.4.4 Radiation-induced lung injury model

Another model of inducing lung injury involves radiation exposure. Within 3 days of exposure to 1200 cGy of total body irradiation, mice have lungs that appear hypocellular with a breakdown of capillaries within the alveolar septae and extravasation of erythrocytes into the alveolar spaces (Liu et al., 2006). Irradiation-induced alveolar epithelial damage is quickly repaired by proliferation of type II alveolar epithelial cells followed by differentiation into type I alveolar epithelial cells (Theise et al., 2002). The notion that bone marrow-derived stem cells have the ability to contribute to repair of the injured alveolar airway epithelium has also been suggested by the transplantation of bone marrow cells retrovirally tagged with green fluorescent protein into irradiated mice (Grove et al., 2002).
1.4.5 Sulfur dioxide-induced lung injury model

Sulfur dioxide (SO$_2$) is a common air pollutant implicated in the initiation of asthmatic symptoms (Langley-Evans et al., 1996). Exposure to SO$_2$ is used as a non-specific injury model in which global repair of the epithelium within the large airways can be assessed. Since SO$_2$ is soluble in water, it is easily absorbed into the wet mucous membranes of the airway upon inhalation (Liu et al., 2006). Exposure to SO$_2$ results in severe epithelial injury within the proximal airways, such as the trachea and large bronchi, whereas the more distal airways, such as the bronchioles, are invariably spared (Asmundsson et al., 1973). This feature makes inhalation of SO$_2$ an important injury model for the proximal airways. While various species of animals have different sensitivities to SO$_2$, mice are the most sensitive and rats are the most tolerant (Liu et al., 2006). Inhalation of SO$_2$ in mice has been shown to destroy most of the pseudostratified airway epithelium in the trachea with complete regeneration occurring within 7 days following injury (Borthwick et al., 2001).

1.4.6 Hyperoxic lung injury models

Exposing the lung to high concentrations of oxygen (O$_2$) or oxidant gases, such as nitrogen dioxide (NO$_2$) or ozone (O$_3$), has been used to generate various hyperoxic lung injury models. Cell death from hyperoxic injury may occur through either apoptotic or non-apoptotic pathways, possibly by the generation of oxygen free radicals (Liu et al., 2006). The biochemical, cellular, and morphologic characterizations of hyperoxic lung injury have been studied extensively (Smith, 1985). After 3 days of inhalation exposure to high concentrations of O$_2$ in mice, mild damage consisting of alveolar septal thickening and increases in alveolar macrophages can be detected (Smith, 1985). With continuous and prolonged exposure to O$_2$, extensive damage can be induced resulting in destruction of the alveolar walls, proteinaceous exudates in alveoli, and large numbers of interstitial and alveolar polymorphonuclear leukocytes (Smith, 1985).

Prolonged inhalation exposure to NO$_2$ at 20–30 ppm in rodents has been shown to result in mild emphysema and a partially reversible decrease in lung elastin and collagen content (Liu et al., 2006). The mechanism by which NO$_2$ damages type I alveolar epithelial cells is believed to be via the oxidation of unsaturated fatty acids within the cell membrane (Evans et al., 1981).
Subacute inhalation exposure to NO$_2$ (e.g. 20 ppm NO$_2$ for 28 days) in mice is also injurious to ciliated cells and results in swelling of the ciliary shaft, focal loss of cilia, and the formation of compound cilia in the airway epithelium (Ranga and Kleinerman, 1981). Such ciliary lesions, however, appear to be reversible upon cessation of NO$_2$ exposure (Ranga and Kleinerman, 1981). In addition to mice, lung injury after acute NO$_2$ intoxication has also been described in a rat model (Meulenbelt et al., 1992a; Meulenbelt et al., 1992b).

1.4.7 Enzyme-induced emphysema model

Transtracheal delivery of elastase or trypsin can induce emphysematous-like changes in the airways of rodents (Dubaybo et al., 1991). An increase in lung volume reflecting a loss of elastic recoil along with damage to the alveolar walls resulting in the appearance of larger but fewer alveoli as well as diminished alveolar surface area are some of the typical changes that have been found to occur (Massaro and Massaro, 1997).

1.4.8 Other models of lung injury

In similarity to the liver, the lung has the capacity to undergo compensatory tissue growth following surgical resection and therefore, unilateral pneumonectomy is a classical model commonly utilized to study the cellular mechanisms of lung tissue regeneration (Hoffman et al., 2010; Nolen-Walston et al., 2008; Park et al., 2006). The ex vivo epithelial tracheal xenograft model is another approach that has historically been used to study progenitor-progeny relationships in the adult proximal airway (Duan et al., 1998; Engelhardt et al., 1991; Engelhardt et al., 1995; Engelhardt et al., 1992; Presente et al., 1997; Sehgal et al., 1996). This model involves seeding isolated airway epithelial cells onto graft tracheas that have been denuded of all endogenous airway epithelia by freeze-thawing (Liu et al., 2006). After the airway stem/progenitor cells are seeded, the tracheal grafts are implanted subcutaneously into immunocompromised hosts, such as nude mice or severely compromised immunodeficient (SCID) mice (Liu et al., 2006). A fully differentiated airway epithelium regenerates approximately 3-4 weeks after transplantation (Filali et al., 2002).
1.4.9 Gender differences in lung injury

Since female sex hormones, such as estrogen, can induce the expression of many drug-metabolizing enzymes in the lung (Ben-Zaken Cohen et al., 2007; Patel, 2005; Siegfried, 2001; Sin et al., 2007), females have the potential to undergo substantially more pulmonary metabolic bioactivation of various xenobiotics into toxic metabolites than males. For instance, studies have shown that hormonal patterns associated with different stages of the estrous cycle can alter naphthalene metabolism in the lungs of cycling virgin female mice (Stelck et al., 2005), and that metabolism of naphthalene occurs much faster and is associated with an earlier onset and greater extent of lung injury in female mice as compared to male mice administered the same dose (Van Winkle et al., 2002). In addition, another study demonstrated that the degree of lung fibrosis is enhanced and is associated with higher mortality rates in female rats as compared to male rats after bleomycin treatment (Gharaee-Kermani et al., 2005). Moreover, the extent of lung fibrosis diminishes substantially in ovariec-tomized female rats, while hormone replacement therapy with estradiol reverses this diminution and restores the pulmonary fibrotic response, indicating a potential role for female sex hormones in influencing the fibrotic response to bleomycin-induced lung injury (Gharaee-Kermani et al., 2005). Similarly, it has been reported that male sex hormones, such as testosterone, may play a role in exacerbating the decline in lung function following bleomycin-induced pulmonary fibrosis in mice (Voltz et al., 2008). Gender differences in O3-induced lung injury and inflammation have also been observed as female mice are generally more susceptible than male mice; however, the observed effect of gender on the pulmonary injurious response to O3 inhalation was found to be both age- and strain-dependent (Vancza et al., 2009).

1.5 Airway epithelial regeneration following naphthalene-induced Clara cell injury

Although cell turnover in the adult lung is relatively slow under normal homeostatic conditions with an estimated number of cells cycling at any one time being about 1% (Boers et al., 1998; Boers et al., 1999), it is capable of extensive repair and regeneration after injury (Warburton et al., 2001). Following naphthalene treatment in mice, the injured and necrotic Clara cells are
replaced by other airway epithelial stem/progenitor cells, which undergo dynamic changes in cell migration, proliferation, and differentiation (Kida et al., 2008; Rawlins and Hogan, 2006; Snyder et al., 2009a). The various cell types as well as their putative stem cell niches, which are currently known to play a role in the pulmonary regenerative response to naphthalene-induced Clara cell ablation, are illustrated in Figure 1-3 and will be discussed in greater detail in the following sections.
Figure 1-3: A graphic representation of the various cell types within their putative stem cell niches throughout the airway epithelium.

Airway epithelial stem cells and their associated niches are illustrated as follows: 1) airway basal stem cells located within the submucosal gland duct; 2) surface airway basal stem cells located at the cartilage-intercartilage junction; 3) vCE stem cells located within the NEB microenvironment; 4) BASCs located at the BADJ. Reproduced with permission of the American Thoracic Society. Copyright (c) 2011 American Thoracic Society. Reproduced from Randell, 2006.
1.5.1 Cell types involved in airway epithelial regeneration

Many different cell types have been shown to partake in the pulmonary regenerative response to naphthalene-induced Clara cell ablation in mice and those identified to date include: airway basal stem cells (Hong et al., 2004a; Hong et al., 2004b), PNECs (Linnoila et al., 2007; Peake et al., 2000; Reynolds et al., 2000b), vCE stem cells (Giangreco et al., 2009; Hong et al., 2001; Rawlins et al., 2009; Reynolds et al., 2000a), BASCs (Giangreco et al., 2002; Kim et al., 2005), ciliated cells (Lawson et al., 2002; Park et al., 2006; Rawlins et al., 2007), and peribronchiolar interstitial cells (Van Winkle et al., 1995; Van Winkle et al., 1997).

1.5.1.1 Basal cells

Basal cells have been shown to function as a multipotent progenitor capable of renewing the naphthalene-injured tracheal and bronchial airway epithelium in mice (Hong et al., 2004a; Hong et al., 2004b). These basal stem/progenitor cells can be found at two main niches within the proximal (i.e. tracheobronchial) airways: 1) the submucosal gland ducts and 2) the cartilage-intercartilage junctions (see Figure 1-3) (Engelhardt, 2001; Liu and Engelhardt, 2008; Rawlins and Hogan, 2006; Stripp and Shapiro, 2006). However, recent experimental evidence derived from lineage-tracing studies in mice has suggested that the tracheal basal cell population may include distinct facultative progenitor cell pools, which are uniformly distributed along the proximal to distal axis of the trachea (i.e. not spatially restricted to the putative stem cell niches) and can generate differentiated progeny, such as Clara-like secretory cells and ciliated cells, to restore the injured airway epithelium after naphthalene-mediated depletion of both Clara-like and ciliated cells (Cole et al., 2010; Ghosh et al., 2011). In addition, the Wnt/β-catenin signaling pathway has been reported to play an important role in regulating both the proliferation and fate determination (i.e. differentiation into either Clara-like cells or ciliated cells) of airway tracheal basal cells during repair after naphthalene-induced injury (Brechbuhl et al., 2011).

1.5.1.2 Pulmonary neuroendocrine cells

It has previously been observed in mice that PNEC hyperplasia, which is characterized by increased numbers of NEBs without significant changes in the number of isolated PNECs, can
occur in response to naphthalene-induced acute airway epithelial injury and that cell proliferation contributes to this observed PNEC response (Stevens et al., 1997). Moreover, in vivo evidence has suggested that the zinc finger transcription factor, growth factor independent-1 (Gfi1), which is required for the differentiation and proper maturation of PNECs, may play an important role in up-regulating PNEC proliferation during airway epithelial repair as gene-targeted deletion of Gfi1 in mice was shown to result in a significant reduction in PNEC proliferation after naphthalene-induced Clara cell injury (Linnoila et al., 2007). However, the timing and extent of Clara cell reconstitution was unaffected by the absence of Gfi1, thus indicating that PNEC hyperplasia may not play a dominant role in the airway regeneration process following naphthalene injury (Linnoila et al., 2007). Additionally, the basic helix-loop-helix transcription factor, achaete-scute homologue-1, which is involved in regulating PNEC differentiation, has been proposed to play a role in maintaining a progenitor phenotype that promotes renewal of both neuroendocrine and epithelial cells during repair after naphthalene-induced lung injury (Jensen-Taubman et al., 2010). Interestingly, it has previously been demonstrated in mice that acute Clara cell damage induced by exposure to naphthalene, which is a prominent carcinogen found in tobacco smoke, can transiently up-regulate expression of the neuronal/PNEC marker, protein gene product 9.5 (PGP9.5), throughout the non-neuroendocrine airway epithelium, thus strengthening the notion of PGP9.5 as a central player in the cellular response to carcinogen-inflicted injury and initiation of tumorigenesis in tobacco-exposed pulmonary epithelium (Poulsen et al., 2008).

While PNECs have been observed to proliferate after selective ablation of Clara cells, they mainly function as a self-renewing progenitor population (see Figure 1-2) and are unable to repopulate the entire airway, thus suggesting that PNECs/NEBs alone are not sufficient or completely indispensable for epithelial renewal (Linnoila et al., 2007; Peake et al., 2000; Reynolds et al., 2000b). Nonetheless, by secreting various mitogenic effectors in a paracrine fashion, the NEBs provide a crucial airway microenvironment that has been identified as one of the major niches responsible for harboring and maintaining other lung progenitor cells capable of contributing towards extensive epithelial regeneration (see Figure 1-3) (Bishop, 2004; Hong et al., 2001; Linnoila, 2006; Rawlins and Hogan, 2006; Reynolds et al., 2000a; Stripp and Reynolds, 2008; Stripp and Shapiro, 2006).
1.5.1.3 Variant CC10/CCSP-expressing cells

The vCE cells have been identified as an important population of label-retaining progenitor cells located within the NEB airway microenvironment that are critical for Clara cell renewal after naphthalene injury (see Figures 1-2 and 1-3) (Bishop, 2004; Hong et al., 2001; Linnoila, 2006; Rawlins and Hogan, 2006; Reynolds et al., 2000a; Stripp and Reynolds, 2008; Stripp and Shapiro, 2006). These vCE cells are like Clara cells in that they retain expression of CC10/CCSP; however, unlike Clara cells, they have been characterized as pollutant-resistant (i.e. naphthalene-resistant) because they lack detectable expression of CYP2F2, thus rendering them unable to generate many of the cytotoxic intermediates derived from naphthalene metabolism that are necessary for inducing cellular injury (Bishop, 2004; Hong et al., 2001; Linnoila, 2006; Rawlins and Hogan, 2006; Reynolds et al., 2000a; Stripp and Reynolds, 2008; Stripp and Shapiro, 2006).

1.5.1.4 Bronchioalveolar stem cells

BASCs are a rare population of pollutant-resistant progenitor cells that are anatomically located at another important airway niche, known as the BADJ (see Figures 1-2 and 1-3) (Giangreco et al., 2002; Kim, 2007a; Kim, 2007b; Kim et al., 2005; Rawlins and Hogan, 2006; Stripp and Reynolds, 2008; Stripp and Shapiro, 2006). These cells have been characterized thoroughly and were shown to express certain hematopoietic stem cell markers, such as the surface glycoprotein, CD34, and the stem cell antigen, Sca-1 (Kim et al., 2005). Moreover, BASCs exhibited self-renewal and were multipotent in clonal assays, further highlighting their stem cell properties (Kim et al., 2005). Interestingly, BASCs were also found to expand in response to transformation with oncogenic K-ras mutation in vitro (i.e. in culture) and in vivo (i.e. in precursors of lung adenocarcinomas of the BADJ) (Kim et al., 2005). In addition, BASCs have been shown to co-express both the Clara cell marker, CC10/CCSP, and the type II alveolar cell marker, SftpC, and have the ability to respond to either naphthalene-induced bronchiolar epithelial injury or bleomycin-induced alveolar epithelial injury by proliferating and giving rise to daughter cells capable of differentiating into either bronchiolar epithelial (i.e. Clara) cells or type II alveolar epithelial cells, respectively (see Figure 1-2) (Kim, 2007b; Kim et al., 2005; Rawlins and Hogan, 2006). A different study, however, has demonstrated with lineage-labeling experiments that the
putative BASC population is not unique in co-expressing both CC10/CCSP and SftpC, as a proportion of type II cells throughout the alveolar epithelium was found to express low levels of CC10/CCSP as well as high levels of SftpC (Rawlins et al., 2009). The data from this study had further suggested that type II cells rather than BASCs are the major stem/progenitor cell for the alveolar epithelium during repair following O2-induced (i.e. hyperoxic) alveolar injury (Rawlins et al., 2009). However, it is also possible that BASCs could contribute to the renewal of type II cells after bleomycin-induced alveolar injury. Also, it is important to acknowledge that others have reported that despite careful evaluation of more than 500 BADJs within the lungs of 22 mice after naphthalene injury, only 2 cells dually positive for both CC10/CCSP and SftpC were detected by double immunofluorescent labeling, thus indicating the rarity of this BASC population (Kida et al., 2008).

1.5.1.5 Ciliated cells

Although highly controversial, it has been proposed that in an effort to repair the naphthalene-injured bronchiolar airway epithelium in mice, ciliated cells undergo squamous metaplasia and cellular spreading, followed by proliferation and transdifferentiation into distinct epithelial cell types (Lawson et al., 2002; Park et al., 2006). However, other investigators had carried out transgenic lineage-tracing experiments in order to follow the fate of ciliated cells in mice after naphthalene-induced Clara cell ablation and showed strong evidence that ciliated cells can transiently change their morphology (i.e. undergo squamous metaplasia) but do not proliferate or transdifferentiate as part of the repair process (Rawlins et al., 2007). The exact role of ciliated cells in airway epithelial repair post naphthalene injury is currently not well defined; however, it is postulated that they may play an important role in temporarily covering up and protecting the denuded basement membrane of the injured bronchiolar airway epithelium until completion of Clara cell reconstitution. This assumption is based on the previously made observations that ciliated epithelial cells remain undamaged and intact within the naphthalene-injured bronchiolar airway epithelium, and can transiently dedifferentiate into a squamous phenotype, internalize and disassemble their cilia, and spread beneath the dying Clara cells to maintain the integrity of the airway epithelium during the repair process (Kida et al., 2008; Lawson et al., 2002; Park et al., 2006; Rawlins et al., 2007).
1.5.1.6 Peribronchiolar interstitial cells

Extensive cell proliferation has previously been detected within the peribronchiolar interstitium during repair of the naphthalene-injured bronchiolar airway epithelium in mice (Van Winkle et al., 1995; Van Winkle et al., 1997). These proliferating interstitial cells are believed to be alveolar macrophages and fibroblast-like cells, and have been reported to interact with the basal lamina of adjacent bronchiolar epithelial cells during the repair process (Van Winkle et al., 1995; Van Winkle et al., 1997). Since the naphthalene-induced model of acute Clara cell injury and subsequent regeneration is not normally associated with excessive inflammation or fibrosis (Atkinson et al., 2007), it is unlikely that the extensive interstitial cell proliferation that occurs during repair of the naphthalene-injured airway epithelium is related to these two pathological processes. The precise nature of this observed increase in peribronchiolar interstitial cell proliferation is currently not known; however, it may represent a particular facet of the airway remodeling response that can occur as part of the repair process following naphthalene-induced bronchiolar epithelial injury (Snyder et al., 2009b).
Hypotheses and Objectives

Introduction

The results presented in this thesis provide ample experimental evidence suggesting that both gender and the epithelium-specific ETS transcription factor, ESE-1, may play important roles in affecting and regulating lung regeneration, respectively, in mice after Clara cell-specific ablation. The goal of the research described in this thesis is two-fold: 1) to determine whether gender plays a major role in influencing the pulmonary regenerative response to naphthalene-induced bronchiolar epithelial injury and 2) to investigate whether ESE-1 is involved in regeneration and repair of the naphthalene-injured bronchiolar airway epithelium. Firstly, characterizing potential gender differences in airway epithelial damage and repair can contribute towards advancing our understanding of the basis for gender differences in the susceptibility to and severity of many different airway-related diseases. Secondly, characterizing the specific role played by ESE-1 in airway epithelial regeneration can potentially enhance our current understanding of how transcription factor-mediated regulation of gene expression may influence the repair process, which can occur in response to extensive airway inflammation and severe epithelial damage within the setting of various pulmonary diseases.

Rationale

Since previous studies have provided convincing data indicating that there are gender-based differences in both naphthalene metabolism and naphthalene-induced lung epithelial injury in mice (Stelck et al., 2005; Van Winkle et al., 2002), it is rational to hypothesize that there are also gender-based differences in lung regeneration and repair following naphthalene-induced injury. Moreover, while the role played by gender in affecting the short-term pulmonary injurious response to acute naphthalene exposure has been well documented, very little is known about the effect of gender on the long-term cellular repair responses. In addition, although several aspects of airway epithelial damage and repair have been studied extensively by many different research groups, the specific roles of various transcription factors in regulating the process of airway epithelial regeneration is a particular facet of this field of research that definitely requires further investigation. Because ESE-1 is an epithelial-specific transcription factor that has previously
been reported to be strongly expressed in certain human lung cancers and high expression levels of Elf3 have been detected in mouse lung tissue during fetal development (Tymms et al., 1997), it is reasonable to hypothesize that Elf3 is involved in lung epithelial regeneration. Furthermore, although previous studies have demonstrated a defect in terminal differentiation of the small intestinal epithelium in Elf3 -/- mice during fetal/postnatal development (Flentjar et al., 2007; Ng et al., 2002), very little is currently known about the role Elf3 may play in repair of the bronchiolar airway epithelium after injury. In addition, the small intestinal epithelium of Elf3 -/- mice has been shown to express reduced protein levels of TGF-β RII (Flentjar et al., 2007; Ng et al., 2002) and reduced protein levels of IL-6 have also previously been detected within the lungs of Elf3 -/- mice during airway inflammation after intranasal instillation of LPS (Wu et al., 2008). Thus, these findings have provided strong in vivo evidence suggesting that Elf3 plays a role in regulating the expression of both TGF-β RII and IL-6, and this coupled with the fact that others have shown a potential involvement of both TGF-β RII and IL-6 in the process of lung injury and repair (Kida et al., 2008; Zhao et al., 1997; Zhao and Shah, 2000) further strengthens the notion that ESE-1 may play an important role in regulating lung regeneration.

Importantly, optimal conditions for utilizing the naphthalene-induced model of airway epithelial injury and repair must first be established before exploring a possible implication of ESE-1 in regeneration of the naphthalene-injured airway epithelium. Therefore, experiments were initially conducted by administering three different doses (i.e. 50 mg/kg, 100 mg/kg, or 200 mg/kg) of naphthalene to both male and female mice, as described in chapter 2. Since the data obtained from these dose-response experiments had indicated that the high dose (i.e. 200 mg/kg) of naphthalene is optimal to specifically ablate Clara cells with a consequently adequate repair response in the lungs of both male and female mice, this dose was used for all subsequent experiments aimed at investigating the role of ESE-1 in repair of the injured bronchiolar airway epithelium in chapter 3. Furthermore, because the findings presented in chapter 2 had also suggested that gender-dependent differences in naphthalene-induced bronchiolar epithelial injury can influence downstream repair kinetics, both genders (i.e. male and female) of both wild-type (Elf3 +/+) and Elf3 -/- mice were also utilized for examining a potential involvement of ESE-1 in regulating repair of the injured bronchiolar epithelium in chapter 3.
Hypotheses

1. There are gender differences in lung repair kinetics post naphthalene-induced Clara cell damage in mice.

2. Elf3 plays a role in regulating lung repair kinetics post naphthalene-induced Clara cell damage in mice.

Objectives

1. To determine if gender is a major factor in influencing the pulmonary regenerative response to naphthalene-induced Clara cell injury in mice.

2. To investigate if Elf3 is involved in regulating the pulmonary regenerative response to naphthalene-induced Clara cell injury in mice.

3. To examine if Elf3 plays a role in regulating TGF-β RII expression during repair of the naphthalene-injured bronchiolar airway epithelium in mice.
Chapter 2

Gender differences in bronchiolar epithelial repair kinetics following naphthalene-induced Clara cell injury

2.1 Abstract

**Background:** Accumulating evidence suggests that gender affects the incidence and severity of several pulmonary diseases. Previous studies have shown gender differences in the susceptibility to naphthalene-induced lung injury in mice where the Clara cell damage was found to occur earlier and to be more extensive in females than in males. However, very little is known about whether there are any gender differences in subsequent lung repair responses.

**Objective of study:** To investigate whether gender plays an important role in influencing the pulmonary regenerative response to naphthalene-induced Clara cell ablation.

**Methods:** Adult male and female mice were injected with a low, medium, or high dose of naphthalene, and lung tissue regeneration was examined by immunohistochemical staining for the cell proliferation marker, Ki-67, and the mitosis marker, phosphohistone-3 (PH-3).

**Results:** Histopathological analysis showed that naphthalene-induced Clara cell necrosis was more prominent in the lungs of female mice as compared to male mice. Cell proliferation and mitosis in both the distal bronchiolar airway epithelium and peribronchiolar interstitium of female mice was significantly greater than that of male mice after treatment with the low and medium doses. However, after treatment with the high dose of naphthalene, lung regeneration was delayed in female mice, while male mice mounted a timely regenerative response.

**Conclusions:** Taken together, these findings show that there are clear gender differences in naphthalene-induced lung injury and repair.

2.2 Introduction

Many respiratory disorders, such as asthma and CF, involve airway inflammation and epithelial cell injury. Subsequent regeneration and repair of the injured lung epithelium is a vital process to help maintain the function and integrity of the airways. Many different laboratories have used naphthalene treatment in experimental animals, which causes Clara cell-specific injury, as a
model to study the molecular and cellular mechanisms of airway epithelial cell regeneration (Kim et al., 2005; Linnoila et al., 2007; Park et al., 2006; Rawlins et al., 2007; Stripp et al., 1995; Van Winkle et al., 1995). Naphthalene is one of the most common polycyclic aromatic hydrocarbons found in tobacco smoke, automobile emissions, pesticides, and the ambient air (Van Winkle et al., 2004; Van Winkle et al., 2001). Conversion of naphthalene to the cytotoxic and reactive metabolite, 1R, 2S-naphthalene oxide, is mediated by the xenobiotic-metabolizing isoenzyme, CYP2F2, which is abundantly expressed in Clara cells, thus making this cell type the primary target for injury in the airways of mice (Buckpitt et al., 2002; Buckpitt et al., 1992; Buckpitt et al., 1995; Lin et al., 2009; Plopper et al., 1992a; Plopper et al., 1992b). After treatment with naphthalene, the injured and necrotic Clara cells are replaced by other airway epithelial cells, which undergo dynamic changes in cell migration, proliferation, and differentiation (Kida et al., 2008; Rawlins and Hogan, 2006; Snyder et al., 2009a). Many different cell types have been shown to partake in the pulmonary regenerative response to naphthalene-induced depletion of Clara cells in mice, and those identified to date include: basal cells (Hong et al., 2004a; Hong et al., 2004b), PNECs (Linnoila et al., 2007; Peake et al., 2000; Reynolds et al., 2000b), BASCs (Giangreco et al., 2002; Kim et al., 2005), ciliated cells (Lawson et al., 2002; Park et al., 2006; Rawlins et al., 2007), peribronchiolar interstitial cells (Van Winkle et al., 1995; Van Winkle et al., 1997), and a pollutant-resistant subpopulation of Clara cells that retain their expression of CC10/CCSP, also known as vCE cells (Hong et al., 2001; Reynolds et al., 2000a).

Substantial epidemiological evidence suggests that gender affects the incidence, susceptibility, and severity of many different pulmonary diseases, such as COPD and lung cancer, with a greater prevalence and worse prognosis in women than in men (Carey et al., 2007). Since female sex hormones, such as estrogen, can induce the expression of many drug-metabolizing enzymes in the lung, females can potentially undergo more site-specific metabolic bioactivation of xenobiotics to toxic metabolites than males (Ben-Zaken Cohen et al., 2007; Patel, 2005; Siegfried, 2001; Sin et al., 2007). This may very well be the case for naphthalene, as a previous study has shown that hormonal patterns associated with different stages of the estrous cycle can alter naphthalene metabolism in the lungs of cycling virgin female mice (Stelck et al., 2005). Furthermore, another study demonstrated that female mice have a more rapid metabolism of naphthalene associated with an earlier onset and greater extent of Clara cell injury as compared
with male mice at the same dose (Van Winkle et al., 2002). However, the aforementioned study only examined gender differences in the acute lung injury response following naphthalene exposure and very little is known about the effect of gender on the long-term cellular repair responses. The goal of this study was to determine whether there is a gender-based difference in the pulmonary regenerative response, which occurs subsequent to naphthalene-induced Clara cell ablation in mice. Defining gender differences in lung injury and repair is important in understanding the basis for gender differences in the susceptibility to various pulmonary diseases.

### 2.3 Materials and Methods

**Animals and treatments**

Adult (8-12 weeks of age) male and female C57BL/6 mice were used in these experiments. Animals were housed five per cage under pathogen-free conditions, and were maintained in an environment with controlled temperature and humidity along with alternating 12 hour light/dark cycles. All animals were fed standard mouse chow and water *ad libitum*. In order to elicit a sufficient range of repair responses in the lungs of male and female mice, dose-response studies were performed by administering three different doses of naphthalene designated as follows: low dose (50 mg/kg body weight), medium dose (100 mg/kg body weight), or high dose (200 mg/kg body weight). Naphthalene (>99% pure; Sigma Chemical Co., St Louis, MO, USA) was dissolved in corn oil (Sigma Chemical Co.) and administered such that the specified doses were in 10 ml/kg body weight. Naphthalene or corn oil alone (10 ml/kg body weight, vehicle control) was administered by a single intraperitoneal injection. Groups of 3-7 mice per gender were sacrificed at different time points (0, 2, 14, and 21 days) after treatment with each dose of naphthalene or at 2 days after corn oil treatment (control group). In order to minimize the influence of diurnal fluctuations in glutathione abundance on the extent of naphthalene injury, all mice were dosed and killed in the morning between 8:00 A.M. and 10:00 A.M. All animals were purchased from Charles River Laboratories (St. Constant, QC, Canada) and maintained as per guidelines of The Hospital for Sick Children (HSC) animal facilities. All the animal studies were reviewed and approved by the HSC institutional animal care committee for humane use of laboratory animals.
Histopathological analysis

Immediately after death, mouse lung tissue was collected for routine histology and was fixed overnight in 10% buffered formalin followed by paraffin embedding. Each mouse was processed so that each paraffin-embedded tissue block contained all of the lobes of the lungs. Lung tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) for examination of naphthalene-induced histopathological tissue changes. H&E-stained mouse lung sections were scored for extent of bronchiolar epithelial (Clara) cell necrosis under a light microscope. The degree of necrosis was estimated semi-quantitatively, and was expressed for each mouse as the mean of ten random fields (1-2 airways per field) at x400 magnification within each section (one section per mouse) classified on a scale of 0-3. Scoring criteria were as follows: 0, no necrosis, defined as no detection of necrotic bronchiolar epithelial cells exfoliated within the airway lumen; 1, mild, defined as only occasional detection of necrotic bronchiolar epithelial cells exfoliated within the airway lumen; 2, moderate, defined as more frequent detection of necrotic bronchiolar epithelial cells exfoliated within the airway lumen; 3, severe, defined as very frequent detection of numerous necrotic bronchiolar epithelial cells within the airway lumen. Data are presented as the mean necrosis score ± standard error (SE) of 3-7 mice per gender at each time point after treatment with each dose of naphthalene.

Immunohistochemical labeling analysis

Serial sections (5 μm) of formalin-fixed and paraffin-embedded mouse lung tissue were also used for immunohistochemical staining for the following: 1) the Clara cell marker: CC10/CCSP; 2) the ciliated cell marker: FoxJ1; 3) the cell proliferation marker: Ki-67; and 4) the mitosis marker: PH-3. Briefly, sections were deparaffinized, rehydrated, and then microwaved in 0.01 M citrate buffer (pH 6.0) for 18.5 minutes at 1000 watts for antigen unmasking. Sections were kept in the hot citrate buffer solution for an additional 20 minutes, washed in water, treated with 3% hydrogen peroxide in methanol for 15 minutes in order to quench endogenous peroxidase activity, and then transferred to phosphate-buffered saline (pH 7.2). Tissue sections were blocked with 10% normal goat serum for 1 hour at room temperature, and then incubated with one of the following primary antibodies: rabbit polyclonal anti-mouse CC10/CCSP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 1:7000 dilution; mouse monoclonal anti-human
FoxJ1 (Seven Hills Bioreagents, Cincinnati, OH, USA) in a 1:6000 dilution; rabbit monoclonal anti-human Ki-67 (Lab Vision Corporation, Fremont, CA, USA) in a 1:200 dilution; or rabbit polyclonal anti-human PH-3 (Upstate Laboratories, Temecula, CA, USA) in a 1:6000 dilution. All primary antibodies were incubated overnight on tissue sections at 4°C, and the optimal antibody concentration that gave positive tissue staining with minimal background staining was determined separately for each antibody using a series of dilutions. Immunohistochemical staining was subsequently carried out using the immunoperoxidase method according to the guidelines for the Vectastain Elite ABC Peroxidase Kit (Vector Laboratories Inc., Burlingame, CA, USA) for the rabbit primary antibodies and the M.O.M. Immunodetection Peroxidase Kit (Vector Laboratories Inc.) for the mouse primary antibody. Sites of peroxidase binding were detected with the chromogenic 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate, and positive staining appeared brown in color. Tissue sections were counterstained with hematoxylin, and sections stained without primary antibody served as negative controls.

Measurement of Ki-67 labeling index and PH-3 mitotic index

The labeling index (LI) was determined with the cell proliferation marker, Ki-67, and was expressed as the percentage of Ki-67 positive nuclei in the distal bronchiolar airway epithelium and in the peribronchiolar interstitium. Distal bronchiolar airways were defined as distal conducting airways with a diameter of \( \leq 250 \mu m \). The distal bronchiolar airway epithelium was defined as the cells located between the basal lamina and the airway lumen, and the peribronchiolar interstitium was defined as the cells located between the basal lamina of the distal bronchiolar airway epithelium and an adjacent blood vessel, alveolus, or bronchiole. The number of Ki-67 positive nuclei and the total number of nuclei in the distal bronchiolar airway epithelium and in the peribronchiolar interstitium were counted separately in five random fields (1-2 airways per field) per section under a light microscope at x400 magnification. The LI was calculated by dividing the number of Ki-67 positive nuclei by the total number of nuclei per section (one section per mouse). Data are presented as means ± SE of 3-7 mice per gender at each time point after treatment with each dose of naphthalene. In order to confirm the gender differences in lung repair kinetics initially observed with the Ki-67 LI data, the mitotic index (MI) was also determined exactly as described above for the LI; however, instead of Ki-67, the mitosis marker, PH-3, was detected.
Statistical analysis

The results of the experiments are expressed as means ± SE. A one-way analysis of variance (ANOVA) was used to evaluate the data followed by Tukey’s post hoc-tests for statistical comparisons between groups at different time points using Prism 4.0 software. Differences were considered to be statistically significant when \( P < 0.05 \).

2.4 Results

Female mice are more susceptible than male mice to naphthalene-induced bronchiolar epithelial (Clara) cell injury independent of the dose

Naphthalene-induced histopathological changes in the lungs of male and female mice were assessed by H&E staining. At day 0 after naphthalene treatment (control group), no histopathological changes were detected and a normal bronchiolar airway epithelium, consisting mainly of columnar non-ciliated (Clara) cells and to a lesser extent ciliated cells, was observed in the lungs of both male (Figure 2-1, panels A, I, and Q) and female (Figure 2-1, panels E, M, and U) mice, as expected. Similarly, a normal airway epithelium with no histopathological changes was also observed in both male and female mice upon treatment with corn oil (data not shown). An exfoliation of numerous injured and necrotic bronchiolar epithelial cells into the airway lumen was the most prominent histopathological change observed, and was detected in female mice by 2 days after injection with the low, medium, or high doses of naphthalene (Figure 2-1, panels F, N, and V), while in male mice, it was observed only after injection with the medium or high doses (Figure 2-1, panels J and R). This sloughing of necrotic cells from the airway epithelium left the basement membrane denuded, with only few uninjured and flattened bronchiolar epithelial cells remaining intact, in both male and female mice upon treatment with the high dose of naphthalene (Figure 2-1, panels R and V). The extent of airway epithelial cell injury and necrosis was dose-dependent with the most drastic changes observed at 2 days after treatment with the high dose in both male and female mice (Figure 2-1, panels R and V). Moreover, bronchiolar epithelial injury was more prominent and occurred to a greater extent within the lungs of female mice (Figure 2-1, panels F, N, and V) as compared to male mice (Figure 2-1, panels B, J, and R) after injection with any of the three different doses (i.e. low dose,
medium dose, or high dose) of naphthalene, and was also confirmed semi-quantitatively by necrosis scoring (Table 2-1). By 2 days after treatment with each dose of naphthalene, the mean necrosis score of the injured bronchiolar airway epithelium peaked in both male and female mice and was significantly greater in female mice as compared to male mice (Table 2-1). Within 14-21 days after injection with each dose of naphthalene, the mean necrosis score returned to baseline control levels (Table 2-1) and the airway epithelium appeared to be fully regenerated and restored with an abundance of columnar non-ciliated (Clara) cells in both male (Figure 2-1, panels C-D, K-L, and S-T) and female (Figure 2-1, panels G-H, O-P, and X) mice. However, few residual necrotic and exfoliated bronchiolar epithelial cells were detected within the airway lumen at 14 days after injection with the high dose in female mice (Figure 2-1, panel W) but not in male mice (Figure 2-1, panel S), thus showing faster recovery from naphthalene-induced airway epithelial cell injury in male mice. The mean necrosis score was also significantly greater in female mice as compared to male mice at 14 days after injection with the high dose of naphthalene (Table 2-1).
Figure 2-1: H&E analysis of bronchiolar epithelial (Clara) cell injury and renewal in male and female mice after treatment with a low, medium, or high dose of naphthalene.

Lung histopathology was examined at 0, 2, 14, and 21 days after injection with (A-H) low dose (50 mg/kg), (I-P) medium dose (100 mg/kg), or (Q-X) high dose (200 mg/kg) of naphthalene. A normal bronchiolar airway epithelium was detected in both male (panels A, I, and Q) and female (panels E, M, and U) control mice at day 0. Injured and necrotic Clara cells, which had exfoliated into the bronchiolar airway lumen, were detected in male mice at 2 days after injection with either the medium or high dose of naphthalene (asterisks in panels J and R, respectively) and in female mice at 2 days after injection with the low, medium, or high dose of naphthalene (asterisks in panels F, N, and V, respectively). Few residual injured and necrotic Clara cells were also detected within the airway lumen in female mice at 14 days after injection with the high dose of naphthalene (asterisks in panel W). See Table 2-1 for necrosis scoring. A fully regenerated airway epithelium was restored within 14-21 days after injection with each dose of naphthalene in both male (panels C-D, K-L, and S-T) and female (panels G-H, O-P, and X) mice. Photomicrographs are representative of 3-7 mice per gender at each time point after treatment with each dose of naphthalene. All scale bars: 50 µm. Magnification, x400.
Table 2-1: Necrosis scoring of male and female mouse lung sections at different time points after treatment with a low, medium, or high dose of naphthalene

<table>
<thead>
<tr>
<th>Time (days) post naphthalene injection</th>
<th>Mean necrosis score/time point after naphthalene injection</th>
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<tbody>
<tr>
<td></td>
<td>Low dose (50 mg/kg)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>2</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>14</td>
<td>0.07±0.03</td>
</tr>
<tr>
<td>21</td>
<td>0.0±0.0</td>
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Histopathological analysis of H&E-stained male and female mouse lung sections at 0, 2, 14, and 21 days after injection with low dose (50 mg/kg), medium dose (100 mg/kg), or high dose (200 mg/kg) of naphthalene, as shown in Figure 2-1, was performed and the extent of bronchiolar epithelial (Clara) cell necrosis was scored. See Materials and Methods for details. Data are presented as the mean necrosis score ± SE of 3-7 mice per gender at each time point after treatment with each dose of naphthalene. *Significantly different from that of male mice at same time point after treatment with same dose of naphthalene (P<0.05). #Significantly different from the day 0 (control) group (P<0.05). The degree of Clara cell necrosis was estimated semi-quantitatively, and was expressed for each mouse as the mean of ten random fields (1-2 airways per field) within each section (one section per mouse) classified on a scale of 0-3. Scoring criteria were as follows: 0, no necrosis, defined as no detection of necrotic bronchiolar epithelial cells exfoliated within the airway lumen; 1, mild, defined as only occasional detection of necrotic bronchiolar epithelial cells exfoliated within the airway lumen; 2, moderate, defined as more frequent detection of necrotic bronchiolar epithelial cells exfoliated within the airway lumen; 3, severe, defined as very frequent detection of numerous necrotic bronchiolar epithelial cells within the airway lumen.
Since the H&E analysis of lung injury had shown that the most drastic histopathological changes occur after treatment with the high dose of naphthalene (Figure 2-1), we decided to focus exclusively on this dose for studying naphthalene-induced changes in Clara cells and ciliated cells.

In order to follow the fate of Clara cells in male and female mice after naphthalene treatment, immunohistochemical staining for the Clara cell marker, CC10/CCSP, was performed (Figure 2-2, panels A-H). A normal bronchiolar airway epithelium, which stained strongly for CC10/CCSP, was detected in both male (Figure 2-2A) and female (Figure 2-2E) control mice at day 0. Injured and necrotic bronchiolar epithelial cells, which had exfoliated into the airway lumen, were more abundant in female mice (Figure 2-2F) as compared to male mice (Figure 2-2B) at 2 days post naphthalene injection. These exfoliated cells also stained very strongly for CC10/CCSP in both male (Figure 2-2B) and female (Figure 2-2F) mice, thus confirming that the naphthalene injury was specific to Clara cells. In addition, the diminished residual airway epithelium, which had remained intact after the massive Clara cell ablation, stained very weakly for CC10/CCSP in both male (Figure 2-2B) and female (Figure 2-2F) mice at 2 days after naphthalene treatment. Residual necrotic and exfoliated Clara cells were also detected within the airway lumen at 14 days post naphthalene injection in female mice only (Figure 2-2G). Within 14-21 days, the naphthalene injury had cleared up, and the airway epithelium now resembled that of the day 0 control mice and stained very strongly for CC10/CCSP in both male (Figure 2-2, panels C-D) and female (Figure 2-2, panel H) mice, thus suggesting Clara cell renewal.

In order to follow the fate of ciliated cells in male and female mice after naphthalene-induced Clara cell injury, immunohistochemical staining for the ciliated cell marker, FoxJ1, was performed (Figure 2-2, panels I-P). In the day 0 control group, a normal bronchiolar airway epithelium, containing several FoxJ1-positive (ciliated) cells as well as FoxJ1-negative (Clara) cells, was detected in both male (Figure 2-2I) and female (Figure 2-2M) mice. By 2 days post naphthalene injection, the injured and exfoliated bronchiolar epithelial cells detected within the airway lumen did not stain for FoxJ1 in both male (Figure 2-2J) and female (Figure 2-2N) mice, thus confirming that ciliated cells were not injured even after treatment with the high dose of naphthalene used in this study. In addition, many of the residual airway epithelial cells, which remained intact after naphthalene-induced Clara cell ablation, stained positively for FoxJ1,
suggesting that ciliated cells can remain behind to possibly help cover up and protect the denuded basement membrane of the injured airway epithelium in both male (Figure 2-2J) and female (Figure 2-2N) mice. Within 14-21 days after injection with naphthalene, the regenerative response was complete and the airway epithelium resembled that of the day 0 control groups in both male (Figure 2-2, panels K-L) and female (Figure 2-2, panels O-P) mice.
Figure 2-2: Immunohistochemical staining for the Clara cell marker, CC10/CCSP, and the ciliated cell marker, FoxJ1, in male and female mouse lungs after naphthalene treatment.

(A-H) Immunostaining for CC10/CCSP in male (A-D) and female (E-H) mice at 0 (A and E), 2 (B and F), 14 (C and G), and 21 (D and H) days after injection with the high dose (200 mg/kg) of naphthalene was performed. At day 0 (control group), a normal bronchiolar airway epithelium, which stained strongly for CC10/CCSP, was detected in both male (panel A) and female (panel E) mice. By 2 days post naphthalene injection, injured and necrotic bronchiolar epithelial cells were detected within the airway lumen and stained very strongly for CC10/CCSP in both male and female mice (asterisks in panels B and F, respectively). These injured CC10/CCSP-positive bronchiolar epithelial cells were also detected within the airway lumen at 14 days post naphthalene injection in female mice only (asterisks in panel G). A fully regenerated airway epithelium, which stained strongly for CC10/CCSP, was restored within 14-21 days after injection with naphthalene in both male (panels C-D) and female (panel H) mice. Positive staining for CC10/CCSP appears brown in color, and sections were counterstained with hematoxylin. Photomicrographs are representative of 3-7 mice per gender at each time point after treatment with naphthalene. Scale bars: 50 μm. Magnification, x400.

(I-P) Immunostaining for FoxJ1 in male (I-L) and female (M-P) mice at 0 (I and M), 2 (J and N), 14 (K and O), and 21 (L and P) days after injection with the high dose (200 mg/kg) of naphthalene was performed. At day 0 (control group), a normal bronchiolar airway epithelium, containing several FoxJ1-positive (ciliated) cells as well as FoxJ1-negative (Clara) cells, was detected in both male (panel I) and female (panel M) mice. By 2 days post naphthalene injection, the injured and exfoliated airway epithelial cells stained negatively for FoxJ1 in both male and female mice (asterisks in panels J and N, respectively), while many of the residually intact airway epithelial cells stained positively for FoxJ1. Within 14-21 days after naphthalene injection, the regenerative response was complete and the airway epithelium resembled that of the respective control groups in both male (panels K-L) and female (panels O-P) mice. Positive staining for FoxJ1 appears brown in color, and sections were counterstained with hematoxylin. Photomicrographs are representative of 3-7 mice per gender at each time point after treatment with naphthalene. Scale bars: 50 μm. Magnification, x400.
Levels of cell proliferation and mitosis within the bronchiolar airway epithelium and peribronchiolar interstitium of female mice are greater than those of male mice during lung repair after treatment with both the low and medium naphthalene doses

The pulmonary regenerative response to naphthalene-induced Clara cell ablation was examined by quantification of cell proliferation in the lungs of male and female mice. Immunohistochemical staining for Ki-67 was performed to determine cell proliferation within both the distal bronchiolar airway epithelium and peribronchiolar interstitium of male and female mice after treatment with the low (Figure 2-3, panels A-H) and medium (Figure 2-4, panels A-H) doses of naphthalene.

In the distal bronchiolar airway epithelium, a low baseline Ki-67 LI level of about 5%-7% was observed for all control mice at day 0 after naphthalene injection and at 2 days after corn oil injection (Figures 2-3I and 2-4I). By 2 days after injection with the low dose of naphthalene, the Ki-67 LI increased to about 22% in female mice, and this value was significantly higher than that for male mice, which had not increased above control levels and was around 9% (Figure 2-3I). At 2 days after injection with the medium dose of naphthalene, the Ki-67 LI had now increased significantly above control levels in male mice but was still significantly less than that of female mice, which increased dramatically to almost 40% (Figure 2-4I). Within 14-21 days after injection with both the low (Figure 2-3I) and medium (Figure 2-4I) doses in both male and female mice, cell proliferation of the bronchiolar airway epithelium had decreased to similar levels as those seen in control mice.

In the peribronchiolar interstitium, a baseline Ki-67 LI level of about 11% was observed for all control mice at day 0 after naphthalene injection and at 2 days after corn oil injection (Figures 2-3J and 2-4J). By 2 days after injection with the low dose of naphthalene, the Ki-67 LI had increased significantly above control levels in male mice (to about 16%); however, it was still significantly less than the LI observed for female mice, which had increased to over 30% (Figure 2-3J). At 2 days after injection with the medium dose of naphthalene, the Ki-67 LI was significantly greater than control levels in male mice, but was still significantly less than that of female mice, which now increased to almost 40% (Figure 2-4J). Within 14-21 days after injection with both the low (Figure 2-3J) and medium (Figure 2-4J) doses, cell proliferation of
the peribronchiolar interstitium in both male and female mice had decreased to similar levels as those seen in control mice.
Figure 2-3: Immunohistochemical staining for the cell proliferation marker, Ki-67, in male and female mouse lungs after treatment with the low dose of naphthalene.

(A-H) Immunostaining for Ki-67 in male (A-D) and female (E-H) mice at 0 (A and E), 2 (B and F), 14 (C and G), and 21 (D and H) days after injection with the low dose (50 mg/kg) of naphthalene was performed. Ki-67 positive nuclei are brown and were detected in both the distal bronchiolar airway epithelium (black arrowheads) and peribronchiolar interstitium (red arrowheads). Sections were counterstained with hematoxylin. All scale bars: 50 μm. Magnification, x400. The percentage of Ki-67 positive cells was quantified in both the bronchiolar epithelium (I) and peribronchiolar interstitium (J), as described in the Materials and Methods section. Results are presented as means ± SE of 3-7 mice per gender at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (vehicle control). *Significantly different from that of male mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
Figure 2-4: Immunohistochemical staining for Ki-67 in male and female mouse lungs after treatment with the medium dose of naphthalene.

(A-H) Immunostaining for Ki-67 in male (A-D) and female (E-H) mice at 0 (A and E), 2 (B and F), 14 (C and G), and 21 (D and H) days after injection with the medium dose (100 mg/kg) of naphthalene was performed. Ki-67 positive nuclei are brown and were detected in both the distal bronchiolar airway epithelium (black arrowheads) and peribronchiolar interstitium (red arrowheads). Sections were counterstained with hematoxylin. All scale bars: 50 μm. Magnification, x400. The percentage of Ki-67 positive cells was quantified in both the bronchiolar epithelium (I) and peribronchiolar interstitium (J), as described in the Materials and Methods section. Results are presented as means ± SE of 3-7 mice per gender at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (vehicle control). *Significantly different from that of male mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
In order to confirm the aforementioned gender differences in lung regeneration initially observed with the Ki-67 LI, immunohistochemical staining for PH-3 was subsequently performed to determine mitosis within both the distal bronchiolar airway epithelium and peribronchiolar interstitium of male and female mice after treatment with the low (Figure 2-5, panels A-H) and medium (Figure 2-6, panels A-H) doses of naphthalene.

In the distal bronchiolar airway epithelium, a low baseline PH-3 MI level of about 3%-4% was observed for all control mice at day 0 after naphthalene injection and at 2 days after corn oil injection (Figures 2-5I and 2-6I). By 2 days after injection with the low dose of naphthalene, the PH-3 MI increased to about 20% in female mice, and this value was significantly higher than that for male mice, which had not increased above control levels and was around 5% (Figure 2-5I). At 2 days after injection with the medium dose of naphthalene, the PH-3 MI had now increased significantly above control levels in male mice, but was still significantly less than that of female mice, which increased dramatically to about 36% (Figure 2-6I). Within 14-21 days after injection with both the low (Figure 2-5I) and medium (Figure 2-6I) doses, mitosis detected within the bronchiolar epithelium of both male and female mice had decreased to similar levels as those seen in control mice.

In the peribronchiolar interstitium, a baseline PH-3 MI level of about 9% was observed for all control mice at day 0 after naphthalene injection and at 2 days after corn oil injection (Figures 2-5J and 2-6J). By 2 days after injection with the low dose of naphthalene, the PH-3 MI had increased significantly above control levels in male mice (to about 14%); however, it was still significantly less than the MI observed for female mice, which increased to almost 30% (Figure 2-5J). At 2 days after injection with the medium dose of naphthalene, the PH-3 MI was significantly greater than control levels in male mice, but was still significantly less than that of female mice, which now increased to about 35% (Figure 2-6J). Within 14-21 days after injection with both the low (Figure 2-5J) and medium (Figure 2-6J) doses, mitosis detected within the peribronchiolar interstitium of both male and female mice had decreased to similar levels as those seen in control mice.
Figure 2-5: Immunohistochemical staining for the mitosis marker, PH-3, in male and female mouse lungs after treatment with the low dose of naphthalene.

(A-H) Immunostaining for PH-3 in male (A-D) and female (E-H) mice at 0 (A and E), 2 (B and F), 14 (C and G), and 21 (D and H) days after injection with the low dose (50 mg/kg) of naphthalene was performed. PH-3 positive nuclei are brown and were detected in both the distal bronchiolar airway epithelium (black arrowheads) and peribronchiolar interstitium (red arrowheads). Sections were counterstained with hematoxylin. All scale bars: 50 μm. Magnification, x400. The percentage of PH-3 positive cells was quantified in both the bronchiolar epithelium (I) and peribronchiolar interstitium (J), as described in the Materials and Methods section. Results are presented as means ± SE of 3-7 mice per gender at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (vehicle control). *Significantly different from that of male mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
Figure 2-6: Immunohistochemical staining for PH-3 in male and female mouse lungs after treatment with the medium dose of naphthalene.

(A-H) Immunostaining for PH-3 in male (A-D) and female (E-H) mice at 0 (A and E), 2 (B and F), 14 (C and G), and 21 (D and H) days after injection with the medium dose (100 mg/kg) of naphthalene was performed. PH-3 positive nuclei are brown and were detected in both the distal bronchiolar airway epithelium (black arrowheads) and peribronchiolar interstitium (red arrowheads). Sections were counterstained with hematoxylin. All scale bars: 50 μm. Magnification, x400. The percentage of PH-3 positive cells was quantified in both the bronchiolar epithelium (I) and peribronchiolar interstitium (J), as described in the Materials and Methods section. Results are presented as means ± SE of 3-7 mice per gender at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (vehicle control). *Significantly different from that of male mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
Kinetics of cell proliferation and mitosis within the bronchiolar airway epithelium and peribronchiolar interstitium of female mice is delayed as compared to that of male mice during lung repair after treatment with the high dose of naphthalene

Cell proliferation and mitosis within both the distal bronchiolar airway epithelium and peribronchiolar interstitium of male and female mice was also examined after treatment with the high dose of naphthalene, as shown in panels A-H in both Figure 2-7 and Figure 2-8.

In the distal bronchiolar airway epithelium, cell proliferation increased to over 20% in male mice by 2 days post naphthalene injection and this was significantly greater than the Ki-67 LI level observed for female mice, which had not significantly increased above control levels at this time point (Figure 2-7I). However, by 14 days after naphthalene injection, the Ki-67 LI had increased to almost 20% in female mice and this was now significantly higher than the level of cell proliferation observed within the airway epithelium of male mice, which had decreased close to that of control mice (Figure 2-7I). By 21 days post naphthalene injection, cell proliferation had decreased to that of control levels (Ki-67 LI of about 6%-7%) in both male and female mice (Figure 2-7I). Similarly to that already observed by analysis of cell proliferation, mitosis had increased to almost 20% within the airway epithelium of male mice by 2 days post naphthalene injection and this was significantly greater than the PH-3 MI level observed for female mice, which had not significantly increased above control levels at this time point (Figure 2-8I). However, by 14 days after naphthalene injection, the PH-3 MI increased to about 15% in female mice and this was now significantly higher than the level of mitosis observed within the airway epithelium of male mice, which had decreased close to that of control mice (Figure 2-8I). By 21 days after naphthalene treatment, mitosis had decreased to that of control levels (PH-3 MI of about 5%) in both male and female mice (Figure 2-8I).

In the peribronchiolar interstitium, cell proliferation increased to about 33% in male mice at 2 days after naphthalene injection and was significantly greater than the Ki-67 LI observed for female mice, which increased to only 24% (Figure 2-7J). However, by 14 days after naphthalene injection, the Ki-67 LI decreased to near control levels in male mice, while interstitial cell proliferation remained significantly above control levels at about 21% in female mice (Figure 2-7J). By 21 days post naphthalene injection, interstitial cell proliferation had decreased to control
levels (Ki-67 LI of about 9%-11%) in both male and female mice (Figure 2-7J). Similarly to that already observed by analysis of cell proliferation, mitosis had increased to about 30% within the peribronchiolar interstitium of male mice by 2 days post naphthalene injection and this was significantly greater than the PH-3 MI observed for female mice, which increased to only about 21% (Figure 2-8J). However, by 14 days after naphthalene injection, the PH-3 MI decreased to near control levels in male mice, while interstitial cell mitosis remained significantly above control levels at about 17% in female mice (Figure 2-8J). By 21 days after naphthalene treatment, interstitial cell mitosis had decreased to control levels (PH-3 MI of about 8%-9%) in both male and female mice (Figure 2-8J).
Male

0 days

2 days

14 days

21 days

Female

I

Percentage of Ki-67 positive bronchial epithelial cells

<table>
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Percentage of Ki-67 positive peribroncholar interstitial cells

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Figure 2-7: Immunohistochemical staining for Ki-67 in male and female mouse lungs after treatment with the high dose of naphthalene.

(A-H) Immunostaining for Ki-67 in male (A-D) and female (E-H) mice at 0 (A and E), 2 (B and F), 14 (C and G), and 21 (D and H) days after injection with the high dose (200 mg/kg) of naphthalene was performed. Ki-67 positive nuclei are brown and were detected in both the distal bronchiolar airway epithelium (black arrowheads) and peribronchiolar interstitium (red arrowheads). Injured and necrotic bronchiolar epithelial (Clara) cells, which had exfoliated into the airway lumen, were also detected in both male and female mice at 2 days post naphthalene treatment (asterisks in panels B and F). Sections were counterstained with hematoxylin. All scale bars: 50 μm. Magnification, x400. The percentage of Ki-67 positive cells was quantified in both the bronchiolar epithelium (I) and peribronchiolar interstitium (J), as described in the Materials and Methods section. Results are presented as means ± SE of 3-7 mice per gender at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (vehicle control). *Significantly different from that of male mice at same time point after naphthalene treatment ($P<0.05$). #Significantly different from both the day 0 and corn oil (control) groups ($P<0.05$).
Figure 2-8: Immunohistochemical staining for PH-3 in male and female mouse lungs after treatment with the high dose of naphthalene.

(A-H) Immunostaining for PH-3 in male (A-D) and female (E-H) mice at 0 (A and E), 2 (B and F), 14 (C and G), and 21 (D and H) days after injection with the high dose (200 mg/kg) of naphthalene was performed. PH-3 positive nuclei are brown and were detected in both the distal bronchiolar airway epithelium (black arrowheads) and peribronchiolar interstitium (red arrowheads). Sections were counterstained with hematoxylin. All scale bars: 50 μm. Magnification, x400. The percentage of PH-3 positive cells was quantified in both the bronchiolar epithelium (I) and peribronchiolar interstitium (J), as described in the Materials and Methods section. Results are presented as means ± SE of 3-7 mice per gender at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (vehicle control). *Significantly different from that of male mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
2.5 Discussion

There are many biological factors that can modulate the epithelial response to toxicants in the airways, including: 1) the metabolic potential at specific sites for bioactivation and detoxification; 2) the nature of the local inflammatory response; 3) age of the organism at the time of exposure; 4) history of previous exposure; 5) species and strain of the organism exposed; and 6) gender of the exposed organism (Plopper et al., 2001). Although previous studies have shown gender differences in naphthalene metabolism and naphthalene-induced lung injury in mice (Stelck et al., 2005; Van Winkle et al., 2002), very little is known about the magnitude of gender on the long-term regenerative responses. In the present study, we have used three different doses of naphthalene in order to obtain a broad range of lung injury and repair responses. We found that female mice were not only more susceptible than male mice to the Clara cell injury observed at 2 days after treatment with a very low dose (i.e. 50 mg/kg) or medium dose (i.e. 100 mg/kg) of naphthalene, but that both cell proliferation and mitosis were substantially more abundant in the distal bronchiolar airway epithelium and in the peribronchiolar interstitium of female mice as compared to male mice. Furthermore, by 14-21 days after treatment with the low and medium doses, the bronchiolar injury had cleared up and a low level of lung tissue regeneration was detected in both male and female mice, thus indicating a timely regenerative response to these two relatively low naphthalene doses. However, after treatment with the high dose (i.e. 200 mg/kg), different lung repair responses were observed. We found that lung regeneration was attenuated and delayed in female mice with bronchiolar epithelial cell proliferation and mitosis not peaking until 14 days, while male mice mounted an adequate and timely regenerative response with cell proliferation and mitosis peaking at 2 days. This delayed lung repair response in female mice may be attributed to the very severe Clara cell damage detected at 2-14 days after treatment with the high dose of naphthalene. Female mice are more sensitive than male mice to naphthalene-induced lung injury and may have required more time to recover from the massive Clara cell ablation, thus causing a delayed regenerative response after treatment with the high dose. Nevertheless, female mice were eventually able to mount an adequate repair response, and lung regeneration was complete by 21 days after naphthalene treatment.
In the present study, we have used immunohistochemical staining for both Ki-67 and PH-3 to assess the levels of cell proliferation and mitosis in the lungs (i.e. the distal bronchiolar airway epithelium and peribronchiolar interstitium) of both male and female mice after naphthalene treatment. The levels of lung cell regeneration measured with these two markers showed different values with the percentage of PH-3 positive cells being slightly lower than the percentage of Ki-67 positive cells. This can be explained by the fact that these two markers are indicative of different stages of the cell cycle. While Ki-67 is a more general cell proliferation marker and is preferentially expressed during all phases of the cell cycle except for quiescence (Takebayashi et al., 2005), PH-3 is a mitosis marker and is specific to M phase only (Hendzel et al., 1997). Therefore, it makes sense that the MI measured with PH-3 would have a lower percentage of positive cells than the LI measured with Ki-67. Nevertheless, the data obtained with both markers showed similar patterns, and the trends in lung regeneration observed in both genders of mice after treatment with the three different doses of naphthalene were conserved. Thus, both the Ki-67 LI data and PH-3 MI data shown in this study provide strong evidence for gender differences in mouse lung regeneration after naphthalene-induced Clara cell depletion.

There are many possible explanations for the gender differences in lung repair observed in this study. For instance, female sex hormones, such as estrogen, may play a role in the pulmonary regenerative response to naphthalene-induced Clara cell ablation. It is known that estrogen can induce the expression of many drug-metabolizing enzymes in the lung (Ben-Zaken Cohen et al., 2007; Patel, 2005; Siegfried, 2001; Sin et al., 2007) and therefore, females have the potential to undergo more metabolic bioactivation of xenobiotics to toxic metabolites than males. Furthermore, studies have shown that hormonal patterns associated with different stages of the estrous cycle can alter naphthalene metabolism in the lungs of female mice (Stelck et al., 2005), and that metabolism of naphthalene occurs much faster and is associated with a greater extent of lung injury in female mice as compared to male mice (Van Winkle et al., 2002). Thus, we can only speculate that the more extensive Clara cell injury in female mice as compared to male mice observed in the aforementioned studies and in our study may be due to the fact that females produce substantially more estrogen than males. Although it has not been directly shown, estrogen may specifically up-regulate the pulmonary expression of CYP2F2, which is the main enzyme responsible for converting naphthalene into its toxic metabolites, and this may be the reason for the previously observed more rapid metabolism of naphthalene in the Clara cells of
female mice as compared to male mice (Van Winkle et al., 2002). Therefore, high estrogen levels may lead to increased naphthalene metabolism, which in turn can subsequently lead to increased injury and repair in the lungs of female mice. In addition, both estrogen receptor-alpha and estrogen receptor-beta are expressed in the airways and upon binding to its receptors, estrogen also has the ability to induce lung cell proliferation and differentiation (Ben-Zaken Cohen et al., 2007). It is currently not known if the gender differences in lung cell proliferation and mitosis observed in our study are due to gender differences in estrogen levels. However, this study provides the novel finding that gender-dependent differences in naphthalene-induced lung injury can subsequently influence downstream repair kinetics.

Various X-linked genes involved in the process of lung repair/regeneration may also play a role in the gender differences observed in this study. A possible candidate is the gene encoding for the gastrin-releasing peptide receptor (GRPR), which is located on the X-chromosome near a cluster of genes that escape X-chromosome inactivation (Patel, 2005; Shriver et al., 2000; Siegfried, 2001). Thus, females have two actively transcribed alleles of the GRPR gene compared with only one in males (Patel, 2005). Moreover, a previous study has reported sex differences in the expression of the GRPR gene where in the absence of smoking, it is expressed more frequently in the airway cells of women than in men, and in response to tobacco exposure, GRPR expression is activated earlier in women as compared to men (Shriver et al., 2000). There is also increased expression of the GRPR gene when human airway cells are exposed to estrogen, suggesting that GRPR could be hormonally regulated (Patel, 2005; Siegfried, 2001). This increased expression of GRPR in women is believed to be a contributing factor to the increased susceptibility of women to tobacco-induced lung cancer (Shriver et al., 2000). Indeed, signaling through the GRPR is highly mitogenic, and we can only speculate that it may account for the higher levels of cell proliferation and mitosis detected in the lungs of female mice as compared to male mice in our study.

Many different cell types have been shown to play a role in lung regeneration after naphthalene-induced Clara cell ablation in mice, and those identified to date include: basal cells (Hong et al., 2004a; Hong et al., 2004b), PNECs (Linnoila et al., 2007; Peake et al., 2000; Reynolds et al., 2000b), BASCs (Giangreco et al., 2002; Kim et al., 2005), ciliated cells (Lawson et al., 2002; Park et al., 2006; Rawlins et al., 2007), peribronchiolar interstitial cells (Van Winkle et al., 1995;
Van Winkle et al., 1997), and a pollutant-resistant subset of CC10/CCSP-expressing stem cells, also known as vCE cells (Giangreco et al., 2009; Hong et al., 2001; Rawlins et al., 2009; Reynolds et al., 2000a).

In the proximal airways, basal cells have been shown to function as a multipotent progenitor capable of renewing the naphthalene-injured tracheal and bronchial epithelium (Hong et al., 2004a; Hong et al., 2004b). However, since we observed that the most drastic changes in cell proliferation and mitosis occur in the distal bronchiolar airway epithelium and peribronchiolar interstitium of the lung and did not focus on cell regeneration in the proximal airways (i.e. trachea and bronchi), basal cells are unlikely to be involved in the gender differences in lung regeneration observed in our study.

In the distal airways, PNECs, vCE cells, BASCs, ciliated cells, and peribronchiolar interstitial cells have all been shown to partake in the lung repair response following naphthalene-induced depletion of Clara cells. PNECs are rare airway epithelial cells organized in a solitary arrangement or as innervated clusters, known as NEBs, and have been proposed to serve various functions, such as regulation of embryonic/fetal lung growth and maturation through the secretion of various neuropeptides and bioactive amines (Linnoila, 2006). Although PNECs/NEBs have been shown to proliferate after selective ablation of Clara cells, they mainly function as a self-renewing progenitor population and are unable to repopulate the entire airway, suggesting that PNECs/NEBs are not sufficient for epithelial renewal (Linnoila et al., 2007; Peake et al., 2000; Reynolds et al., 2000b). However, vCE cells are an important population of label-retaining progenitor cells that are critical for Clara cell renewal following naphthalene-induced injury (Bishop, 2004; Hong et al., 2001; Linnoila, 2006; Rawlins and Hogan, 2006; Reynolds et al., 2000a; Stripp and Reynolds, 2008; Stripp and Shapiro, 2006). BASCs are another population of progenitor cells involved in renewal of the terminal bronchiolar airway epithelium after naphthalene-induced Clara cell injury (Giangreco et al., 2002; Kim, 2007a; Kim, 2007b; Kim et al., 2005; Rawlins and Hogan, 2006). Since we focused on repair of the distal bronchiolar airway epithelium after naphthalene treatment, it is quite possible that the gender differences in cell proliferation and mitosis observed in our study may involve vCE cells and/or BASCs. As studies have shown that ciliated epithelial cells do not proliferate during repair of the naphthalene-injured mouse airway epithelium (Kida et al., 2008; Rawlins et al., 2007), it is
unlikely that the gender differences in bronchiolar epithelial cell proliferation observed in our study involve ciliated cells. However, we did observe that in both male and female mice, ciliated cells remain intact and uninjured after the naphthalene-induced Clara cell damage and exfoliation, possibly in an effort to help cover up and protect the denuded basement membrane of the injured bronchiolar airway epithelium. It has also been previously shown that peribronchiolar interstitial cells proliferate in response to naphthalene-induced Clara cell injury in mice, and these proliferating interstitial cells are believed to be fibroblast-like cells and/or alveolar macrophages (Van Winkle et al., 1995; Van Winkle et al., 1997). In the present study, we found clear gender differences in cell proliferation and mitosis detected within the peribronchiolar interstitium following naphthalene administration; however, since our studies of cell regeneration were at the light microscope level, we could not further classify the dividing interstitial cells in our mice.

In summary, we have demonstrated that gender plays an important role in the pulmonary regenerative response to naphthalene-induced Clara cell ablation in mice. The mechanisms involved in lung repair may include various distinctive steps, and have been studied in several laboratories (Kim et al., 2005; Linnoila et al., 2007; Park et al., 2006; Rawlins et al., 2007; Stripp et al., 1995; Van Winkle et al., 1995) but further studies are required in order to understand the exact role that gender and sex hormones may play in various stages of cellular regeneration.
ESE-1 plays a role in regulating bronchiolar epithelial repair kinetics following naphthalene-induced Clara cell injury

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3.1 Abstract

**Background:** ESE-1 is a member of the ESE subfamily within the ETS family of transcription factors, and is strongly expressed in epithelial-rich tissues, such as small intestine, colon, and uterus, as well as in certain lung cancers and lung cancer-derived cell lines. High expression levels of the murine homolog for the human ESE-1 gene, Elf3, have also previously been detected within mouse lung tissue during fetal development. Although previous studies have shown a defect in terminal differentiation of the small intestinal epithelium in Elf3 -/- mice during fetal/neonatal development, very little is known about the role Elf3 may play in repair of the airway epithelium after injury.

**Objective of study:** To investigate whether Elf3 is involved in regeneration of the bronchiolar airway epithelium after naphthalene-induced Clara cell injury.

**Methods:** Naphthalene was administered to both Elf3 +/+ and Elf3 -/- mice, and subsequent lung tissue injury and repair responses were examined by histopathological, immunohistochemical labeling, and western blotting analyses.

**Results:** Histopathological analysis revealed no significant difference in the extent of naphthalene-induced Clara cell necrosis between Elf3 +/+ and Elf3 -/- mice. In the bronchiolar airway epithelium of Elf3 -/- mice, there was a substantial delay in the kinetics of cell proliferation and mitosis along with Clara cell renewal, whereas, in the peribronchiolar interstitium, there was a significantly greater level of cell proliferation and mitosis in Elf3 -/- mice than in Elf3 +/+ mice. Lastly, the intensity of immunopositive signal for TGF-β RII, which is a well-known transcriptional target gene of Elf3 and is involved in the induction of epithelial cell differentiation, was significantly lower in the airway epithelium of Elf3 -/- mice as compared to Elf3 +/+ mice.

**Conclusions:** Taken together, these data suggest that Elf3 plays an important role in the regulation of lung cell proliferation and differentiation during repair of the injured bronchiolar airway epithelium, and this may be in part related to the regulation of TGF-β RII expression.
3.2 Introduction

Although the molecular and cellular mechanisms of airway epithelial injury and repair have been studied extensively and well documented, the role of various transcription factors in regulating the process of airway epithelial regeneration is a particular facet of this field of research that requires further investigation. ESE-1 is a member of the ESE subfamily of ETS transcription factors and is strongly expressed in epithelial-rich tissues, such as small intestine, colon, and uterus (Oettgen et al., 1997a; Tymms et al., 1997). Interestingly, expression of human ESE-1 was also shown to occur in some lung cancers, such as large-cell carcinoma and adenocarcinoma, and in lung cancer-derived cell lines, such as A549 (Tymms et al., 1997). In addition, even though low levels of expression of the murine homolog for the human ESE-1 gene, Elf3, have been detected in adult mouse lung, very high levels of Elf3 expression have been reported in developing fetal mouse lung (Tymms et al., 1997). It should also be acknowledged that although low expression levels for Elf3 have previously been observed in adult mouse lung, this may only be the case under basal conditions as quantitative real-time RT-PCR analysis of Elf3 mRNA expression in cultured primary airway epithelial cells isolated from adult mouse lung tissue has shown that expression of Elf3 can be significantly induced after stimulation with proinflammatory cytokines (Kushwah et al., unpublished data).

Mice with a null mutation of Elf3 have previously been generated through targeted gene disruption and the resultant homozygous mutant mice exhibit a distinct phenotype in the small intestine during fetal/neonatal development, which includes severe morphological alterations in tissue architecture manifested by poor villus formation along with improper morphogenesis of the microvilli and defective terminal differentiation of absorptive enterocytes and mucus-secreting goblet cells (Flentjar et al., 2007; Ng et al., 2002). Furthermore, the enterocytes within the small intestinal epithelium of Elf3 −/− mice also express reduced protein levels of TGF-β RII, which is a potent inhibitor of cell proliferation and an inducer of epithelial cell differentiation (Flentjar et al., 2007; Ng et al., 2002). Indeed, many in vitro studies have also established that the TGF-β RII gene is a definite transcriptional target of Elf3 and that Elf3 transactivates the TGF-β RII gene promoter by binding to two adjacent ETS binding sites (Agarkar et al., 2009; Agarkar et al., 2010; Chang et al., 2000; Choi et al., 1998; Kim et al., 2002; Kopp et al., 2004; Lee et al.,
2003). Interestingly, reduced protein levels of the proinflammatory cytokine, IL-6, have previously been detected in the lungs of Elf3 -/- mice as compared to their wild-type littermates after intranasal instillation of LPS (Wu et al., 2008), thus suggesting a possible role for Elf3 in the regulation of IL-6 expression. In addition, both TGF-β RII and IL-6 have been shown to be involved in the process of lung injury and repair (Kida et al., 2008; Zhao et al., 1997).

Since Elf3 is an epithelial-specific transcription factor that is highly expressed during fetal lung development and plays a role in the regulation of TGF-β RII and IL-6 expression, the goal of this study was to investigate whether Elf3 is involved in repair of the injured bronchiolar airway epithelium after naphthalene treatment in mice. The findings from this study show that although the degree of naphthalene-induced Clara cell injury is not significantly different between Elf3 +/+ and Elf3 -/- mice, the kinetics of airway epithelial cell proliferation and mitosis as well as Clara cell renewal is delayed in Elf3 -/- mice as compared to Elf3 +/+ mice. Furthermore, it is also shown from this study that Elf3 -/- mice express substantially reduced protein levels of TGF-β RII in the airway epithelium both basally and during repair after naphthalene injury. Thus, Elf3 may play an important role as a transcription factor regulating the expression of certain genes (e.g. TGF-β RII) that are involved in controlling the kinetics of epithelial cell proliferation and differentiation during the process of lung regeneration.

3.3 Materials and Methods

Animals and treatments

Mice with a null mutation of Elf3 were generated on a C57BL/6 genetic background as previously described (Ng et al., 2002) and the homozygous mutant mice as well as their wild-type littermates were used in this study. Adult (8-12 weeks of age) male and female mice were housed five per cage under pathogen-free conditions, and were maintained in an environment with controlled temperature and humidity along with alternating 12 hour light/dark cycles. All animals were fed standard mouse chow and water ad libitum. Naphthalene (>99% pure; Sigma Chemical Co., St Louis, MO, USA) was dissolved in corn oil (Sigma Chemical Co.) and administered at a dose of 200 mg/kg body weight. Naphthalene or corn oil alone (10 ml/kg body weight, vehicle control) was administered by a single intraperitoneal injection. The dose of
naphthalene used in this study was chosen based on previous dose-response studies, which showed that the 200 mg/kg dose is sufficient to specifically ablate Clara cells with a subsequently adequate repair response in both male and female mice (Oliver et al., 2009). Groups of 3-5 mice were sacrificed at different time points (0, 1, 2, 5, 14, and 21 days) after treatment with naphthalene or at 2 days after corn oil treatment (control group). In order to minimize the influence of diurnal fluctuations in glutathione abundance on the extent of naphthalene injury, all mice were dosed and killed in the morning between 8:00 A.M. and 10:00 A.M. All the animal studies were reviewed and approved by the HSC institutional animal care committee for humane use of laboratory animals.

**Histopathological analysis**

H&E-stained mouse lung tissue sections were scored for extent of naphthalene-induced Clara cell necrosis, as described previously in chapter 2 of this thesis. Data are presented as the mean necrosis score ± SE of 3-5 mice per group at each time point after naphthalene treatment.

**Immunofluorescence/Immunohistochemical labeling analysis**

Serial sections of formalin-fixed and paraffin-embedded mouse lung tissue were also used for immunofluorescent/immunohistochemical staining for the following: 1) the Clara cell marker: CC10/CCSP; 2) the ciliated cell marker: β-Tubulin IV; 3) the cell proliferation marker: Ki-67; 4) the mitosis marker: PH-3; and 5) TGF-β RII.

For immunofluorescent staining, sections (5 µm) were deparaffinized, rehydrated, and then microwaved in 0.01 M citrate buffer (pH 6.0) for 18.5 minutes at 1000 watts for antigen unmasking. Sections were kept in the hot citrate buffer solution for an additional 20 minutes, washed in water, and then transferred to phosphate-buffered saline (pH 7.2). Tissue sections were blocked with 10% normal donkey serum for 1 hour at room temperature, and then incubated with the following primary antibodies: goat polyclonal anti-mouse CC10/CCSP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 1:1000 dilution; mouse monoclonal anti-β-Tubulin IV (Sigma) in a 1:100 dilution; rabbit monoclonal anti-human Ki-67 (Lab Vision Corporation, Fremont, CA, USA) in a 1:100 dilution; and rabbit polyclonal anti-human TGF-β RII (Santa
Cruz Biotechnology) in a 1:200 dilution. All primary antibodies were incubated on tissue sections for 2 hours at room temperature, and the optimal antibody concentration that gave positive tissue staining with minimal background staining was determined separately for each antibody using a series of dilutions. CC10/CCSP was detected with a donkey anti-goat IgG secondary antibody conjugated to an Alexa 488 fluorophore (Invitrogen, Burlington, ON, Canada). β-Tubulin IV was detected with a donkey anti-mouse IgG secondary antibody conjugated to an Alexa 647 fluorophore (Invitrogen). In order to reduce nonspecific binding of the anti-mouse secondary antibody to endogenous mouse IgG antibodies, a Mouse-on-Mouse blocking step was employed before the serum blocking step by incubating tissue sections with F(ab')2 fragment donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in a 1:50 dilution for 1 hour at room temperature followed by overnight incubation at 4°C and then washing extensively. Ki-67 was detected with a donkey anti-rabbit IgG secondary antibody conjugated to an Alexa 555 fluorophore (Invitrogen). TGF-β RII was detected with a donkey anti-rabbit IgG secondary antibody conjugated to an Alexa 488 fluorophore (Invitrogen). All secondary antibodies were incubated on tissue sections in a 1:500 dilution for 1 hour at room temperature. Tissue sections were counterstained with DAPI, and sections stained without primary antibody served as negative controls. Immunofluorescent images were captured at x300 magnification on a Quorum spinning disk confocal microscope (Leica DMIRE2 inverted fluorescence microscope) equipped with a Hamamatsu back-thinned EM-CCD camera and Yokogawa scan head. The unit is also equipped with 4 separate diode-pumped solid state laser lines (Spectral Applied Research: 405nm, 491nm, 561nm, 638nm), an ASI motorized XY stage, an Improvision Piezo Focus Drive, and a 1.5x magnification lens coupler. The equipment is driven by Improvision Volocity acquisition software, and powered by an Apple Power Mac G5. Acquisition settings were optimized to obtain maximal signal in immunostained sections with minimal background in negative control sections.

For colorimetric immunohistochemical staining, everything was initially done exactly as already described above for immunofluorescent staining; however, after antigen unmasking, sections were also treated with 3% hydrogen peroxide in methanol for 15 minutes in order to quench endogenous peroxidase activity. Tissue sections were blocked with 10% normal goat serum for 1 hour at room temperature, and then incubated with one of the following primary antibodies: rabbit monoclonal anti-human Ki-67 (Lab Vision Corporation) in a 1:200 dilution or rabbit
polyclonal anti-human PH-3 (Upstate Laboratories, Temecula, CA, USA) in a 1:6000 dilution. Primary antibodies were incubated overnight on tissue sections at 4°C, and immunohistochemical staining was carried out using the immunoperoxidase method according to the guidelines for the Vectastain Elite ABC Peroxidase Kit (Vector Laboratories Inc., Burlingame, CA, USA). Sites of peroxidase binding were detected with the chromogenic DAB substrate, and positive staining appeared brown in color. Tissue sections were counterstained with hematoxylin, and sections stained without primary antibody served as negative controls.

**Quantification of immunoreactivity against CC10/CCSP and TGF-β RII**

Using Improvision Volocity image analysis software, the fluorescent intensity of immunopositive signal for both CC10/CCSP and TGF-β RII was individually measured in the distal bronchiolar airway epithelium on unprocessed raw data images captured at x300 magnification. More specifically, it was the mean fluorescent intensity of immunopositive signal that was measured, which is the average intensity of all the pixels within the area (i.e. bronchiolar epithelium) analyzed in an image. Distal bronchiolar airways were defined as distal conducting airways with a diameter of ≤ 250 μm and the bronchiolar airway epithelium was defined as the cells located between the basal lamina and the airway lumen. It should be noted that the intensity of immunoreactivity was measured only within the intact and undamaged bronchiolar epithelium and that injured and necrotic epithelial cells, which had detached from the basal lamina and exfoliated into the airway lumen, were not included in the analysis. This analysis was performed in a similar manner as previously described (Chen et al., 2008) with few modifications. Since every microscope slide contained two serial sections of the same mouse lung tissue sample (i.e. one section was used for immunostaining and the other section was used as a negative control), the fluorescent intensity of both immunopositive signal and auto-fluorescence could be measured in the exact same airways analyzed. Because the degree of background auto-fluorescence can vary in different sections, the data was normalized as a ratio by dividing the fluorescent intensity value obtained for immunopositive signal by the fluorescent intensity value obtained for auto-fluorescence. The normalized values were then averaged from all of the airways analyzed in images taken from five random fields (1-2 airways per field) per section (one section per mouse) to produce a mean airway fluorescent intensity value for each mouse sample. The values for each mouse in every group were then averaged, and the data are
presented as means ± SE of 3-5 mice per group at each time point after treatment with naphthalene.

**Measurement of Ki-67 LI and PH-3 MI**

Both the Ki-67 LI and PH-3 MI were measured within both the distal bronchiolar airway epithelium and peribronchiolar interstitium of Elf3 +/+ and Elf3 -/- mice, as described previously in chapter 2 of this thesis. Data are presented as means ± SE of 3-5 mice per group at each time point after treatment with naphthalene.

**Western blotting analysis**

Equal amounts of protein derived from mouse lung tissue homogenates were loaded onto a 15% SDS-polyacrylamide gel, were separated by electrophoresis, and were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blot was next blocked in TBST with 5% non-fat milk and 2% FBS, and then incubated with rabbit polyclonal anti-mouse CC10/CCSP antibody (Santa Cruz Biotechnology) in a 1:500 dilution for overnight at 4°C. After washing thoroughly in TBST, the blot was then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in a 1:3000 dilution for 1 hour at room temperature. Detection of protein was performed with ECL chemiluminescence reagents (Amersham Pharmacia Biotech). After detection of CC10/CCSP protein, the blot was stripped, blocked again, and reprobed with rabbit polyclonal anti-human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) antibody (Trevigen Inc., Gaithersburg, MD, USA) in a 1:2000 dilution for overnight at 4°C. The respective protein expression levels of the housekeeping gene, G3PDH, were detected and used as an internal reference for gel loadings.

**Quantitative real-time RT-PCR analysis**

Total RNA was isolated from mouse lung tissue samples using TRIzol Reagent (Invitrogen) following the manufacturer’s instructions, and then was further cleaned up by RNASpin mini column including on-column DNase digestion (GE Healthcare, Mississauga, ON, Canada). For
TaqMan real-time RT-PCR, total RNA (1 µg) was reverse transcribed using random hexamers and SuperScript II reverse transcriptase (Invitrogen) following the manufacturer’s protocols. The resulting templates (50 ng cDNA) were used for each real-time PCR reaction (ABI 7500, Applied Biosystems, Foster City, CA, USA). Primer and TaqMan probe sequences for mouse Elf3 were as follows: forward: 5’-CTCCTGCTCCTCCGACTAC-3’, reverse: 5’-CCGCTCGCTAGTCCAGCTT-3’, and probe: 5’-ACTTGGTGTTGACCCCTGA-3’. For relative quantification, PCR signals were compared between groups after normalization using 18S rRNA (Ribosomal RNA Control Reagents, ABI) as an internal reference and fold change was calculated as previously described (Wu et al., 2008).

**Statistical analysis**

The results of the experiments are expressed as means ± SE. A one-way ANOVA was used to evaluate the data followed by Tukey’s post hoc-tests for statistical comparisons between groups at different time points using Prism 4.0 software. Differences were considered to be statistically significant when $P < 0.05$.

**3.4 Results**

Since it has been well established that there are sex differences in naphthalene metabolism and naphthalene-induced acute lung injury (Van Winkle et al., 2002) as well as subsequent repair (Oliver et al., 2009), both male and female mice were utilized in this study. Therefore, all data reported herein will first compare lung injury/repair responses between male Elf3 +/+ mice and male Elf3 -/- mice and then between female Elf3 +/+ mice and female Elf3 -/- mice.

**Absence of Elf3 does not affect the severity of naphthalene-induced bronchiolar epithelial injury in Elf3 -/- mice**

Naphthalene-induced histopathological changes were assessed by analysis of H&E-stained lung tissue sections, and the findings from male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-1) are described here first. At day 0 of naphthalene treatment, no histopathological changes were
detected and a normal bronchiolar airway epithelium was observed in the lungs of both Elf3 +/- mice (Figure 3-1A) and Elf3 -/- mice (Figure 3-1G) as expected for uninjured control groups. Similarly, a normal airway epithelium with no histopathological changes was also observed in both Elf3 +/- and Elf3 -/- mice upon treatment with corn oil (data not shown). An exfoliation of numerous injured and necrotic bronchiolar epithelial cells into the airway lumen was detected in both Elf3 +/- mice (Figure 3-1B) and Elf3 -/- mice (Figure 3-1H) by day 1 post naphthalene injection. This sloughing of necrotic cells from the airway epithelium left the basement membrane denuded with some uninjured and flattened epithelial cells remaining intact. The excessive airway epithelial cell injury and exfoliation persisted at day 2 post naphthalene injection and began to clear up by day 5 with just few residual necrotic bronchiolar epithelial cells detected within the airway lumen in both Elf3 +/- mice (Figure 3-1, panels C-D) and Elf3 -/- mice (Figure 3-1, panels I-J). By day 14 post naphthalene treatment, the bronchiolar airway epithelium appeared to be almost completely restored with just some visible areas of denuded basement membrane remaining and by day 21, regeneration of the airway epithelium was observed to be complete in both Elf3 +/- mice (Figure 3-1, panels E-F) and Elf3 -/- mice (Figure 3-1, panels K-L).

The extent of naphthalene-induced airway epithelial cell necrosis was measured semi-quantitatively by scoring the H&E-stained lung tissue sections. For the day 0 and corn oil (control) groups of both Elf3 +/- and Elf3 -/- mice, the mean necrosis score was at a low baseline level of 0 (Figure 3-1M) as expected for uninjured control animals. The mean necrosis score was significantly greater than control levels and at a maximum at 1-2 days post naphthalene injection, and began to decrease thereafter returning to near baseline control levels by 14-21 days in both Elf3 +/- and Elf3 -/- mice (Figure 3-1M). The mean necrosis score was not significantly different at any time point when comparing Elf3 +/- and Elf3 -/- mice (Figure 3-1M), indicating that the naphthalene-induced bronchiolar epithelial injury occurs to the same extent in both Elf3 +/- and Elf3 -/- mice.
Figure 3-1: Histopathological analysis of naphthalene-induced bronchiolar epithelial injury in male (Elf3 +/+ and Elf3 -/-) mice

Histopathological analysis of H&E-stained lung tissue sections from Elf3 +/+ (A-F) and Elf3 -/- (G-L) mice was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. A normal uninjured bronchiolar airway epithelium was detected in both Elf3 +/+ (panel A) and Elf3 -/- (panel G) mice at day 0. Injured and necrotic bronchiolar epithelial cells, which had exfoliated into the airway lumen, were detected in both Elf3 +/+ (asterisks in panels B-D) and Elf3 -/- (asterisks in panels H-J) mice at 1-5 days post naphthalene injection. Bronchiolar airways had recovered from the naphthalene injury by 14-21 days in both Elf3 +/+ (panels E-F) and Elf3 -/- (panels K-L) mice; however, some visible areas of denuded basement membrane were observed in both Elf3 +/+ (arrow in panel E) and Elf3 -/- (arrow in panel K) mice at day 14. Photomicrographs are representative of 3-5 mice per group at each time point after treatment with naphthalene. All scale bars: 50 μm. Magnification, x400. The extent of naphthalene-induced bronchiolar epithelial cell necrosis was estimated semi-quantitatively in both Elf3 +/+ and Elf3 -/- mice, and is expressed as the mean necrosis score (panel M). The degree of necrosis was expressed for each mouse as the mean of ten random fields (1-2 airways per field) within each section (one section per mouse) classified on a scoring scale of 0-3. See Materials and Methods section in chapter 2 of this thesis for detailed description of necrosis scoring criteria. Data are presented as the mean necrosis score ± SE of 3-5 mice per group at each time point after naphthalene treatment or at 2 days after treatment with corn oil (control). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
The naphthalene-induced histopathological changes observed in the lungs of female (Elf3 +/+ and Elf3 -/-) mice were consistent with that observed in male (Elf3 +/+ and Elf3 -/-) mice, as described above, with the exception that the bronchiolar epithelial injury and necrosis was more severe in the female mice (Figure 3-2). Therefore, the mean necrosis score values were higher in female (Elf3 +/+ and Elf3 -/-) mice (Figure 3-2M) than in the male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-1M) at all time points after injection with naphthalene. Most importantly, the overall trend observed in male mice, in which the naphthalene-induced airway epithelial injury occurs to the same extent in both Elf3 +/+ and Elf3 -/- mice, was conserved among female mice as the mean necrosis score was not significantly different at any time point when comparing Elf3 +/+ and Elf3 -/- mice (Figure 3-2M).
Figure 3-2: Histopathological analysis of naphthalene-induced bronchiolar epithelial injury in female (Elf3 +/+ and Elf3 -/-) mice

Histopathological analysis of H&E-stained lung tissue sections from Elf3 +/+ (A-F) and Elf3 -/- (G-L) mice was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. A normal uninjured bronchiolar airway epithelium was detected in both Elf3 +/+ (panel A) and Elf3 -/- (panel G) mice at day 0. Injured and necrotic bronchiolar epithelial cells, which had exfoliated into the airway lumen, were detected in both Elf3 +/+ (asterisks in panels B-D) and Elf3 -/- (asterisks in panels H-J) mice at 1-5 days post naphthalene injection. Bronchiolar airways had recovered from the naphthalene injury by 14-21 days in both Elf3 +/+ (panels E-F) and Elf3 -/- (panels K-L) mice; however, some visible areas of denuded basement membrane were observed in both Elf3 +/+ (arrows in panel E) and Elf3 -/- (arrows in panel K) mice at day 14. Photomicrographs are representative of 3-5 mice per group at each time point after treatment with naphthalene. All scale bars: 50 μm. Magnification, x400. The extent of naphthalene-induced bronchiolar epithelial cell necrosis was estimated semi-quantitatively in both Elf3 +/+ and Elf3 -/- mice, and is expressed as the mean necrosis score (panel M). The degree of necrosis was expressed for each mouse as the mean of ten random fields (1-2 airways per field) within each section (one section per mouse) classified on a scoring scale of 0-3. See Materials and Methods section in chapter 2 of this thesis for detailed description of necrosis scoring criteria. Data are presented as the mean necrosis score ± SE of 3-5 mice per group at each time point after naphthalene treatment or at 2 days after treatment with corn oil (control). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
Delayed Clara cell renewal kinetics within the bronchiolar airway epithelium of Elf3 -/- mice following naphthalene-induced Clara cell depletion

In order to follow the fate of Clara cells during naphthalene-induced bronchiolar epithelial injury and repair, immunofluorescent staining for the Clara cell marker, CC10/CCSP, was performed and the data from male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-3) are presented here first. At day 0 of naphthalene treatment, strong expression of CC10/CCSP was detected within the bronchiolar airway epithelium of both Elf3 +/+ mice (Figure 3-3A) and Elf3 -/- mice (Figure 3-3G) as expected for uninjured control groups. Similarly, strong expression of CC10/CCSP was also observed within the airway epithelium of both Elf3 +/+ and Elf3 -/- mice upon treatment with corn oil (data not shown). Injured and necrotic bronchiolar epithelial cells, which had exfoliated into the airway lumen, stained positively for CC10/CCSP in both Elf3 +/+ mice (Figure 3-3, panels B-C) and Elf3 -/- mice (Figure 3-3, panels H-I) at 1-2 days after naphthalene injection, confirming that the naphthalene-induced airway epithelial injury was specific to Clara cells. In addition, signal for CC10/CCSP was substantially diminished within the residually intact bronchiolar airway epithelium of both Elf3 +/+ mice (Figure 3-3, panels B-C) and Elf3 -/- mice (Figure 3-3, panels H-I) at 1-2 days post naphthalene injection. By 5-14 days post naphthalene treatment, signal for CC10/CCSP began to increase within the airway epithelium of Elf3 +/+ mice (Figure 3-3, panels D-E) to a greater extent than within that of Elf3 -/- mice (Figure 3-3, panels J-K), suggesting delayed Clara cell renewal in Elf3 -/- mice. However, signal for CC10/CCSP within the airway epithelium of both Elf3 +/+ mice (Figure 3-3F) and Elf3 -/- mice (Figure 3-3L) at day 21 was similar to that observed within the airway epithelium of uninjured control mice at day 0 (Figure 3-3, panels A and G), indicating completion of Clara cell reconstitution in both Elf3 +/+ and Elf3 -/- mice by 21 days post naphthalene injection.

In order to quantify the amount of CC10/CCSP immunoreactivity, the fluorescent intensity of immunopositive signal was measured within the intact and undamaged bronchiolar airway epithelium, and was expressed as the mean CC10/CCSP immunofluorescence (IF) normalized to auto-fluorescence, as described in the Materials and Methods section. By 1-2 days after naphthalene injection, the mean CC10/CCSP IF in both Elf3 +/+ and Elf3 -/- mice had decreased significantly below that in their respective day 0 and corn oil (control) groups (Figure 3-3M). Moreover, the mean CC10/CCSP IF was not significantly different between Elf3 +/+ and Elf3 -/-
mice at 1-2 days post naphthalene injection (Figure 3-3M), further indicating that there is no considerable difference in the extent of naphthalene-induced Clara cell depletion when comparing Elf3 +/- and Elf3 -/- mice. At 5-14 days post naphthalene injection, the mean CC10/CCSP IF increased at a faster rate and was significantly greater in Elf3 +/- mice than in Elf3 -/- mice (Figure 3-3M), suggesting a considerable delay in the kinetics of Clara cell renewal within the bronchiolar airway epithelium of Elf3 -/- mice. Western blot analysis of CC10/CCSP expression in lung tissue homogenates at day 14 post naphthalene treatment showed a similar trend with relatively more expression in Elf3 +/- mice than in Elf3 -/- mice (Figure 3-3N). Furthermore, at day 14, the mean CC10/CCSP IF in Elf3 -/- mice was still significantly lower than that in their respective day 0 and corn oil (control) groups, while Elf3 +/- mice had already recovered as their mean CC10/CCSP IF was not significantly different than control levels (Figure 3-3M). However, by day 21 after treatment with naphthalene, Elf3 -/- mice eventually recovered as their mean CC10/CCSP IF had increased substantially and was no longer significantly different than control levels (Figure 3-3M).
**Figure 1**

**A**-**F**: Day 0, 1, 2, 5, 14, and 21 images for Elf3+/+ mice.

**G**-**L**: Day 0, 1, 2, 5, 14, and 21 images for Elf3-/- mice.

**M**: Bar graph showing the Mean CC10/CCSP IF (normalized ratio) for Elf3+/+ and Elf3-/- mice over days 0-21 post naphthalene injection.

**N**: Western blot images for CC10/CCSP and G3PDH showing proteins at 10 kDa and 38 kDa, respectively, for Elf3+/+ and Elf3-/- mice.
Figure 3-3: Immunofluorescent labeling and western blot analyses of CC10/CCSP protein expression in male (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Immunofluorescent staining for CC10/CCSP (green) in Elf3 +/+ (A-F) and Elf3 -/- (G-L) mouse lung tissue sections was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. Sections were counterstained with DAPI (blue) for detection of all cells. At day 0, a normal uninjured bronchiolar airway epithelium, which stained strongly for CC10/CCSP, was detected in both Elf3 +/+ (panel A) and Elf3 -/- (panel G) mice. By 1-2 days post naphthalene injection, an exfoliation of injured and necrotic Clara cells into the airway lumen was observed in both Elf3 +/+ (asterisks in panels B-C) and Elf3 -/- (asterisks in panels H-I) mice. Clara cell regeneration was almost completely restored by day 14 in Elf3 +/+ mice (panel E); however, regeneration was substantially delayed in Elf3 -/- mice and did not reach completion until day 21 (panel L). Photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. All scale bars: 50 μm. Magnification, x300. The fluorescent intensity of CC10/CCSP immunoreactivity was measured within the intact and undamaged bronchiolar airway epithelium of both Elf3 +/+ and Elf3 -/- mice, and is expressed as a normalized ratio of immunopositive signal to auto-fluorescence (panel M). See Materials and Methods section for a more detailed description of how this analysis was performed. Data are presented as the mean CC10/CCSP IF ± SE of 3-5 mice per group at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (control). *Significantly different from that of Elf3 -/- mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05). In an attempt to confirm the data from the immunofluorescent labeling analysis, western blot analysis of CC10/CCSP protein expression in lung tissue homogenates from both Elf3 +/+ and Elf3 -/- mice was also performed at day 14 post naphthalene injection (panel N). Detection of the housekeeping protein, G3PDH, was used as an internal reference for gel loadings.
The histological changes in Clara cells observed in female (Elf3 +/+ and Elf3 -/-) mice (Figure 3-4) were consistent with that observed in male (Elf3 +/+ and Elf3 -/-) mice, as described above. However, since they are more susceptible than male mice to naphthalene toxicity, female mice have more extensive naphthalene-induced Clara cell injury and necrosis than male mice. Therefore, the mean CC10/CCSP IF levels decreased to a greater degree in female (Elf3 +/+ and Elf3 -/-) mice (Figure 3-4M) than in the male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-3M) within 1-2 days after injection with naphthalene. Most importantly, the key finding made in the male Elf3 -/- mice (i.e. delayed Clara cell renewal kinetics) was conserved among female Elf3 -/- mice, as the mean CC10/CCSP IF increased at a faster rate and was significantly greater in female Elf3 +/+ mice than in female Elf3 -/- mice at 5-14 days post naphthalene injection (Figure 3-4M). However, just as observed in male Elf3 -/- mice, female Elf3 -/- mice eventually recovered by day 21 as their mean CC10/CCSP IF increased to a similar level as that detected within the airway epithelium of their respective day 0 and corn oil (control) groups (Figure 3-4M).
Figure 3-4: Immunofluorescent labeling of CC10/CCSP in female (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Immunofluorescent staining for CC10/CCSP (green) in Elf3 +/+ (A-F) and Elf3 -/- (G-L) mouse lung tissue sections was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. Sections were counterstained with DAPI (blue) for detection of all cells. At day 0, a normal uninjured bronchiolar airway epithelium, which stained strongly for CC10/CCSP, was detected in both Elf3 +/+ (panel A) and Elf3 -/- (panel G) mice. By 1-2 days post naphthalene injection, an exfoliation of injured and necrotic Clara cells into the airway lumen was observed in both Elf3 +/+ (asterisks in panels B-C) and Elf3 -/- (asterisks in panels H-I) mice. At 5-14 days post naphthalene injury, immunopositive signal for CC10/CCSP increased within the regenerating airway epithelium of Elf3 +/+ mice (panels D-E) to a greater degree than in Elf3 -/- mice (panels J-K). However, Clara cell regeneration was not completely restored until day 21 in both Elf3 +/+ (panel F) and Elf3 -/- (panel L) mice. Photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. All scale bars: 50 µm. Magnification, x300. The fluorescent intensity of CC10/CCSP immunoreactivity was measured within the intact and undamaged bronchiolar airway epithelium of both Elf3 +/+ and Elf3 -/- mice, and is expressed as a normalized ratio of immunopositive signal to auto-fluorescence (panel M). See Materials and Methods section for a more detailed description of how this analysis was performed. Data are presented as the mean CC10/CCSP IF ± SE of 3-5 mice per group at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (control). *Significantly different from that of Elf3 -/- mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
Ciliated cells remain intact to cover the denuded basement membrane of the naphthalene-injured bronchiolar airway epithelium in both Elf3 +/+ and Elf3 -/- mice

Since the signal for CC10/CCSP had diminished substantially within the residually intact bronchiolar airway epithelium at 1-2 days after naphthalene exposure in both Elf3 +/+ mice (panels B-C in both Figure 3-3 and Figure 3-4) and Elf3 -/- mice (panels H-I in both Figure 3-3 and Figure 3-4), it was hypothesized that ciliated epithelial cells remained intact after the naphthalene-induced Clara cell ablation and could temporarily cover the denuded basement membrane of the injured airway epithelium until completion of Clara cell regeneration. In order to test this hypothesis, double immunofluorescent staining for the Clara cell marker, CC10/CCSP, and the ciliated cell marker, β-Tubulin IV, was performed and the results from male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-5) are described here first. In the uninjured day 0 control groups, a normal bronchiolar airway epithelium, consisting mainly of CC10/CCSP-positive (Clara) cells and β-Tubulin IV-positive (ciliated) cells, was observed in both Elf3 +/+ mice (Figure 3-5A) and Elf3 -/- mice (Figure 3-5C) as expected. At day 2 post naphthalene injection, the injured and necrotic bronchiolar epithelial cells, which had exfoliated into the airway lumen, stained positively for CC10/CCSP but not for β-Tubulin IV in both Elf3 +/+ mice (Figure 3-5B) and Elf3 -/- mice (Figure 3-5D), further confirming that the naphthalene-induced airway epithelial injury was specific to Clara cells and that ciliated epithelial cells were not injured by the dose of naphthalene used in this study. More importantly, many of the residually intact airway epithelial cells stained positively for β-Tubulin IV in both Elf3 +/+ mice (Figure 3-5B) and Elf3 -/- mice (Figure 3-5D), suggesting that ciliated cells can remain behind to help cover up and possibly protect the denuded basement membrane of the injured bronchiolar airway epithelium following naphthalene-induced Clara cell ablation. All of the findings described above for male (Elf3 +/+ and Elf3 -/-) mice were also consistent with the findings obtained from analysis of the female (Elf3 +/+ and Elf3 -/-) mice (Figure 3-6).
Figure 3-5: Double immunofluorescent labeling of CC10/CCSP and β-Tubulin IV in male (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Double immunofluorescent staining for both CC10/CCSP (green) and β-Tubulin IV (red) in Elf3 +/+ (A-B) and Elf3 -/- (C-D) mouse lung tissue sections was performed at 0 (A and C) and 2 (B and D) days post naphthalene injection. Sections were counterstained with DAPI (blue) for detection of all cells. At day 0, a normal uninjured bronchiolar airway epithelium, containing many CC10/CCSP-positive (Clara) and β-Tubulin IV-positive (ciliated) cells, was detected in both Elf3 +/+ (panel A) and Elf3 -/- (panel C) mice. At day 2, numerous injured and necrotic Clara cells, which had detached from the basement membrane and exfoliated into the airway lumen, were detected in both Elf3 +/+ (asterisks in panel B) and Elf3 -/- (asterisks in panel D) mice, whereas ciliated cells were observed as intact and undamaged epithelial cells covering the denuded basement membrane of the injured airway epithelium in both Elf3 +/+ (panel B) and Elf3 -/- (panel D) mice. Photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 µm. Magnification, x300.
Figure 3-6: Double immunofluorescent labeling of CC10/CCSP and β-Tubulin IV in female (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Double immunofluorescent staining for both CC10/CCSP (green) and β-Tubulin IV (red) in Elf3 +/+ (A-B) and Elf3 -/- (C-D) mouse lung tissue sections was performed at 0 (A and C) and 2 (B and D) days post naphthalene injection. Sections were counterstained with DAPI (blue) for detection of all cells. At day 0, a normal uninjured bronchiolar airway epithelium, containing many CC10/CCSP-positive (Clara) and β-Tubulin IV-positive (ciliated) cells, was detected in both Elf3 +/+ (panel A) and Elf3 -/- (panel C) mice. At day 2, numerous injured and necrotic Clara cells, which had detached from the basement membrane and exfoliated into the airway lumen, were detected in both Elf3 +/+ (asterisks in panel B) and Elf3 -/- (asterisks in panel D) mice, whereas ciliated cells were observed as intact and undamaged epithelial cells covering the denuded basement membrane of the injured airway epithelium in both Elf3 +/+ (panel B) and Elf3 -/- (panel D) mice. Photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 μm. Magnification, x300.
Kinetics of cell proliferation and mitosis is delayed within the bronchiolar airway epithelium and increased within the peribronchiolar interstitium of Elf3 -/- mice during repair following naphthalene-induced Clara cell damage

The pulmonary regenerative response to naphthalene-induced Clara cell injury was examined by quantification of cell proliferation and mitosis within the distal bronchiolar airway epithelium and peribronchiolar interstitium of both Elf3 +/+ and Elf3 -/- mice. Immunohistochemical staining for Ki-67 was first performed in order to detect lung cell proliferation and the data from male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-7) are presented here first.

In the bronchiolar airway epithelium, a low basal Ki-67 LI level of about 5%-8% was observed for both Elf3 +/+ and Elf3 -/- mice within the uninjured day 0 and corn oil (control) groups (Figure 3-7M). In Elf3 +/- mice, the Ki-67 LI had increased significantly above baseline control levels by day 2, peaked at day 5, and then returned to near baseline control levels by day 14 post naphthalene injection (Figure 3-7M). In Elf3 -/- mice, however, the Ki-67 LI did not increase significantly above baseline control levels until day 5, peaked at day 14, and then returned to near baseline control levels by day 21 post naphthalene injection (Figure 3-7M). Furthermore, the Ki-67 LI measured within the airway epithelium of Elf3 +/- mice was significantly higher than that of Elf3 -/- mice at days 2 and 5 post naphthalene injection, whereas at day 14, the Ki-67 LI was significantly higher in Elf3 -/- mice than in the Elf3 +/- mice (Figure 3-7M). Collectively, these observations suggest delayed cell proliferation kinetics within the regenerating bronchiolar airway epithelium of Elf3 -/- mice as compared to Elf3 +/- mice. Immunohistochemical staining for Ki-67 was also performed with female (Elf3 +/- and Elf3 -/-) mouse lung tissue sections (Figure 3-8, panels A-L) and the overall trend, in which airway epithelial cell proliferation is delayed in Elf3 -/- mice, was conserved as shown with the LI data (Figure 3-8M).

In the peribronchiolar interstitium of male (Elf3 +/- and Elf3 -/-) mice, a basal Ki-67 LI level of about 11%-15% was observed for the uninjured day 0 and corn oil (control) groups (Figure 3-7N). In Elf3 +/- mice, the Ki-67 LI had increased significantly above baseline control levels by day 1, peaked at day 5, and then returned to baseline control levels by day 21 post naphthalene injection (Figure 3-7N). In Elf3 -/- mice, the Ki-67 LI also increased significantly above baseline...
control levels at day 1 and peaked at day 5, but was still significantly higher than baseline control levels at day 21 post naphthalene injection (Figure 3-7N). In addition, the Ki-67 LI measured within the peribronchiolar interstitium of Elf3 -/- mice was significantly greater than that of Elf3 +/+ mice at almost all time points (i.e. days 2-21) after treatment with naphthalene (Figure 3-7N). Taken together, these data suggest that interstitial cell proliferation is augmented within the lungs of Elf3 -/- mice as compared to Elf3 +/+ mice during repair following naphthalene-induced Clara cell ablation. The Ki-67 LI was also measured within the peribronchiolar interstitium of female (Elf3 +/+ and Elf3 -/-) mice (Figure 3-8N) and the results were consistent with that described above for the male mice.
Figure 3-7: Immunohistochemical labeling of Ki-67 in male (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Immunohistochemical staining for Ki-67 in Elf3 +/+ (A-F) and Elf3 -/- (G-L) mouse lung tissue sections was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. Ki-67 positive nuclei are brown in color, and were detected in both the distal bronchiolar airway epithelium (open arrows) and in the peribronchiolar interstitium (closed arrows). Sections were counterstained with hematoxylin for detection of all nuclei and photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 µm. Magnification, x400. The percentage of Ki-67 positive cells was quantified in both the distal bronchiolar airway epithelium (panel M) and in the peribronchiolar interstitium (panel N), and results are presented as means ± SE of 3-5 mice per group at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (control). *Significantly different from that of Elf3 -/- mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
Immunohistochemical staining for Ki-67 in Elf3 +/- (A-F) and Elf3 -/- (G-L) mouse lung tissue sections was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. Ki-67 positive nuclei are brown in color, and were detected in both the distal bronchiolar airway epithelium (open arrows) and in the peribronchiolar interstitium (closed arrows). Sections were counterstained with hematoxylin for detection of all nuclei and photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 µm. Magnification, x400. The percentage of Ki-67 positive cells was quantified in both the distal bronchiolar airway epithelium (panel M) and in the peribronchiolar interstitium (panel N), and results are presented as means ± SE of 3-5 mice per group at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (control). *Significantly different from that of Elf3 -/- mice at same time point after naphthalene treatment ($P<0.05$). #Significantly different from both the day 0 and corn oil (control) groups ($P<0.05$).
In order to confirm the aforementioned differences in lung cell proliferation initially observed with the Ki-67 LI data, immunohistochemical staining for the mitosis marker, PH-3, was subsequently performed to determine the MI within the distal bronchiolar airway epithelium and peribronchiolar interstitium of both male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-9) and female (Elf3 +/+ and Elf3 -/-) mice (Figure 3-10). As expected, all of the findings obtained with the Ki-67 LI data were confirmed with the PH-3 MI data.
Figure 3-9: Immunohistochemical labeling of PH-3 in male (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Immunohistochemical staining for PH-3 in Elf3 +/+ (A-F) and Elf3 -/- (G-L) mouse lung tissue sections was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. PH-3 positive nuclei are brown in color, and were detected in both the distal bronchiolar airway epithelium (open arrows) and in the peribronchiolar interstitium (closed arrows). Sections were counterstained with hematoxylin for detection of all nuclei and photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 µm. Magnification, x400. The percentage of PH-3 positive cells was quantified in both the distal bronchiolar airway epithelium (panel M) and in the peribronchiolar interstitium (panel N), and results are presented as means ± SE of 3-5 mice per group at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (control). *Significantly different from that of Elf3 -/- mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
Fig. 3-10: Immunohistochemical labeling of PH-3 in female (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Immunohistochemical staining for PH-3 in Elf3 +/+ (A-F) and Elf3 -/- (G-L) mouse lung tissue sections was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. PH-3 positive nuclei are brown in color, and were detected in both the distal bronchiolar airway epithelium (open arrows) and in the peribronchiolar interstitium (closed arrows). Sections were counterstained with hematoxylin for detection of all nuclei and photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 μm. Magnification, x400. The percentage of PH-3 positive cells was quantified in both the distal bronchiolar airway epithelium (panel M) and in the peribronchiolar interstitium (panel N), and results are presented as means ± SE of 3-5 mice per group at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (control). *Significantly different from that of Elf3 -/- mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
In order to better characterize the proliferating cells detected within the regenerating bronchiolar airway epithelium following naphthalene injury, triple immunofluorescent staining for CC10/CCSP, β-Tubulin IV, and Ki-67 was performed with lung tissue sections from both male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-11) and female (Elf3 +/+ and Elf3 -/-) mice (Figure 3-12). Since airway epithelial cell proliferation was at a maximum at day 5 in both sexes of Elf3 +/+ mice and at day 14 in both sexes of Elf3 -/- mice, the data from the triple immunofluorescent labeling analysis are presented for these two time points only. Many of the Ki-67-positive cells detected within the regenerating bronchiolar airway epithelium were dually positive for CC10/CCSP in both sexes of both Elf3 +/+ and Elf3 -/- mice (Figure 3-11 and Figure 3-12), suggesting that many of these proliferating airway epithelial cells are CC10/CCSP-expressing progenitor cells. Some CC10/CCSP-positive cells that were not dually positive for Ki-67 were also detected within the airway epithelium of both sexes of both Elf3 +/+ and Elf3 -/- mice (Figure 3-11 and Figure 3-12), and these cells may represent nascent Clara cells that emerged as a result of the Clara cell reconstitution occurring subsequent to the naphthalene-induced injury. In addition, none of the β-Tubulin IV-positive cells detected within the airway epithelium were dually positive for Ki-67 in both sexes of both Elf3 +/+ and Elf3 -/- mice (Figure 3-11 and Figure 3-12), indicating that ciliated cells do not proliferate in response to naphthalene-induced Clara cell ablation.
Figure 3-11: Triple immunofluorescent labeling of CC10/CCSP, β-Tubulin IV, and Ki-67 in male (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Triple immunofluorescent staining for CC10/CCSP (green), β-Tubulin IV (red), and Ki-67 (purple) in Elf3 +/+ (A-B) and Elf3 -/- (C-D) mouse lung tissue sections was performed at 5 (A and C) and 14 (B and D) days post naphthalene injection. Sections were counterstained with DAPI (blue) for detection of all cells. Numerous cells dually positive for both CC10/CCSP and Ki-67 were detected within the regenerating bronchiolar airway epithelium of both Elf3 +/+ and Elf3 -/- mice (as shown in insets), whereas no cells dually positive for both β-Tubulin IV and Ki-67 were detected in both Elf3 +/+ and Elf3 -/- mice. Photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 μm. Magnification, x300.
Figure 3-12: Triple immunofluorescent labeling of CC10/CCSP, β-Tubulin IV, and Ki-67 in female (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Triple immunofluorescent staining for CC10/CCSP (green), β-Tubulin IV (red), and Ki-67 (purple) in Elf3 +/+ (A-B) and Elf3 -/- (C-D) mouse lung tissue sections was performed at 5 (A and C) and 14 (B and D) days post naphthalene injection. Sections were counterstained with DAPI (blue) for detection of all cells. Numerous cells dually positive for both CC10/CCSP and Ki-67 were detected within the regenerating bronchiolar airway epithelium of both Elf3 +/+ and Elf3 -/- mice, whereas no cells dually positive for both β-Tubulin IV and Ki-67 were detected in both Elf3 +/+ and Elf3 -/- mice. Photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 μm. Magnification, x300.
Elf3 -/- mice express reduced levels of TGF-β RII in the bronchiolar airway epithelium during both steady-state and repair after naphthalene injury

Since TGF-β RII is a well known transcriptional target gene of Elf3 and is involved in the induction of epithelial cell differentiation (Agarkar et al., 2009; Agarkar et al., 2010; Chang et al., 2000; Choi et al., 1998; Flentjar et al., 2007; Kim et al., 2002; Kopp et al., 2004; Lee et al., 2003; Ng et al., 2002), expression of TGF-β RII was examined within the naphthalene-injured and regenerating bronchiolar airway epithelium by performing immunofluorescent staining with lung tissue sections from both male (Elf3 +/+ and Elf3 -/-) mice (Figure 3-13, panels A-L) and female (Elf3 +/+ and Elf3 -/-) mice (Figure 3-14, panels A-L). In order to quantify the amount of TGF-β RII immunoreactivity, the fluorescent intensity of immunopositive signal was measured within the intact and undamaged bronchiolar airway epithelium, and was expressed as the mean TGF-β RII IF normalized to auto-fluorescence, as described in the Materials and Methods section. In both male and female Elf3 +/+ mice, the mean TGF-β RII IF had increased significantly above day 0 and corn oil (control) levels at day 14 and then returned to near steady-state control levels by day 21 post naphthalene injection (Figure 3-13M and Figure 3-14M), indicating that expression of TGF-β RII is induced within the regenerating and differentiating airway epithelium at around day 14. In both male and female Elf3 -/- mice, however, the mean TGF-β RII IF remained at a low basal level and never changed significantly from control levels at all time points after treatment with naphthalene (Figure 3-13M and Figure 3-14M), thus suggesting that Elf3 is involved in the induction of TGF-β RII observed within the airway epithelium of Elf3 +/- mice at day 14. Furthermore, the mean TGF-β RII IF was significantly lower in the airway epithelium of Elf3 -/- mice as compared to Elf3 +/- mice both basally (i.e. at day 0 and after injection with corn oil) and at all time points after injection with naphthalene (Figure 3-13M and Figure 3-14M). Additionally, Elf3 mRNA expression was examined within the lungs of male Elf3 +/- mice both basally and during repair after naphthalene injury by performing quantitative real-time RT-PCR analysis (Figure 3-13N). Although there was not a statistically significant change in Elf3 expression at any time point after naphthalene injection, there was a slight induction at day 14 (Figure 3-13N) and this coincides with the same time point as when an induction of TGF-β RII expression was observed in Elf3 +/- mice (Figure 3-13M). Taken together, these findings potentially identify Elf3 as a major in vivo regulator of TGF-β RII expression in the bronchiolar airway epithelium of the lung during both the steady-state and
repair after naphthalene injury. However, the fact that TGF-β RII expression was not completely absent and was detected at low levels within the airway epithelium of Elf3 -/- mice suggests that other transcription factors may also be involved in regulating TGF-β RII expression.
Immunofluorescent staining for TGF-β RII (green) in Elf3 +/+ (A-F) and Elf3 -/- (G-L) mouse lung tissue sections was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. Sections were counterstained with DAPI (blue) for detection of all cells. Immunopositive signal for TGF-β RII detected within the bronchiolar airway epithelium of Elf3 +/+ mice was more intense than that of Elf3 -/- mice. At day 14, the intensity of immunopositive signal for TGF-β RII observed in Elf3 +/+ mice increased substantially compared to that seen in the respective day 0 control mice, whereas in Elf3 -/- mice, it did not change much compared to their respective control mice. Photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 μm. Magnification, x300. The fluorescent intensity of TGF-β RII immunoreactivity was measured within the intact and undamaged bronchiolar airway epithelium of both Elf3 +/+ and Elf3 -/- mice, and is expressed as a normalized ratio of immunopositive signal to auto-fluorescence (panel M). See Materials and Methods section for a more detailed description of how this analysis was performed. Elf3 mRNA expression levels were quantified within the lungs of Elf3 +/+ mice by performing real-time RT-PCR analysis (panel N). Data are presented as means ± SE of 3-5 mice per group at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (control). *Significantly different from that of Elf3 -/- mice at same time point after naphthalene treatment (P<0.05). †Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
Figure 3-14: Immunofluorescent labeling of TGF-β RII in female (Elf3 +/+ and Elf3 -/-) mouse lungs after naphthalene treatment

Immunofluorescent staining for TGF-β RII (green) in Elf3 +/+ (A-F) and Elf3 -/- (G-L) mouse lung tissue sections was performed at 0 (A and G), 1 (B and H), 2 (C and I), 5 (D and J), 14 (E and K), and 21 (F and L) days post naphthalene injection. Sections were counterstained with DAPI (blue) for detection of all cells. Immunopositive signal for TGF-β RII detected within the bronchiolar airway epithelium of Elf3 +/+ mice was more intense than that of Elf3 -/- mice. At day 14, the intensity of immunopositive signal for TGF-β RII observed in Elf3 +/+ mice increased substantially compared to that seen in the respective day 0 control mice, whereas in Elf3 -/- mice, it did not change much compared to their respective control mice. Photomicrographs are representative of 3-5 mice per group at each time point after naphthalene treatment. Scale bars: 50 µm. Magnification, x300. The fluorescent intensity of TGF-β RII immunoreactivity was measured within the intact and undamaged bronchiolar airway epithelium of both Elf3 +/+ and Elf3 -/- mice, and is expressed as a normalized ratio of immunopositive signal to auto-fluorescence (panel M). See Materials and Methods section for a more detailed description of how this analysis was performed. Data are presented as the mean TGF-β RII IF ± SE of 3-5 mice per group at each time point after treatment with naphthalene or at 2 days after treatment with corn oil (control). *Significantly different from that of Elf3 -/- mice at same time point after naphthalene treatment (P<0.05). #Significantly different from both the day 0 and corn oil (control) groups (P<0.05).
3.5 Discussion

The principal aim of this study was to investigate the role of the epithelium-specific ETS transcription factor, ESE-1, in regulating the process of airway epithelial regeneration following Clara cell-specific injury by utilizing an Elf3 -/- mouse model. Regulation of gene expression is a very important aspect of many pathophysiological processes and there have only been few studies examining the role of various transcription factors in regulating the pulmonary regenerative response to naphthalene-induced Clara cell damage (Jensen-Taubman et al., 2010; Kida et al., 2008; Linnoila et al., 2007; Park et al., 2006; Zemke et al., 2009; Zhang et al., 2008). In the present study, our findings suggest that Elf3 may play an important role in regulating airway epithelial repair kinetics, as the rate of bronchiolar epithelial cell proliferation and mitosis as well as Clara cell renewal was delayed in Elf3 -/- mice after treatment with naphthalene. The absence of Elf3 had no observable effect on the extent of Clara cell injury in Elf3 -/- mice, as measured by the mean necrosis score between Elf3 +/- and Elf3 -/- mice at any time point after naphthalene exposure. Therefore, the observed differences in bronchiolar epithelial repair kinetics between Elf3 +/- and Elf3 -/- mice may not be due to differences in the extent of initial injury. This is a reasonable assumption, as Elf3 is not known to regulate the expression of genes which encode for proteins involved in protection against the cytotoxicity of various xenobiotics. However, Elf3 has been shown to be involved in regulating the expression of genes involved in controlling epithelial cell proliferation and differentiation during embryonic/fetal development and neoplasia (Chang et al., 2000; Flentjar et al., 2007; Jedlicka and Gutierrez-Hartmann, 2008; Kageyama et al., 2006; Kwon et al., 2009; Lee et al., 2003; Ng et al., 2002; Yoshida et al., 2000). Therefore, the delayed kinetics of cell proliferation/mitosis and Clara cell reconstitution observed within the bronchiolar airway epithelium of Elf3 -/- mice is more likely due to changes in gene expression for proteins involved in regulating epithelial cell proliferation and/or differentiation during repair after naphthalene-induced Clara cell ablation. The best known candidate is the gene encoding for TGF-β RII, as several studies have clearly shown that Elf3 can bind to the promoter and regulate transcription of the TGF-β RII gene (Agarkar et al., 2009; Agarkar et al., 2010; Chang et al., 2000; Choi et al., 1998; Kim et al., 2002; Kopp et al., 2004; Lee et al., 2003). Moreover, it has also been reported that Elf3 -/- mice express reduced protein levels of TGF-β RII in the developing small intestinal epithelium as compared to Elf3 +/- mice (Ng et al., 2002). While TGF-β RII is a potent inhibitor of cell proliferation, it is very important for the induction
of epithelial cell differentiation (Flentjar et al., 2007; Ng et al., 2002). Interestingly, Elf3 -/- mice also exhibit defective terminal differentiation of the small intestinal epithelium during fetal/neonatal development (Flentjar et al., 2007; Ng et al., 2002). In our study, we found that TGF-β RII levels were significantly lower within the bronchiolar airway epithelium of Elf3 -/- mice than within that of Elf3 +/- mice during both the steady-state and regeneration after naphthalene injury. In addition, TGF-β RII levels in Elf3 +/- mice were significantly higher at day 14 post naphthalene injection than their respective steady-state control (day 0 and corn oil) levels, suggesting that expression of TGF-β RII is induced during repair and differentiation (occurring around day 14) of the injured airway epithelium and that the TGF-β signaling pathway may play an important role in this process. Furthermore, the fact that TGF-β RII levels in Elf3 -/- mice never significantly changed from their respective control levels at any time point after naphthalene exposure further strengthens the notion that Elf3 is a major player involved in the regulation of TGF-β RII expression within the regenerating and differentiating airway epithelium. However, it must be acknowledged that although TGF-β RII expression was substantially reduced in Elf3 -/- mice, it was not completely absent and could still be detected at low levels, thus suggesting that regulation of TGF-β RII expression is complex and other transcription factors may also be involved. It must also be recognized that although regeneration of the airway epithelium was delayed, Elf3 -/- mice eventually recovered with adequate Clara cell restitution by day 21 post naphthalene injury, further implying that other transcription factors involved in regulating the injury-repair process may compensate for the absence of Elf3 in Elf3 -/- mice.

In this study, we examined repair of the naphthalene-injured bronchiolar airway epithelium in both sexes of both Elf3 +/- and Elf3 -/- mice. Sex differences in naphthalene metabolism as well as naphthalene-induced Clara cell injury and subsequent repair have been well documented (Oliver et al., 2009; Van Winkle et al., 2002) and therefore, it is important to study these processes separately in male and/or female mice. In order to obtain an adequate understanding of any possible role for Elf3 in the pulmonary regenerative response to naphthalene-induced Clara cell depletion, we utilized both sexes of Elf3 +/- and Elf3 -/- mice and all of the findings originally made in male (Elf3 +/- and Elf3 -/-) mice were also confirmed in female (Elf3 +/- and Elf3 -/-) mice. It must be discussed, however, that a major caveat of the Elf3 -/- mouse model utilized in this study is the absence of Elf3 during lung development. Thus, the possibility of
compromised lung development in these Elf3 -/- mice cannot be ruled out as it has been previously reported in the literature that Elf3 is highly expressed in the developing fetal mouse lung (Tymms et al., 1997). Therefore, one cannot exclude the notion of a possible impairment of lung development resulting in a potential reduction of progenitor cells in adult Elf3 -/- mice, which could play a role in the delayed repair kinetics observed in these mice following naphthalene injury. On the contrary, previous histological examination of fetal/postnatal lung tissue had failed to detect any gross abnormalities in Elf3 -/- mice (Ng et al., 2002), thus suggesting no major defects of lung development in these mice. However, it is also possible that subtle defects in lung development may have not yet been uncovered in Elf3 -/- mice. Future studies focused on examining progenitor cells during lung development in these Elf3 -/- mice as well as airway epithelial repair after conditionally ablating Elf3 in adult mice are required in order to delineate these possibilities.

In this study, we also found significantly higher levels of cell proliferation and mitosis in the peribronchiolar interstitium of Elf3 -/- mice than in Elf3 +/+ mice. This exaggerated level of cell proliferation/mitosis observed within the peribronchiolar interstitium of Elf3 -/- mice may represent a compensatory mechanism due to the delayed kinetics of cell proliferation/mitosis within the bronchiolar epithelium. It has previously been shown that peribronchiolar interstitial cells can proliferate in response to naphthalene-induced Clara cell damage in mice (Van Winkle et al., 1995; Van Winkle et al., 1997). These proliferating interstitial cells are believed to be alveolar macrophages and fibroblast-like cells, and have been reported to interact with the basal lamina of adjacent bronchiolar epithelial cells during the repair process (Van Winkle et al., 1995; Van Winkle et al., 1997). Interestingly, delayed airway epithelial repair can promote fibroblast proliferation and fibrosis in other models of lung injury (Adamson et al., 1990; Adamson et al., 1988). In our study, histological analysis of Masson’s trichrome staining did not show any evidence of pulmonary fibrosis in the lungs of both Elf3 +/- and Elf3 -/- mice after naphthalene exposure (data not shown). This is in agreement with the fact that this naphthalene-induced model of acute airway epithelial injury and subsequent repair is not normally associated with excessive inflammation or fibrosis (Atkinson et al., 2007). Therefore, we speculate that the vast amount of cell proliferation and mitosis detected within the peribronchiolar interstitium of Elf3 -/- mice in our study may represent a particular facet of an exaggerated airway remodeling response occurring subsequent to the naphthalene-induced epithelial cell injury.
In the present study, we show that in both Elf3 +/+ and Elf3 -/- mice, ciliated cells remain undamaged and intact, and potentially play a role in temporarily covering and protecting the denuded basement membrane of the naphthalene-injured bronchiolar airway epithelium until completion of Clara cell regeneration. We also demonstrate that in both Elf3 +/+ and Elf3 -/- mice, ciliated cells do not proliferate in response to naphthalene-induced Clara cell ablation. Although we used the ciliated cell marker, β-Tubulin IV, which is expressed relatively late in the differentiation of ciliated cells, others have also previously shown that ciliated cells do not proliferate during repair of the naphthalene-injured bronchiolar epithelium using the ciliated cell marker, FoxJ1, which is expressed earlier than β-Tubulin IV in the differentiation of ciliated cells (Kida et al., 2008). These findings are in accordance with a previous study, which utilized transgenic lineage tracing experiments in order to follow the fate of ciliated cells and provided strong evidence that ciliated cells do not proliferate or transdifferentiate into different epithelial cell types during repair of the naphthalene-injured mouse airway epithelium (Rawlins et al., 2007). In addition, we found that many of the proliferating (i.e. Ki-67-positive) cells detected within the naphthalene-injured and regenerating airway epithelium were also dually positive for CC10/CCSP in both Elf3 +/+ and Elf3 -/- mice. We speculate that these cells may represent a naphthalene-resistant subpopulation of CC10/CCSP-expressing progenitor cells, which are also known as vCE cells. Others have previously characterized these vCE cells as immature progenitor and/or stem cells capable of simultaneously expressing CC10/CCSP and undergoing cell division, thereby contributing to the eventual reconstitution of mature and well-differentiated Clara cells within the bronchiolar airway epithelium following naphthalene injury (Hong et al., 2001; Reynolds et al., 2000a). Furthermore, these vCE cells are harbored and maintained within a microenvironment or niche, which is provided by PNECs/NEBs through the paracrine secretion of various neuropeptides and bioactive amines (Linnoila, 2006). It has also previously been shown that acute naphthalene toxicity results in PNEC/NEB hyperplasia in mice (Stevens et al., 1997) and after examination of PNECs/NEBs in our study, we found no observable difference in the extent of PNEC/NEB hyperplasia between Elf3 +/+ and Elf3 -/- mice after naphthalene exposure (data not shown).

In summary, the data reported here from this study clearly show that Elf3 plays an important role in regulating airway epithelial repair kinetics following Clara cell-specific injury and ablation in
mice. These findings potentially indicate a possible involvement of human ESE-1 in regulating gene expression in the context of repair occurring in response to airway inflammation and subsequent epithelial injury in the setting of various pulmonary diseases, such as asthma and COPD. In addition, the findings obtained from this study contribute to the field of ETS biology by revealing a novel role for the epithelium-specific ETS transcription factor,Elf3, in controlling airway epithelial cell differentiation in mice. These findings are also unique when compared to prior studies utilizing knockout mouse models for other ETS genes in which no epithelial defects have been described (Bartel et al., 2000). Although a defect in terminal differentiation of the small intestinal epithelium during embryonic development has previously been demonstrated in Elf3 -/- mice (Ng et al., 2002), this is the first study to describe abnormal kinetics of cell proliferation and differentiation during repair of the injured bronchiolar airway epithelium in these mice. Thus, regulation of TGF-βRII expression by Elf3 during repair of airway epithelial injury potentially has major implications for this process, as the TGF-β signal transduction pathway is a major player for inducing epithelial cell differentiation. While it has not been directly shown, a positive feedback loop involving both TGF-βRII and Elf3 may occur whereby activation of TGF-βRII may lead to an induction of Elf3 gene expression followed by a subsequent induction of TGF-βRII gene expression by Elf3. The cellular and molecular mechanisms of airway epithelial regeneration and repair are very complex and have been studied extensively in several laboratories; however, further studies are required in order to elucidate the exact role of ESE-1 and other ETS transcription factors in this process.
Summary and Future Directions
4.1 Summary

The findings presented in this thesis are derived from experimentation that was mainly focused on elucidating the role of the epithelium-specific ETS transcription factor, ESE-1, in regeneration and repair of the airway epithelium after extensive injury. Thus, we utilized the naphthalene-induced model of Clara cell-specific damage and subsequent repair to investigate a possible participation of ESE-1 in regulating lung regeneration. However, certain conditions for using this model should be optimized accordingly, and therefore, dose-response studies were initially performed by administering three different doses (i.e. 50 mg/kg, 100 mg/kg, or 200 mg/kg) of naphthalene to both male and female mice. The intended goal of these studies was two-fold: 1) to elicit a broad range of lung injury/repair responses for determining the optimal dose of naphthalene to use in future experiments and 2) to determine whether gender plays a dominant role in influencing the pulmonary regenerative response to naphthalene-induced injury.

As reported in chapter 2, we found that while the extent of naphthalene-induced Clara cell injury and necrosis is dose-dependent in both male and female mice, female mice are more susceptible to naphthalene injury and undergo a greater degree of Clara cell damage than male mice independent of the dose. In addition, we provided evidence suggesting that ciliated cells remain residually intact within the bronchiolar airway epithelium of both male and female mice following massive Clara cell destruction induced by treatment with the high dose (i.e. 200 mg/kg) of naphthalene. This observation is believed to occur in an effort of ciliated cells to temporarily cover up and help protect the denuded basement membrane of the injured bronchiolar airway epithelium until completion of Clara cell reconstitution. Importantly, we also discovered that the respective levels of both cell proliferation and mitosis are considerably greater within the distal bronchiolar airway epithelium and peribronchiolar interstitium of female mice than within that of male mice during repair after treatment with either a very low dose (i.e. 50 mg/kg) or medium dose (i.e. 100 mg/kg) of naphthalene. Moreover, by 14-21 days after treatment with the low and medium doses, the Clara cell injury and exfoliation had cleared up and a low level of lung tissue regeneration was detected in both male and female mice, thus indicating a timely regenerative response to these two relatively low naphthalene doses. After treatment with the high dose, however, different lung repair responses were observed. We found that the kinetics of cell proliferation and mitosis within the distal bronchiolar airway epithelium
and peribronchiolar interstitium as well as cell differentiation (i.e. Clara cell renewal) is delayed in female mice, while male mice mount an adequate and timely regenerative response. This delayed lung repair response in female mice is thought to be attributed to the very severe Clara cell damage detected after treatment with the high dose. Indeed, female mice are more sensitive to naphthalene toxicity than male mice and may require additional time to recover from the more extensive Clara cell injury and ablation, thus resulting in a delayed regenerative response to treatment with the high dose of naphthalene in female mice. Nevertheless, female mice are eventually able to recover with a completed pulmonary regenerative response by 21 days post naphthalene exposure. Taken together, these findings indicate that there are gender-based differences in the extent of lung epithelial injury and ablation as well as downstream regeneration and repair, and these respective differences should be taken into consideration when designing experiments aimed at examining the mechanisms of epithelial cell proliferation and differentiation occurring within the setting of this naphthalene-induced model of acute Clara cell damage and subsequent repair.

In order to determine whether the epithelial-specific transcription factor, ESE-1, is involved in regulating the pathological process of airway epithelial regeneration following naphthalene-induced Clara cell depletion, we next exploited a mouse knockout model of human ESE-1 (i.e. Elf3 -/- mice). Since the results derived from the dose-response experiments described in chapter 2 had demonstrated that the high dose of naphthalene is optimal to specifically injure Clara cells with a consequently adequate repair response in the lungs of both male and female mice, this dose was used for subsequent studies focusing on the role of ESE-1 in repair of the damaged bronchiolar airway epithelium. Moreover, because the data presented in chapter 2 had also indicated that there are gender differences in both naphthalene-induced injury and downstream repair kinetics, both sexes of both Elf3 +/+ and Elf3 -/- mice were utilized for exploring a potential involvement of ESE-1 in regulating lung epithelial regeneration. Importantly, all of the findings originally made in male (Elf3 +/+ and Elf3 -/-) mice were also confirmed in female (Elf3 +/+ and Elf3 -/-) mice.

As reported in chapter 3, we found that the respective rates of cell proliferation and mitosis as well as cell differentiation (i.e. Clara cell renewal) are delayed within the distal bronchiolar airway epithelium of Elf3 -/- mice as compared to Elf3 +/+ mice during repair of naphthalene-
induced Clara cell damage. In contrast, we detected a significantly greater level of cell proliferation and mitosis within the peribronchiolar interstitium of Elf3 -/- mice than that detected within the interstitium of Elf3 +/+ mice during the repair response to naphthalene injury. This exaggerated airway remodeling response observed within the pulmonary interstitium of Elf3 -/- mice is believed to represent a compensatory mechanism due to the delayed kinetics of Clara cell renewal within the airway epithelium. Collectively, these data provide substantial in vivo evidence suggesting that ESE-1 plays an important role in the regulation of lung cell proliferation and differentiation during repair of the injured bronchiolar airway epithelium.

Importantly, we also determined that the absence of Elf3 has no observable effect on the extent of naphthalene-induced injury in Elf3 -/- mice, as there is no significant difference in the degree of Clara cell necrosis between Elf3 +/+ and Elf3 -/- mice after naphthalene exposure. Therefore, since the severity of naphthalene-induced Clara cell damage is not affected by the absence of Elf3 in Elf3 -/- mice, the observed differences in the kinetics of airway epithelial repair between Elf3 +/+ and Elf3 -/- mice are most likely not due to differences in the extent of initial injury. Rather, the delayed kinetics of cell proliferation and mitosis as well as Clara cell renewal observed within the bronchiolar airway epithelium of Elf3 -/- mice is more likely due to changes in gene expression for proteins involved in controlling epithelial cell proliferation and/or differentiation during repair after injury. Indeed, we also discovered that Elf3 -/- mice express substantially reduced levels of TGF-β RII, which is a well-known transcriptional target gene of Elf3 and is involved in regulating epithelial cell differentiation, as compared to Elf3 +/+ mice during both basal steady-state conditions and regeneration after naphthalene-induced injury within the bronchiolar airway epithelium. In addition, we found that the expression levels of TGF-β RII are significantly higher within the airway epithelium of Elf3 +/+ mice at day 14 post naphthalene exposure than within that of their respective steady-state control mice at day 0, thus suggesting that TGF-β RII expression is induced during the process of cellular differentiation that occurs within the regenerating airway epithelium at around day 14 and that the TGF-β signaling pathway may play an important role in this process. Furthermore, TGF-β RII expression levels in Elf3 -/- mice never significantly changed from their respective steady-state control levels at any time point after naphthalene exposure, thus strengthening the notion that the induction of TGF-β RII expression observed in Elf3 +/+ mice at day 14 may at least in part be mediated by Elf3. Taken together, these findings suggest that Elf3 functions as a major player in
regulating TGF-β RII expression within the regenerating and differentiating airway epithelium after Clara cell-specific injury and ablation.

Lastly, we also found that in both Elf3 +/+ and Elf3 -/- mice, ciliated cells remain behind possibly to help cover and protect the denuded basement membrane of the bronchiolar airway epithelium after drastic Clara cell depletion induced by naphthalene treatment. In addition, we demonstrated that in both Elf3 +/+ and Elf3 -/- mice, ciliated airway epithelial cells do not proliferate during the pulmonary regenerative response to naphthalene-induced Clara cell ablation. Instead, we discovered that many of the proliferating cells express CC10/CCSP during regeneration of the naphthalene-injured airway epithelium in both Elf3 +/+ and Elf3 -/- mice, and it is thought that these cells may represent a specific subset of naphthalene-resistant, CC10/CCSP-expressing progenitor cells, such as vCE cells. Importantly, since these last few findings summarized here occur to a similar extent in both Elf3 +/+ and Elf3 -/- mice, they are most likely not dependent on Elf3.
4.2 Future Directions

4.2.1 Determination of whether sex hormones play a dominant role in influencing the pulmonary regenerative response to naphthalene-induced Clara cell injury and ablation

The results presented in chapter 2 of this thesis provide a considerable amount of experimental evidence suggesting that there are gender-based differences in the pulmonary regenerative response to naphthalene-induced Clara cell injury in mice. Although gender-dependent differences in initial lung injury are believed to affect downstream repair kinetics, the exact role of sex hormones in influencing the regenerative process after naphthalene injury has not been well established. Future studies focused on examining repair of naphthalene-induced Clara cell damage in both sexes of surgically castrated mice (i.e. ovariectomized female mice and gonadectomized male mice) will help to reveal if sex hormones play a major role in influencing the pulmonary regenerative response to airway epithelial injury. In addition, hormone replacement therapy experiments involving subcutaneous implantation of pellets containing female sex hormones (e.g. estrogen) into ovariectomized female mice as well as intact male mice or male sex hormones (e.g. testosterone) into gonadectomized male mice as well as intact female mice prior to inducing Clara cell injury with naphthalene treatment can further clarify the extent to which sex hormones may influence the process of airway epithelial regeneration and repair.

4.2.2 Determination of whether X-linked genes play an important role in influencing the process of lung regeneration following Clara cell-specific injury

While the findings described in this thesis indicate that there are gender differences in lung repair following naphthalene-induced injury with relatively higher levels of cell proliferation and mitosis observed within both the bronchiolar airway epithelium and peribronchiolar interstitium of female mice as compared to male mice, the potential contribution of various X-linked genes in influencing this process is currently not well known. Since there are gender differences in the number of X-chromosomes with females possessing two copies and males possessing only one, there can also potentially be gender differences in the expression levels of certain X-chromosome linked genes, particularly those that encode for proteins involved in regulating the process of lung regeneration after injury. A possible candidate is the gene encoding for GRPR,
which is located on the X-chromosome and is able to escape X-chromosome inactivation. Thus, under certain conditions, there can potentially be gender differences in expression of the GRPR gene with females having two actively transcribed alleles of the gene compared with only one in males. Indeed, the GRPR-mediated signal transduction pathway is very mitogenic and is involved in regulating many physiologically-related processes, such as lung development, and may also play a role in influencing the gender differences in lung regeneration described in this thesis. Future experiments aimed at assessing the expression levels of various X-linked genes, such as GRPR, within the lungs of both male and female mice during repair of naphthalene-induced Clara cell damage can contribute towards revealing the extent to which gender-based differences in lung regeneration may be influenced by gender-based differences in the expression of certain X-linked genes.

4.2.3 Characterization of progenitor cells within the airway epithelium of Elf3 -/- mice during both lung development and lung repair after injury

A fundamental limitation of the Elf3 -/- mouse model utilized for the research presented in chapter 3 of this thesis is the absence of Elf3 in these mice during lung development. Since Elf3 is strongly expressed in Elf3 +/+ mouse lung tissue during fetal development, there is the potential for several defects of lung development to occur in the absence of functional Elf3 in Elf3 -/- mice. For instance, a potential abnormality in lung development can subsequently result in a potential reduction of airway epithelial progenitor cells in Elf3 -/- mice during adulthood, and this could possibly be related to the delayed kinetics of airway epithelial repair observed in these mice following treatment with naphthalene. Although no major defects in lung tissue development have previously been detected in Elf3 -/- mice, it is still possible that some subtle defects may have not yet been revealed in these mice. Future studies focused on characterizing airway epithelial progenitor cells in Elf3 -/- mice during both lung development and repair after injury as well as examining airway epithelial regeneration after conditionally ablating Elf3 in adult Elf3 +/+ mice are needed to further explore these proposed possibilities.
4.2.4 Dissection of ESE-1-mediated regulation of TGF-β RII expression as well as the TGF-β signal transduction pathway during regeneration and repair of the injured airway epithelium

While the data obtained from the analysis of TGF-β RII expression in both Elf3 +/+ and Elf3 -/- mice as shown in chapter 3 of this thesis supports the notion that ESE-1 functions as a transcriptional regulator of TGF-β RII expression within the bronchiolar airway epithelium during both steady-state conditions and repair after injury, the exact molecular mechanisms of this regulation are not well defined. Therefore, further mechanistic experiments involving: 1) an in depth analysis of the putative molecular interactions between ESE-1 and other transcriptional regulatory proteins at the TGF-β RII gene promoter and 2) an evaluation of a potential activation of specific components of the TGF-β signal transduction pathway, such as phosphorylation of the SMAD transcription factor proteins and their subsequent regulation of target gene expression, during regeneration and repair of the injured airway epithelium can enhance our current understanding of the mechanisms by which ESE-1 regulates both TGF-β RII gene expression and downstream SMAD-mediated activation of target gene transcription.

4.2.5 Identification of other essential target genes regulated by ESE-1 during lung regeneration and repair after injury

Although some of the results presented in this thesis demonstrate that the ETS transcription factor, ESE-1, plays an important role in regulating the pulmonary regenerative response to naphthalene-induced airway epithelial injury by controlling TGF-β RII expression, other potential target genes that are regulated by ESE-1 and play a role in the process of lung repair after injury need to be identified. Thus, a broad microarray analysis of changes in gene expression within both Elf3 +/+ and Elf3 -/- mice during lung regeneration after Clara cell-specific injury with naphthalene treatment can help to identify key target genes that are potentially regulated by ESE-1 and further advance our understanding of the molecular mechanisms by which ESE-1 contributes to the regulation of this important pathological process.
4.2.6 Examination of a potential involvement of ESE-1 in regulating lung repair and remodeling in the context of clinically relevant models of human lung diseases

The studies described in chapter 3 of this thesis focused on investigating the role of ESE-1 in regulating lung regeneration exclusively in the context of the naphthalene-induced model of Clara cell damage and subsequent repair using an Elf3 -/- mouse model. However, future experimentation using these Elf3 -/- mice to investigate a potential involvement of ESE-1 in regulating the pathophysiological process of lung repair and remodeling in the context of more clinically relevant models, such as the enzyme-induced emphysema model, various murine asthma and CF models, the bleomycin-induced model of alveolar injury and pulmonary fibrosis, and unilateral pneumonectomy, can help to discern if ESE-1 plays a similar role within the setting of related human lung diseases.
4.3 Conclusions

The principal aims of the research presented in this thesis was to first optimize various conditions for utilizing the naphthalene-induced model of airway epithelial injury and repair, followed by exploiting this model to investigate whether ESE-1 plays a role in regulating the process of lung regeneration. We have shown that although the extent of naphthalene-induced Clara cell injury is dose-dependent in both male and female mice, female mice are more sensitive than male mice to the injury independent of the dose. As a result, lung cell proliferation and mitosis occurs to a greater extent in female mice than in male mice at low naphthalene doses, whereas at higher doses, there is a delayed pulmonary regenerative response in female mice as compared to that observed in male mice. Thus, we conclude that gender is a very important factor affecting not only the pulmonary injurious response to naphthalene exposure but also the succeeding regenerative response, as there are gender-based differences in both naphthalene-induced Clara cell injury and downstream repair kinetics. Future work, however, will need to determine the exact mechanism by which gender influences the process of lung injury and repair.

The findings reported in this thesis suggest that ESE-1 plays an important role in regulating the rate of lung cell proliferation and differentiation during repair of Clara cell-specific damage following naphthalene exposure. Furthermore, since we had found that TGF-β RII expression levels are significantly lower within the bronchiolar airway epithelium of Elf3 -/- mice than within that of Elf3 +/+ mice during regeneration after naphthalene injury, ESE-1 may regulate the pulmonary regenerative response to injury in part by regulating TGF-β RII expression. Thus, we have provided a hint of downstream effectors but because ESE-1 is a transcription factor, multiple pathways may be involved. Further mechanistic studies are required in order to elucidate the exact mechanism of ESE-1 in controlling the pathophysiological process of lung regeneration after injury. Overall, our findings indicate a potential involvement of ESE-1 in regulating gene expression in the context of repair, which can occur in response to severe airway inflammation and subsequent epithelial injury within the setting of many different airway-related diseases, such as asthma and CF. In addition, the data obtained from the studies described in this thesis contribute to the field of ETS biology by revealing a novel role for the epithelium-specific ETS transcription factor, ESE-1, in controlling airway epithelial cell differentiation during the regenerative process following excessive injury.
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