ABERRANT TRANSFORMING GROWTH FACTOR β1-ANGIOTENSIN II CROSSTALK IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE PARENCHYMAL FIBROBLASTS

by

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Abstract

**Rationale:** The airflow limitation in Chronic Obstructive Pulmonary Disease (COPD) is caused by small airways obstruction and/or obliteration and emphysematous destruction of parenchymal tissue. Our group previously characterized a gene expression signature for emphysema that included members of the TGFβ1 and Angiotensin (ANG) II signaling family. Further, we have shown that isolated parenchymal lung fibroblasts from severe COPD patients are unable to contract collagen 1α1 efficiently, but this impairment is reversed with the addition of TGFβ1 or the tripeptide Gly-His-Lys-Copper (GHK-Cu). Thus, we hypothesize that dysregulated ANG II-TGFβ1 crosstalk within the lung fibroblasts of COPD patients disrupts normal wound repair processes leading to disease.

**Methods:** Parenchymal fibroblasts from ex-smokers with normal lung function (n=9), moderate COPD (GOLD II, n=5) and very severe COPD (GOLD IV, n=5) were treated with TGFβ1 (10 ng/mL), ANG II (100 nM) or GHK-Cu (100 nM) to evaluate the relationship between TGFβ1 and ANG II signaling and the potential of GHK-Cu as a therapeutic. Gene expression analysis using NanoString was performed on lung tissues from healthy (n=3) and COPD GOLD IV (n=3) donors. Comparisons were made using student’s t-test, paired t-tests or ANOVA.

**Results:** TGFβ1 gene expression was reduced in COPD tissues (P<0.05), and exogenous TGFβ1 downregulated angiotensin receptor type 1 (AT1R) expression in fibroblasts from healthy control (P<0.05) but not COPD. This discrepant response was not due to inactive TGFβ1 signaling or changes in AT1R mRNA expression, and GHK-Cu was unable to correct this signaling defect. Expression of AT1R was significantly lower in COPD GOLD IV tissues (P<0.0001), and exogenous ANG II increased TGFβ1 production of parenchymal fibroblasts derived from all donor groups albeit levels were lower from COPD derived fibroblasts. Collagen 1α1 mRNA was increased in COPD lung tissue (P<0.0001), and the production of collagen 1α1 in response to exogenous TGFβ1 was increased in COPD derived fibroblasts (P<0.05).

**Conclusion:** TGFβ1-ANG II crosstalk is defective in COPD parenchymal fibroblasts which could contribute to the development of emphysema through loss of cell responsiveness to TGFβ1, an important mediator in tissue repair.
Preface

- The study was performed in the Centre for Heart Lung Innovation at St. Paul’s Hospital, University of British Columbia in collaboration with the University Medical Centre Groningen (UMCG), Groningen, Netherlands and the St. Joseph Healthcare, Hamilton, Ontario, Canada.

- Dr. Tillie-Louise Hackett was responsible for conception of the research program.

- Tracee Wee and Dr. Tillie-Louise Hackett contributed to the hypothesis, design and objectives of this study.

- The Groningen cohort of parenchymal fibroblasts were isolated and provided by Dr. Wim Timens, Prof. Dirkje S. Postma and Dr. Corry-Anke Brandsma.

- The Hamilton cohort lung explant tissue was provided by Dr. Parameswaran Nair and the technical support of Katherine Radford. Parenchymal fibroblasts from this tissue were isolated and maintained by Tracee Wee and Furquan Shaheen.

- Tracee Wee, Furquan Shaheen and Dr. Tillie-Louise Hackett all helped in performing the cell culture experiments for this study.

- Marc Sze extracted RNA from lung tissues that were sent to NanoString for gene expression analysis.

- Tracee Wee performed all the molecular work and analysis under the guidance of Dr. Tillie-Louise Hackett.

- Tracee Wee wrote the manuscript. Dr. Tillie-Louise Hackett provided edits and final approval for the work.
Ethics approval: This study was approved by the Research Ethics Board of all institutions including the University of British Columbia (#H0-50110).
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List of Abbreviations

A1AT    Alpha-1 antitrypsin
ACE     Angiotensin-converting enzyme
ANG     Angiotensin
ARB     Angiotensin II receptor blocker
AT1R    Angiotensin II receptor type 1
AT2R    Angiotensin II receptor type 2
BAL     Bronchoalveolar lavage
CMap    Connectivity Map
COPD    Chronic obstructive pulmonary disease
CTGF    Connective tissue growth factor
DMEM    Dulbecco’s modified Eagle medium
ECM     Extracellular matrix
EMT     Epithelial-mesenchymal transition
ERK MAPK Extracellular signal-regulated kinase mitogen-activated protein kinase
FBS     Fetal bovine serum
FEV₁    Forced expiratory volume in 1 second
FVC     Forced vital capacity
GHK-Cu  Gly-His-Lys-Copper
GM-CSF  Granulocyte-macrophage colony stimulating factor
GOLD    Global Initiative for Obstructive Lung Disease
GPCR    G-protein coupled receptor
IL-1β   Interleukin-1β
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>JNK</td>
<td>JUN N-terminal kinase</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent TGFβ-binding protein</td>
</tr>
<tr>
<td>MDCT</td>
<td>Multi-detector computed tomography</td>
</tr>
<tr>
<td>Micro-CT</td>
<td>Micro-computed tomography</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PD4</td>
<td>Phosphodiesterase-4</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGFβ-activated kinase 1</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TGFβRI</td>
<td>Transforming growth factor receptor type I</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>Transforming growth factor receptor type II</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of matrix metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor associated factor 6</td>
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</table>
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Dedication

I dedicate this work to my parents, Ki Lip and Terry Wee and my sister, Kathleen Wee, for their unwavering love, support and understanding. They have helped me through the worst of times, and I never would have gotten to this point in my life without them.
Chapter 1: Introduction

1.1 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible airflow limitation that is caused by emphysematous destruction of the gas-exchange surface (emphysema) and/or small airway remodeling and obstruction (1-3). In COPD patients, this causes symptoms such as dyspnea, chronic cough and sputum production that greatly impacts the quality of life of people suffering from the disease, and inevitably limits their ability to perform various daily life activities (2-6).

The diagnosis of COPD is confirmed by spirometry, which captures lung function values such as the volume of air that can be forcibly exhaled in 1 second (Forced Expiratory Volume in 1 second or FEV$_1$), and the total volume of air that can be exhaled by an individual from a full inhale and exhale (Forced Vital Capacity or FVC). Both FEV$_1$ and the ratio of FEV$_1$/FVC are often expressed as a percentage of the predicted value for an individual based on their sex, height, weight, age and ethnicity. These measures are commonly used parameters in the diagnosis of COPD, as patients have airflow obstruction when they have an FEV$_1$/FVC ratio of <70% and a post-bronchodilator FEV$_1$ of <80% of the predicted value. COPD is then further categorized by the severity of lung function obstruction according to the Global Initiative for Obstructive Lung Disease (GOLD) guidelines. In this scale: GOLD grade 1 is the mildest form of disease with an FEV$_1$ of $\geq$80% of the predicted value; GOLD grade 2 is a moderate form with an FEV$_1$ between 50-79% of the predicted value; GOLD grade 3 is the severe form with an FEV$_1$ between 30-49% of the predicted value; and GOLD grade 4 is the most severe form of the disease with an FEV$_1$ of <30% of the predicted value (Table 1.1) (3). The disease therefore
causes progressive lung function decline that cannot be reversed by any current therapies for COPD.

<table>
<thead>
<tr>
<th>GOLD Grade</th>
<th>Severity</th>
<th>FEV$_1$/FVC</th>
<th>FEV$_1$</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Mild</td>
<td>&lt;0.70</td>
<td>≥80% predicted</td>
</tr>
<tr>
<td>II</td>
<td>Moderate</td>
<td>&lt;0.70</td>
<td>50-79% predicted</td>
</tr>
<tr>
<td>III</td>
<td>Severe</td>
<td>&lt;0.70</td>
<td>30-49% predicted</td>
</tr>
<tr>
<td>IV</td>
<td>Very severe</td>
<td>&lt;0.70</td>
<td>&lt;30% predicted</td>
</tr>
</tbody>
</table>

Table 1.1 COPD disease severity grades as outlined by the GOLD guidelines.

1.1.1 Epidemiology

According to World Health Organization global estimates, COPD is the fourth leading cause of death in the world, with 65 million people suffering from moderate to severe COPD globally (7). In 2005 alone, over 3 million people died of COPD (7). Thus due to the high mortality rate, total deaths from the disease are projected to increase by more than 30% in the next 10 years, indicating that COPD will be the third leading cause of death worldwide by 2030 (7).

In Canada alone, 4% of the population suffer with COPD and the disease ranks as the fourth leading cause of death in the country (8). Due to the debilitating consequences of the disease, this results in a significant socioeconomic burden with estimated annual costs for COPD hospitalizations being $1.5 billion per year (9).

The disease prevalence is highest in the elderly population and studies have shown that COPD typically afflicts individuals over the age of 40, with the greatest prevalence in people over 60 years of age (3, 5). Furthermore, former smokers are more likely to develop COPD than non-smokers thus despite the rise in the smoking prevalence of women, it is still more prevalent in men and therefore a greater disease prevalence is observed in males (2, 3).
1.1.2 Risk factors associated with COPD

As described, COPD is a complex disease, and previous studies have shown that genetics and the environment play a critical role in disease development. These studies have elaborated on the contribution of genetics to disease development, particularly with the discovery of the defect in the alpha-1 antitrypsin (A1AT) gene. The A1AT gene defect is estimated to account for approximately 5% of COPD incidences but is often not diagnosed appropriately as only 3-5% of patients with the deficiency have been identified (10). A1AT deficiency is most commonly observed in people of European descent and the defect causes systemic effects on patients as the body is unable to curb the enzymatic activity of neutrophil elastase in the body, leading to the destruction of lung tissues (4, 6, 10).

The environment also plays a significant role in disease development, as exposure to inhaled particles and noxious fumes from cigarette smoke, indoor and outdoor pollution (through biomass fuel exposure), and occupational exposures can lead to COPD (3, 5). The main risk factor for COPD is direct or indirect cigarette smoke exposure, with 20% of smokers developing the disease (5, 6). The importance of cigarette smoke inhalation is further highlighted by the finding that smokers with A1AT deficiency develop emphysema earlier than non-smokers with the deficiency (10).

1.1.3 Pathology of COPD

COPD is a mixture of pathological changes in the large airways (chronic bronchitis), small airways (obstruction and remodeling), and parenchyma (emphysema), which all differ in the degree of pathology in each individual (6), and these differences cannot be fully determined by
spirometry. However, many histological studies have characterized the different pathologies, thereby broadening our understanding of the disease process.

1.1.3.1 Chronic bronchitis

Chronic bronchitis is a major indicator of COPD, which is clinically diagnosed as a productive cough every day for at least three months in two consecutive years (1, 3, 11, 12). However, only about 34% of COPD patients experience this symptom (13). The pathology is due to chronic inflammation of the large airways (>2mm in diameter), causing structural changes or remodeling of the airway. These changes include squamous cell metaplasia, and goblet cell and submucosal gland hyperplasia which together contribute to mucus hypersecretion (14). Although sputum produced with chronic bronchitis can obstruct the airway, it has been shown not to be a cause of airflow limitation in COPD (1, 11, 15). However, the presence of chronic bronchitis in young adults who smoke is associated with increased risk of developing COPD (16, 17), and COPD patients who have severe airflow limitation with chronic bronchitis have been associated with rapid lung function decline, with increased risk of hospitalization compared to COPD patients without chronic bronchitis (1, 11).

1.1.3.2 Small airway disease

Chronic inflammation in COPD also leads to remodeling of the small airways (<2mm in diameter) such that the airway lumen narrows from both airway wall thickening and mucus hypersecretion (15, 18) (Fig. 1.1).
Figure 1.1 Small airway remodeling and obstruction in COPD.
(A) Normal small airway (B) with a mucus plug containing few immune cells. (C) Remodeled small airway with inflammation as shown by mucus in the lumen containing many immune cell infiltrates. With small airway remodeling, (D) all layers of tissue around the airway are thickened (D), which narrows the airway lumen and contributes to airflow limitation.

All components of the airway wall contribute to the airway wall thickening. Squamous and goblet cell metaplasia lead to increased numbers of goblet cells in the epithelium which not only increases airway wall thickness, but also forms mucus plugs in the lumen (18, 19) creating greater airflow limitation (Figure 1.1B). Airway smooth muscle area is increased in the small
airways which correlates with the degree of airflow limitation, and together with fibrosis of the airway walls, causes further airway thickening (Figures 1.1C and 1.1D) (18, 20). These structural alterations have been implicated as the main source of airflow obstruction in COPD, and thus the severity of small airway disease has been associated with lung function decline and disease severity (1, 11, 18, 21, 22).

Small airway narrowing has been documented pathologically for many years (23), however the relationship with emphysematous lesions has been speculative. Recently, McDonough and his colleagues have identified that with increasing disease severity, there is greater loss of numbers of terminal bronchioles in patient lungs with end-stage COPD compared to control lungs (24). Moreover, they observed that there is loss of terminal bronchioles in regions of lung with no emphysematous destruction, suggesting that loss of terminal bronchioles precedes the development of emphysema (24).

1.1.3.3 Emphysema

Emphysema can be classified into two different subtypes based on which area of the acinus is affected, namely centrilobular and panlobular emphysema (Figure 1.2) (25-27). Centrilobular emphysema is most commonly associated with cigarette smoke exposure, and the pathology often begins in the upper lobes and is progressively observed throughout the lung with increasing disease severity (centre, Figure 1.2) (27). In contrast, panlobular emphysema is most commonly associated with patients suffering from the genetic defect resulting in A1AT deficiency (26). The destruction in panlobular emphysema is found uniformly in the lung acinus, and generally initiates from the lower lobes and spreads to the rest of the lungs (right, Figure 1.2) (25, 27).
Figure 1.2 Centrilobular and panlobular emphysema.

Emphysematous destruction of the lungs predominantly occurs after the terminal bronchioles (TB). The normal acinar structure (left) has intact respiratory bronchioles (RB), alveolar ducts (AD) and alveolar sacs (AS). In centrilobular emphysema (middle), which is primarily due to cigarette smoke exposure, destruction of the gas exchange surface originates from the RB and progresses distally from the TB. In contrast, the destruction in panlobular emphysema (right) is observed throughout the entire respiratory zone.

The presence of emphysema causes loss of elastic recoil (4) of the lung parenchyma which leads to reduced expiratory airflow driving pressure and loss of radial traction to support the alveolar walls, leading to gas trapping and airway collapse during expiration (4, 28). Clinically, emphysema can be evaluated in COPD patients using high-resolution multi-detector computed tomography (MDCT) (4, 26) however due to costs and radiation exposure, this is not a standard of care. Additionally, the current resolution of MDCT scans are still unable to detect emphysema early or visualize small airways less than 2.3 mm in diameter which have been shown to be the major sites of airflow obstruction in COPD (21).

Recently, the use of laboratory micro-computed tomography (micro-CT) has been used to visualize lung tissues at the micron level to quantify the degree of emphysema by measuring the
mean linear intercept (Lm), which quantifies airspace size and numbers of terminal bronchioles. Specifically, the use of micro-CT to measure emphysema has been validated by McDonough et al., who used micro-CT to determine the total number of terminal bronchioles per milliliter of lung tissue, and extended the measure to the total number of terminal bronchioles per entire lung volume using pre-operative MDCT scans (24). However, micro-CT does have limitations as it is restricted to processed tissue samples due to the size of the field of view of the micro-CT scanner, and the high radiation exposure the samples are subjected to for resolution at the micron level.

1.1.4 Chronic inflammation in COPD

COPD is characterized by chronic inflammation of the airways and lung parenchyma (1, 11, 14, 29). The inflammation is strongly associated to cigarette smoke exposure, with evidence that there is a sustained, albeit altered signature of inflammation in the airways of ex-smokers who have quit smoking for at least one year (30, 31), with molecular changes in lung tissues lasting over 25 years after smoking cessation (32). Although lung and airway inflammation occurs in all smokers, only a small subset of smokers develop COPD (33), suggesting that there are specific factors mediating the susceptibility of these smokers to COPD disease development.

1.1.4.1 Inflammatory cells and mediators

Cigarette smoke inhalation causes inflammation in the lungs characterized by infiltration of inflammatory cells such as neutrophils, macrophages and CD8+ T cells into the small airways and lung parenchyma to prevent infection, and repair the damage caused by exposure to the noxious substances. As the primary phagocytic cell of the immune system, macrophages are
found throughout the body to sample for pathogens. Cigarette smoke extract has been shown to directly activate macrophages (34), suggesting that it could be an important interface with the immune system in COPD. Studies have shown that smokers with COPD not only have more alveolar macrophages than non-smokers (35) and healthy smokers (33), but they appear to survive longer with the increased expression of anti-apoptotic proteins (36). The prolonged survival of macrophages may have a great impact on the disease pathology as they release many inflammatory mediators such as various interleukins, tumour necrosis factor (TNF)-α, reactive oxygen species (ROS) and elastolytic enzymes including cathepsins and matrix metalloproteinases (MMPs) (14, 37), which can all induce lung injury. Moreover, macrophages from COPD patients have been shown to secrete more of these factors compared to macrophages from non-smoking controls, and this ability is further amplified with smoke exposure (14, 38-40). Studies have shown that not only are macrophages found in sites of alveolar destruction (41), but there is also a strong correlation between disease severity and airway and lung tissue macrophage abundance (41, 42), suggesting an important role for macrophages in COPD pathophysiology.

Neutrophils have commonly been associated with COPD with the observed increase of numbers in sputum and bronchoalveolar lavage (BAL) in COPD patients compared to healthy smokers (14, 30, 43). This increase has been associated with COPD disease severity and lung function decline (42-44). Neutrophils produce oxidants and a plethora of enzymes including neutrophil elastase, MMP-8, MMP-9, proteinase-3 and cathepsin G (14, 45) that can degrade lung extracellular matrix components. The presence of tissue neutrophils can also promote mucus hypersecretion since serine proteases can stimulate goblet cell and submucosal gland
activity (14, 46), contributing to more airflow obstruction in addition to its infiltration of the airways.

T-lymphocytes have also been shown to play a role in COPD, with increased numbers of T lymphocytes found in patient lungs (41, 45, 47). Although both types of T cells (CD4+ and CD8+) are increased in COPD lungs, the total increase is predominantly due to elevated CD8+ cells (14). The increase in CD8+ cells can cause significant damage to the epithelium and promote the development of emphysema since they produce granzyme B, TNF-α and perforins, which are all factors capable of inducing apoptosis or lysis of alveolar epithelial cells (29, 48, 49). Furthermore, CD8+ T cells collected from the sputum of patients with COPD have greater perforin expression and cytotoxic activity compared to CD8+ T cells from healthy smokers and non-smokers (50), suggesting a greater capacity to damage lung tissues.

1.1.4.2 Protease-anti-protease imbalance and oxidative stress

A protease-anti-protease imbalance has classically been thought to be the cause of the connective tissue degradation observed in emphysema. The recruitment and activation of inflammatory cells in response to smoke exposure, particularly for neutrophils and macrophages, result in the copious release of serine proteases such as neutrophil elastase, cathepsins and various MMPs to degrade the extracellular matrix to travel to the injury site (14). However, these proteases can also damage functional lung tissue and therefore, it is critical for these enzymes to be inactivated by their respective anti-proteases, namely A1AT for neutrophil elastase and tissue inhibitors of matrix metalloproteinases (TIMPs) for MMPs (4). In A1AT deficient patients, there is inadequate production of A1AT from the liver due to a spectrum of genetic defects (10), and
therefore neutrophil elastase function is not adequately inhibited. In addition, in susceptible smokers, the large influx of immune cells into the large and small airways corresponds with elevated protease levels that may overload the normal inactivating capacity of circulating anti-proteases. Both instances result in excessive protease activity that destroys lung tissue leading to emphysematous lesions.

Oxidative stress, whether produced directly by oxidants inhaled from cigarette smoke, or indirectly through the release of ROS by neutrophils and macrophages in response to cigarette smoke, can cause further damage to lung tissues (4, 14). Moreover, ROS can inhibit anti-protease activity by modifying the elastase-binding site, induce endothelial and epithelial apoptosis, and increase chemokine and cytokine production (4, 14, 51), thereby exacerbating the inflammation and subsequent tissue damage. Together, constant exposure to cigarette smoke leads to a cycle of injury and repair that can cause tissue remodeling and destruction.

1.2 Lung tissue repair
Lung injury activates repair mechanisms to prevent infection and further damage. When the epithelium is damaged due to environmental insults such as cigarette smoke, the surviving epithelial cells produce inflammatory mediators such as TNF-α, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-1β to recruit inflammatory cells to the damaged area (14, 52). Resident and recruited inflammatory cells such as macrophages contribute to the creation of a provisional matrix with the release of fibronectin (53, 54) to provide a surface for epithelial cells to de-differentiate and migrate over the site of injury (52, 55, 56). Epithelial cells also produce growth factors such as TGFβ2, fibroblast growth factor, insulin-like growth factor,
and provisional matrix proteins, to activate subepithelial fibroblasts and augment the wound repair process (57). In normal conditions, resident subepithelial lung fibroblasts are responsible for the homeostasis of the tissue by extracellular matrix (ECM) production and turnover of ECM by proteases such as MMP-2 and -9 (52). Once activated by epithelial released factors, fibroblasts are stimulated to migrate to the injury site, proliferate and differentiate into myofibroblasts. Myofibroblasts are potent producers of ECM proteins including fibronectin and collagen 1 (58). Collagen 1 fibres are formed by a triple helix composed of two α1 chains and one α2 chain (59). Myofibroblasts mediate wound closure by remodeling the strands of collagen into thick helical fibrillar collagen, and adhesion to fibrillar collagen through integrin-mediated mechanisms, particularly via integrin β1 (60). Attachment to the collagen fibres allows myofibroblasts to generate mechanical tension using their actin cytoskeleton formed as stress fibres, to contract collagen and promote wound closure (58, 61). In addition to their function in wound closure, fibroblasts also produce growth factors such as plasma-derived growth factor, insulin-like growth factor-1 and TGFβ1, forming a positive feedback loop as TGFβ1 also induces myofibroblast differentiation (61). Furthermore, TGFβ1 elicits a potent repair and regeneration response in epithelial cells to initiate restitution of the epithelium by proliferation and differentiation. Once the repair process is complete, resolution of the wound involves apoptosis of the myofibroblasts and resolution of the granular tissue (58, 62). In fibrotic lung diseases such as idiopathic pulmonary fibrosis (IPF), regulation of myofibroblast apoptosis is thought to be lost leading to persistent over-expression of repair molecules such as TGFβ1 leading to fibrosis (63). Therefore, regulation of pleiotropic cytokines such as TGFβ1 are important for normal tissue homeostasis.
1.3 Molecular determinants of COPD pathogenesis

Currently, there are several theories believed to play a significant role in the pathogenesis of COPD tissue destruction, which are protease-antiprotease activity imbalance, oxidative stress, abnormal angiogenesis, autoimmune responses, chronic inflammation, apoptosis and tissue remodeling processes that destroy lung ECM. Many groups have endeavoured to understand the molecular mechanisms that result in the pathology by gene expression profiling of lung tissue from patients with or without COPD, based on lung function measurements or disease severity (64-67). However, whole organ function cannot be used to compare regional differences in the anatomical lesions that develop in COPD, when trying to assess gene expression changes. To specifically identify the molecular alterations involved in COPD disease development, our group performed gene expression analysis on lung tissues with assessed tissue pathology using micro-CT, which used Lm as a measure of emphysema in the adjacent tissue, to create a gene expression profile for emphysematous disease (60). The study identified 127 genes that were significantly associated with emphysema severity, including genes expressed by infiltrating immune cells that were significantly upregulated with increasing emphysematous destruction, and genes involved in tissue repair such as the TGFβ pathway, actin organization and integrin signaling were significantly downregulated (60).

In addition to obtaining a gene expression profile for emphysema severity, the study utilized the profile to find compounds that could reverse the gene expression pattern of progressive emphysema using the Connectivity Map (CMap). CMap is a publicly available bioinformatics tool that contains a database of microarray experiments from cancer cell lines treated with therapeutic compounds. With this tool, the 127 genes that were significantly different with emphysema severity were used as a query to determine if in the CMap database
there was a therapy that could significantly reverse this gene signature, and that molecule was the tripeptide Gly-His-Lys-Copper (GHK-Cu) (60). In skin repair research, GHK-Cu has been shown to promote wound healing through various mechanisms, including cellular proliferation, ECM production, anti-oxidant and anti-protease mechanisms (68-70). Within the lung, fibroblasts are the major interstitial cells involved in maintaining the lung tissue integrity by preserving the appropriate connective tissue in the lungs. In the study, the authors demonstrated that the inherent defects in collagen contraction and remodeling in parenchymal fibroblasts from COPD patients can be restored through the addition of exogenous TGFβ1 or GHK-Cu (60). Together these data further support the hypothesis that a wound-healing-like process, related to the TGF pathway, is diminished as a function of emphysema progression and further suggests the potential of GHK-Cu as a therapeutic in the treatment of COPD. Thus in Aim 3, we will determine how GHK-Cu modifies the TGFβ1–ANG II crosstalk.

1.4 Transforming growth factor-β1 (TGFβ1) signaling

The TGFβ superfamily of signaling molecules has been established to play a significant role in tissue repair (71, 72), but dysregulation of this family has also been shown to be pivotal in tissue fibrosis. TGFβ has three isoforms 1, 2 and 3, but most of our understanding on the roles of TGF signaling in homeostasis and disease are based on studies of TGFβ1 (72). TGFβ1 is a pleiotropic growth factor produced by various cells such as macrophages, epithelial cells and fibroblasts, and is involved in many cellular processes including apoptosis, cell differentiation, proliferation and extracellular matrix remodeling (71, 73). The production of TGFβ1 involves its secretion into the extracellular milieu in a latent form bound to the latency-associated peptide (LAP) that further associates with the latent TGFβ-binding protein (LTBP) (71, 74). TGFβ1 is activated by
either integrin-mediated mechanisms via αvβ3, αvβ5, αvβ6 or αvβ8, or through integrin-independent mechanisms including proteases, changes in pH, ROS, ANG II and thrombospondin-1 (63, 74, 75, 95, 99). Once activated, TGFβ1 is free to signal by binding to the Transforming growth factor receptors type I (TGFβRI) and II (TGFβRII). TGFβ1 initially binds to a homodimer of TGFβRII resulting in the recruitment of the TGFβRI homodimer (76-78). Through autophosphorylation, TGFβRII is able to transphosphorylate the TGFβRI to activate either the canonical SMAD signaling pathway, the main pathway thought to be involved in fibrosis, or the non-canonical signaling pathways which include p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase MAPK (ERK MAPK), RHO, JUN N-terminal kinase (JNK) and phosphoinositide 3-kinase (PI3K) (Fig. 1.2) (63, 71).

As shown in Figure 1.2 during canonical SMAD signaling, activation of the TGFβRII/TGFβRI complex leads to the recruitment and phosphorylation of SMAD2 and SMAD3 by phosphorylated TGFβRI (79). Together with SMAD4, the heterotrimer translocates into the nucleus and initiates gene transcription by binding to SMAD response elements (Fig. 1.2) (78-80). SMAD signaling is regulated by the inhibitory SMADs, SMAD6 and SMAD7, by competitively binding to TGFβRI and/or by recruiting ubiquitin ligases or phosphatases to target the receptor for degradation, thereby terminating signaling (63).
Figure 1.3 TGFβ1 canonical and non-canonical signaling pathways.
When TGFβ1 is active, it binds to the TGFβRII homodimer thereby recruiting the TGFβRI homodimer to the complex. Transphosphorylation of TGFβRI by TGFβRII allows it to activate various TGFβ1 signaling pathways. One of the main pathways activated is the canonical SMAD signaling pathway (orange), through phosphorylation of SMAD2 and SMAD3. Together with SMAD4, the complex is able to translocate to the nucleus and activate gene transcription. Other pathways activated by TGFβ1 are the non-canonical signaling pathways (pink): p38 MAPK, ERK MAPK, RHO, JNK, and PI3K.

The activation of TGFβRII and TGFβRI homodimers by TGFβ1 can also lead to the recruitment and phosphorylation of various kinases and adaptor molecules for activating the non-canonical pathways. TGFβ1 activates both JNK and p38 through the recruitment of TNF receptor associated factor 6 (TRAF6) to the TGFβRI/II complex and the phosphorylation of TGFβ-
activated kinase 1 (TAK1) (Figure 1.2) (81). At this point, the signaling pathway diverges leading to JNK/JUN or p38 activation which can both induce apoptosis and fibroblast differentiation to myofibroblasts (82, 83). For ERK MAPK activation, the assembly of the adaptor proteins SH2, Grb2 and Sos leads to activation of Ras and the phosphorylation of ERK1/2 (Figure 1.2) (84), which has been shown to have essential roles in cell proliferation and differentiation (82). PI3K signaling can also promote cell differentiation and proliferation (85), however the precise mechanisms by which TGFβ receptors lead to the phosphorylation of the downstream effector AKT is not known. RHO or Rho-like GTPases can also be activated by TGFβ1 via multiple mechanisms (82). Although each pathway seems to have distinct effects, the crosstalk of the different pathways is essential to respond appropriately to the TGFβ1 signal.

1.4.1 TGFβ signaling in COPD

The role of TGFβ in wound repair is complex and plays a role in most steps of the wound healing process. Gene expression studies on emphysema disease development and histological assessment of lungs from patients with COPD have suggested that TGFβ plays an important role in the disease (64, 65, 86, 87). However, the exact mechanism by which TGFβ mediates the pathological development in cigarette smoke-induced COPD is still unknown as there have been an abundance of conflicting data that indicate either excessive or deficient TGFβ activity is involved in COPD disease progression. While TGFβ1 does not seem to change in bronchial biopsies of patients with COPD (88), the small airway epithelium and monocytes have been shown to increase TGFβ1 production in COPD (86, 87). However, there have also been evidence of diminished TGFβ1 activity in COPD macrophages and emphysematous lesions (60, 89). Thus taken together, the intensity of TGFβ activity may be cell- and compartment-specific and
abnormalities in the system lead to a dysregulated wound repair phenotype with aberrant ECM deposition in each region. This would then explain the variable pathologies in COPD lungs with either fibrotic large and small airways, and emphysematous lesions.

During wound repair, the production of TGFβ1 induces epithelial plasticity in epithelial cells to confer a more migratory phenotype which includes diminished expression of intracellular adhesion proteins to allow for migration over the wound site through an interplay between the SMAD, ERK MAPK, JNK/p38 MAPK and RHO pathways (57, 82, 90, 91). In fibroblasts, TGFβ1 SMAD signaling not only mediates fibroblast transition to myofibroblast, it also induces production of connective tissue growth factor (CTGF), collagen 1, fibronectin and tenascin (92, 93). Furthermore, TGFβ1 mediates fibroblast survival via the activation of PI3K signaling (85).

Since TGFβ1 has broad effects in various physiological processes and regulates many cellular activities, its functions are tightly regulated at various levels, including its production. One of the factors that can influence the production of TGFβ1 is angiotensin II (ANG II) (94, 95). Since we found the TGFβ family of signaling molecules which includes ANG II signaling pathways to be significantly downregulated in emphysematous lesions, the interaction between these pathways may play a significant role in the development of emphysema.

1.5 Renin-angiotensin system (RAS) and angiotensin II signaling

The renin-angiotensin system (RAS) is more commonly associated with the cardiovascular system in its ability to regulate blood pressure through vasoconstriction or vasodilation of blood vessels, and induce production of aldosterone by the adrenal glomerulosa (96). The active peptides of the RAS pathway are derived from angiotensinogen, which is produced by the liver and cleaved by renin from the kidneys to convert it into the decapeptide angiotensin (ANG) I
As ANG I travels via the circulation through the lung, angiotensin-converting enzyme (ACE)-1, produced primarily by endothelial cells, lung epithelial cells, and macrophages cleaves ANG I into the octapeptide hormone ANG II (Fig. 1.3) (97-99) which can then signal through its receptors ANG II receptors type 1 and type 2 (AT1R and AT2R, respectively). ANG II can be further processed into other active RAS ligands: angiotensin III, angiotensin IV and angiotensin 1-7 (Fig. 1.3) (97, 100). Despite the lack of evidence on the levels of ACE in COPD BAL fluid, increased levels of ACE in BAL fluid have been observed in interstitial lung diseases such as IPF and sarcoidosis (101, 102). Additionally, as there is an increasing indication for marked activation of RAS in COPD with regards to COPD co-morbidities (103), this signaling pathway warrants further study in the context of pulmonary biology.

**Figure 1.4 The renin-angiotensin system.**
The active peptides in the RAS pathway are derived from angiotensinogen produced by the liver, which is cleaved by renin from the kidneys into the decapeptide ANG I. As ANG I transits through the lungs, ACE-1 produced by the lungs cleaves ANG I into the active octapeptide hormone ANG II. ANG II can be processed further into the active RAS ligands ANG III, ANG IV and ANG 1-7.
The commonly known bioactive peptide of RAS, ANG II, signals through two G-protein coupled receptors (GPCRs) AT1R and AT2R to mediate its effects. Both receptors have a high affinity for ANG II, but mediate opposing effects. As the name suggests, activation of each receptor results in G-protein activation, thereby activating the downstream signaling molecules. AT1R mediates the vasoconstrictive and cell proliferative effects of ANG II and can activate the JAK-STAT, p38 MAPK, JNK and ERK MAPK signaling pathways (Fig. 1.4) (98, 104, 105). In contrast, AT2R promotes vasodilative effects of ANG II and inhibits cell proliferation by the production of nitric oxide, and the activation of protein tyrosine phosphatases (PTPs), which inhibits ERK MAPK pathway activation (Fig. 1.4) (104, 106, 107).

In the production of ANG II in the lungs, particularly by the epithelium, ACE mRNA has been shown to be expressed by both columnar and squamous airway epithelial cells (108). However in culture, only squamous cells are capable of secreting ANG II (108). Squamous cell metaplasia is one of the major pathological changes in COPD due to cigarette smoke exposure and has been previously correlated with severity of airflow obstruction (22). Thus, it suggests that there is a potential dysregulation of ANG II production in the airway epithelium which could be contributing to the progression of the disease pathology.
Figure 1.5 ANG II signaling through AT1R and AT2R.
ANG II can bind to either one of its receptors, AT1R or AT2R, to activate signaling. Both receptors are GPCRs and activation leads to recruitment of various signaling molecules to activate the p38 MAPK, JAK-STAT, JNK/JUN, PI3K and ERK MAPK pathways for AT1R, while AT2R activation induces nitric oxide production and activates protein tyrosine phosphatases that antagonize ERK MAPK signaling.
1.6  TGFβ1 and ANG II signaling crosstalk

TGFβ1 has important functions in tissue morphogenesis and repair, and thus has been implicated to play a significant role in the pathogenesis of many diseases including asthma, IPF and other fibrotic diseases (88, 92, 109) due to either an excess or deficiency in its activity. The diverse disease pathologies demonstrate the importance of regulating the levels of TGFβ1. There have been many studies documenting the crosstalk between TGFβ1 and ANG II signaling pathways in various organ systems (94, 95, 110-113) to promote tissue remodeling and potentially, fibrosis. In cardiac myocytes, fibroblasts and lung fibroblasts, ANG II has been shown to increase TGFβ1 production through AT1R signaling (94, 95), while TGFβ1 upregulates AT1R expression through the ERK MAPK pathway (113). Moreover, TGFβ1 can induce angiotensinogen production in lung fibroblast cell lines via transcriptional activation by JunD and HIF-1α (95, 112). Together, this creates a paracrine feedback loop between the two systems. However, since we found that both ANG II and TGFβ genes are downregulated in emphysema, we propose that the crosstalk between TGFβ1 and ANG II may be dysregulated in disease and promote disease progression.

1.6.1  Potential role for TGFβ1-ANG II crosstalk in COPD

The systemic effects of COPD, such as activation of the RAS, which can lead to ischemic heart disease and hypertension, have been established to contribute to the disease morbidity and mortality (2, 3). Therefore, the use of medications targeting the angiotensin-converting enzymes (ACE inhibitors) and ANG II receptors (ANG II receptor blockers or ARBs) have been in clinical use to treat co-morbid cardiovascular disease in COPD patients. Although most studies evaluating the effect of ACE inhibitors and ARBs have shown no effect on the lung function
decline associated with COPD (114-116), one study has shown that the AT1R antagonist can reduce lung hyperinflation in COPD patients (117) and current clinical trials are on ongoing. Moreover, in a mouse model of emphysema, Raupach et al. have demonstrated the ability of the AT1R inhibitor irbesartan to reduce emphysema and improve lung compliance (118) while a mouse model of Marfan syndrome treated with neutralizing antibodies against TGFβ show marked reduction in the air space enlargement observed in the animals (119). In the airways, AT1R is predominantly expressed by interstitial cells in the subepithelium while AT2R expression is observed in the epithelium and the expression of both these receptors are significantly greater in COPD, with AT1R expressed ten-fold greater in disease (120), which could suggest dysregulated ANG II signaling in COPD and therefore, crosstalk with TGFβ1.

1.7 Current therapies for COPD

Currently, there are no interventions that can reverse the progressive decline in lung function that occurs in patients with COPD. However, there are therapies that can either relieve the symptoms of the disease or slow the inevitable lung function decline.

1.7.1 Smoking cessation

Smoking cessation is the most effective therapy for current smokers with COPD. It has been shown that smoking cessation at any point of a COPD patient’s life significantly reduces the rapid decline in lung function associated with disease (121). However, smoking cessation is difficult for smokers due mainly to the addiction to nicotine which induces the release of various neurotransmitters that can regulate pleasure and mood. A wealth of nicotine replacement products are therefore available in addition to pharmacological interventions such as varenicline,
which is a partial agonist of a receptor believed to mediate nicotine dependence, and buproprion, a non-tricyclic antidepressant that can reduce nicotine cravings (122, 123). Both medications have been shown to improve smoking cessation strategies for patients with tobacco dependence (122-124).

### 1.7.2 Bronchodilators and corticosteroids

Although the airflow limitation in COPD is not fully reversible, bronchodilators are an important therapy for patients with mild to severe COPD to improve lung function due to the ability of these medications to alleviate bronchoconstriction. The two commonly prescribed classes of bronchodilators include beta$_2$-agonists, which activate beta$_2$-adrenergic receptors to increase cyclic AMP levels, and anticholinergics, which antagonize the muscarinic receptors to promote airway smooth muscle relaxation (3). While the long-acting forms of both drugs have been shown to improve FEV$_1$ and lung volume deficiencies, they have not been shown to affect lung function decline and mortality with long term use (2, 125, 126). The combination use of long acting beta$_2$-agonists and anticholinergics have been found most effective with reduced side effects, compared to single compound formulations (127, 128). Due to the chronic inflammation in the airways of patients with COPD, inhaled corticosteroids are prescribed to patients with COPD and are most effective in patients who have FEV$_1$ <60% predicted (129, 130). In combination with long-acting beta$_2$-agonists, there is further improvement of lung function and reduction of exacerbation frequency (129, 130). However, both bronchodilators and inhaled corticosteroids do not prevent the inevitable decline in lung function and do not affect patient mortality (3, 129).
1.7.3 Phosphodiesterase-4 inhibitors

Phosphodiesterase-4 (PD4) inhibitors, such as roflumilast, reduce inflammation by inhibiting the breakdown of cyclic AMP, but are indicated for use with at least one long-acting bronchodilator (3). It has been shown to improve FEV$_1$ in patients treated with salmeterol or tiotropium and reduce exacerbations (131, 132). However, the use of PD4 inhibitors has many adverse effects compared to inhaled medications including nausea, diarrhea and headache (131, 132).

1.7.4 Surgical treatment

Due to hyperinflation of the lung in severe end-stage COPD, removal of emphysema bulla, referred to as lung volume reduction surgery, can improve respiratory muscle mechanical efficiency and increase lung elastic recoil to improve expiratory flow of the remaining healthy lung tissue (133). Although lung volume reduction surgery has been shown to be effective in COPD patients with emphysema in the upper lobes and with low exercise tolerance, it can actually lead to a higher mortality rate in patients with severe emphysema than pharmacological treatments (134). For end-stage COPD patients the only remaining treatment is lung transplantation, which can improve significantly the patient’s quality of life (135), but comes with many risks including acute organ rejection and opportunistic infections.

1.8 Copper tripeptide Gly-His-Lys as a candidate therapeutic for COPD

In a previous study, our group identified the naturally-occurring tripeptide GHK-Cu as a candidate molecule for the treatment of emphysema. GHK-Cu has been shown to have wound healing properties, particularly in skin research as it has been shown to induce wound contraction, angiogenesis, cell proliferation and increase production of antioxidant enzymes and
expression of integrins in skin wound repair models (68-70). We found that the addition of GHK-Cu to COPD parenchymal fibroblasts was able to reverse their inability to remodel and contract collagen 1 (60), which are important fibroblast functions in the repair process. Therefore, this novel therapeutic could be able to reverse the emphysematous lesions.

1.9 Hypothesis and specific aims

There is much evidence to support the critical role of TGFβ1 on COPD pathogenesis (60, 86, 136). Since we found that both ANG II and TGFβ1 signaling are downregulated in emphysematous lesions and there is an aberrant repair response in primary parenchymal fibroblasts derived from COPD lungs, we hypothesize that the TGFβ1-ANG II crosstalk is dysregulated in lung fibroblasts of COPD patients, thereby disrupting normal wound repair and leading to disease.

To study the dysregulated crosstalk, our aims were to:

1) Determine the integrity of both the TGFβ1 and ANG II signaling pathways in COPD derived parenchymal fibroblasts.

2) Investigate the dysregulation of crosstalk between TGFβ1 and ANG II signaling in COPD derived fibroblasts.

3) Assess the potential of GHK in repairing TGF-ANG II crosstalk in COPD derived parenchymal fibroblasts.
Chapter 2: Materials and methods

2.1 Primary fibroblast isolation and culture

Primary parenchymal fibroblasts were isolated from lung tissue of ex-smokers that were undergoing lung resection surgery for cancer at University Medical Centre Groningen (UMCG), Groningen, Netherlands and St. Joseph Healthcare, Hamilton, Ontario. These samples were obtained through the kind assistance of our collaborators Prof. D.S. Postma, Dr. W. Timens and Dr. C.A. Brandsma (UMCG), and Dr. P. Nair (Hamilton). This study was approved by the Research Ethics Board of all institutions including the University of British Columbia (#H0-50110). Patients were defined by the Global Initiative for Obstructive Lung Disease (GOLD) using pre-operative spirometry measurements to define ex-smokers with normal lung function (FEV1/FVC >0.70), or COPD (FEV1/FVC <0.70) that was further stratified into mild GOLD I (≥ 80% predicted FEV1), moderate GOLD II, (50-79% predicted FEV1), severe GOLD III (30-49% predicted FEV1) or very severe GOLD IV (<30% predicted FEV1) COPD (3) as listed in Table 2.1.

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<td>Healthy (n=9)</td>
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<td>COPD GOLD II (n=5)</td>
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<td>COPD GOLD IV (n=5)</td>
<td>58.8 ± 1.5</td>
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<td>2M/3F</td>
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</table>

Table 2.1 Primary parenchymal fibroblast donor information.

M = male, F = female, FEV1 = forced expiratory volume in 1 sec, FVC = forced vital capacity, COPD = chronic obstructive pulmonary disease, GOLD = Global Initiative for Obstructive Lung Disease
Parenchymal fibroblasts were isolated from the normal margins of lung tissue using our previously described outgrowth technique (137). Briefly, parenchymal tissues were aseptically dissected into 2-3 mm² fragments and 5-6 fragments were seeded per well of a six-well tissue culture plate (BD Falcon) in the growth medium Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 1x antibiotic-antimycotic (Gibco), and incubated at 37°C with 5% CO₂. Over the course of 6 days, fibroblasts proliferated and migrated out of the tissue fragments on to the tissue culture plastic. After 7 days, the tissue fragments were removed, and fibroblasts were maintained in culture until confluent in the growth medium. For maintenance, cells were grown in 75 cm² flasks with ventilated cap in 11 mL growth media and were fed with fresh growth media every other day. At 80-90% confluency, cells were passaged with 3 mL 0.25% Trypsin-EDTA (Gibco, Life Technologies) and neutralized with 6 mL of growth media. The cells were pelleted at 1000 rpm for 5 min and seeded into a new flask or into 6-well tissue culture plates for experiments.

2.2 Primary fibroblast cell culture experiments

To examine the regulation of TGFβ1-ANG II crosstalk, primary parenchymal fibroblasts at passages 2-5 were seeded at 5 x 10⁴ cells/well in six-well tissue culture plates and grown to confluency at 37°C with 5% CO₂. To determine the signaling pathways activated by TGFβ1 and ANG II, fibroblasts were treated with either media alone, TGFβ1 (10 ng/ml, Peprotech), ANG II (100 nM, R&D Systems) or GHK-Cu (100 nM, Sigma) in DMEM supplemented with 10% FBS for 24 h prior to collection of mRNA or 48 h before harvesting protein lysates.

To determine the effect of serum concentration on the TGFβ1 response, parenchymal fibroblasts intended for low serum treatments were grown to confluency before an overnight
serum-starvation with DMEM supplemented with 1% FBS or 10% FBS DMEM. Fibroblasts were then treated with or without 10 ng/mL TGFβ1 in DMEM supplemented with either 1% FBS or 10% FBS for 24 h and 48 h for collection of mRNA and protein, respectively.

For protein extraction, cells were rinsed with sterile 1x PBS (HyClone, Thermo Scientific) then lysed in 120 µL protein lysis buffer containing 5x proteinase and 1x phosphatase inhibitor cocktails (Sigma) with 0.3 mM phenylmethanesulfonyl fluoride and collected using a cell scraper (Sarstedt).

For RNA extractions cells were lysed in 350 µL buffer RLT containing β-mercaptoethanol, and samples were extracted using cell scrapers then mRNA was processed according to the Qiagen RNeasy kit protocol (see method 2.5).

2.3 Denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot

Equal concentrations of protein lysates or supernatants, determined using a BCA Bradford protein assay (Bio-Rad) were centrifuged at 13000 rpm at 4°C, then mixed with SDS sample loading buffer (containing β-mercaptoethanol and bromophenol blue from a 4x stock solution) and boiled for 5 min. Before gel electrophoresis, a 10% SDS-polyacrylamide separating gel and a 4% SDS-containing stacking gel were made. Once loaded, samples were initially run at 90 V for 10-15 minutes in 1x running buffer containing glycine and SDS, to allow samples to resolve in the stacking gel, and then run for 1 - 1.5 h at 150 V to separate proteins by molecular weight in the separating gel. Subsequently, the proteins in the gels were transferred onto 0.45 µm nitrocellulose membranes (GE Healthcare Life Sciences) using 1x transfer buffer containing methanol at 100 V for 3 h, keeping the entire transfer box on ice. Protein transfer was ascertained
by Ponceau staining of the nitrocellulose membranes, followed by a TBS wash to destain the membranes. The membranes were then blocked with 1x Tris buffered saline (TBS) with 1% Casein (Bio-Rad) for 1 h rocking at room temperature to prevent non-specific binding of antibodies. Primary antibodies listed in Table 2.2 were then diluted to the required concentration in 1x TBS with 1% Casein and 0.1% Tween-20, and incubated with the nitrocellulose membranes overnight on a rocker at 4°C. After primary antibody incubation, membranes were washed 3 times with 1x TBS with 0.1% Tween-20 (1x TBST) prior to addition of the secondary antibodies. The Alexa Fluor 680 goat anti-rabbit IgG (1:2500 dilution, Life Technologies) and the anti-mouse IgG IRDye800 conjugated antibody (1:2500 dilution, Rockland Immunochemicals) were used to detect the primary rabbit or mouse anti-human antibodies, respectively. The secondary antibodies were diluted in 1x TBS with 1% Casein, 0.1% Tween-20 and 0.02% SDS and incubated for 1 h while rocking at room temperature. The membranes then were washed and after the last wash kept in 1x TBS before being scanned on the LI-COR Odyssey scanner (LI-COR Inc., Lincoln, NE). Densitometry values for the protein bands were obtained using the commercially available Odyssey version 2.1 software package. All values were normalized to the densitometry values of the housekeeping protein β-tubulin for each sample. To determine the activity of signaling molecules, the ratios of the normalized phosphorylated form to the normalized total form of each signaling protein were calculated.
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Table 2.2 Primary antibodies used for immunoblotting in denaturing conditions.

2.4 Non-denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot

To measure collagen production by parenchymal fibroblasts, cell culture supernatants were loaded in equal concentrations, after being prepared in non-denaturing sample loading buffer containing bromophenol blue, without β-mercaptoethanol or boiling. Before loading, a 7.5% SDS-polyacrylamide separating gel and 4% SDS-containing stacking gel were made. Samples were run and transferred to nitrocellulose membranes by Western blot as described in the methods above (section 2.3). The primary antibody used for the detection of collagen 1α1 and 1α2 isoforms was a rabbit polyclonal antibody (Abcam, ab3410, 1:1000) and the densitometry values were normalized to the β-tubulin densitometry values in the corresponding lysates.
2.5 RNA isolation

Purified mRNA was isolated according to the Qiagen RNeasy Mini kit protocol. Briefly, lysates were thawed on ice and vigorously vortexed. The lysates were mixed with equal volumes of 70% ethanol and spun through a RNeasy spin column for 30 sec at 17 x 1000g. The column was washed with buffer RW1 and twice with buffer RPE, supplemented with ethanol, prior to elution of the RNA with 30 μL RNase-free water. Total purified RNA was quantified using the NanoDrop 8000 UV-Vis Spectrophotometer.

2.6 Complementary DNA (cDNA) synthesis

cDNA was created with 1 μg purified mRNA according to the SuperScript® II Reverse Transcriptase protocol. Briefly, diluted total RNA was mixed with Random Hexamers (Life Technologies) and deoxynucleotide triphosphate (Life Technologies) and heated to 65°C for 5 min. Samples were then chilled on ice and briefly spun down. The 5x First-Strand buffer (Life Technologies) and 0.1 M dithiothreitol (Life Technologies) were then added and heated to 25°C for 2 min prior to the addition of SuperScript II Reverse Transcriptase. The samples were then incubated at 25°C for 10 min, followed by 50 min of extension at 40°C, and a final enzyme inactivation at 70°C for 15 min. Samples were stored at -80°C prior to use for qPCR.

2.7 Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed according to the TaqMan® Gene Expression Assay protocols. Briefly, each cDNA sample was mixed with 20x TaqMan® Gene Expression Assay and 2x TaqMan® Master Mix in 20 μL reaction volumes prepared in duplicate on a 384-well reaction plate, and thermal cycled as with a standard run on the Applied Biosystems ViiA7 Real-Time PCR
Machine as follows: hold at 95°C for 10 min then 40 cycles between 15 sec at 95°C and 1 min at 60°C. The ABI TaqMan assay (Applied Biosystems, Foster City, CA, USA) used for assessing AT1R mRNA expression was Hs00258938_m1, and two TaqMan assays for housekeeping genes (GAPDH, Hs99999904_m1; HPRT1, Hs99999909_m1) were run in parallel with the angiotensin receptor gene assays to determine the best housekeeping gene to normalize with, and GAPDH was found to be the most consistent gene in the experiments. Gene expression was calculated using the comparative CT method. CT values were obtained as the threshold cycle number when gene expression was first detected. The expression of AT1R was then normalized to GAPDH acquire the ΔΔCT values.

2.8 TGFβ1 enzyme-linked immunosorbent assay (ELISA)

Supernatants were collected from fibroblasts treated with ANG II for 48 h to measure TGFβ1 production using the TGFβ1 E_{max}® ImmunoAssay System (Promega, Madison, WI), as per the manufacturer’s instructions. Briefly, assay plates were coated with the capture antibody overnight for 4°C, then blocked for 35 min at 37°C. Then each sample was diluted 1:2.5 and acidified for 15 min at room temperature with 1N HCl to activate TGFβ1, then neutralized with 1N NaOH to return the pH to approximately 7.6. After washing the plates, the serially diluted standards were loaded with the acidified samples diluted 1:2 for a final dilution at 1:5 and incubated shaking at room temperature for 90 min. The plates were then washed and the detection antibody was incubated for 2 h with shaking at room temperature. After washing the plates, the TGFβ HRP conjugate was incubated for 2 h at room temperature. After further washing, TMB was added and incubated for 15 min at room temperature to visualize TGFβ1, then the reaction was stopped with 1N HCl. Absorbance was then measured at 450 nm using a
Spectra Rainbow Microplate Reader (SLT Lab Instruments). All plate washing steps were done three times using wash buffer composed of 20 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.05% (v/v) Tween 20.

2.9 Whole lung sample acquisition and processing

Lungs tissues were obtained from COPD GOLD IV patients (n=3) who underwent double lung transplantation at the University of Pennsylvania and donor lungs without COPD (n=3) were received from the Gift of Life Organ Procurement Organization in Philadelphia after no suitable recipients were identified for the organs (Table 2.3). Written informed consent to receive the lungs and the associated clinical and radiological data for the research study was obtained from patients and the next of kin for donated lungs released for research.

Lungs were obtained as described previously (60). Briefly, after surgical removal the bronchial stump was cannulated and slowly inflated with compressed air to a transpulmonary pressure of 30 cmH\textsubscript{2}O for alveolar recruitment, and then held at a transpulmonary pressure of 10 cmH\textsubscript{2}O while frozen in liquid nitrogen vapor. Each lung was cut into 2 cm thick slices from the apex to the base to obtain 8 or more lung slices. A tissue core of 2 cm in diameter was taken from each slice of lung to acquire a total of 8 tissue cores per donor.

<table>
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</tbody>
</table>

Table 2.3 Donor demographics for lung tissue used for gene expression

M = male, F = female, COPD = chronic obstructive pulmonary disease, GOLD = Global Initiative for Obstructive Lung Disease
2.10 NanoString sample preparation and analysis

RNA was isolated from each core using the Qiagen RNeasy Mini kit according to the protocol outlined above, then sent to NanoString Technologies (NanoString Technologies, Inc.) for gene expression analysis. Data acquired from NanoString Technologies were analyzed according to the nCounter™ Data Analysis Guidelines. Briefly, the geometric mean for each sample was taken for the positive spike-in RNA hybridization controls to estimate the overall probe hybridization efficiency per sample. The geometric means were averaged to obtain a normalization reference for all samples. Further normalization was done to account for RNA content variability in each sample using the five housekeeping genes: ACTB, CLTC, PGK1, POLR1B, RPLP0. After multiplying the normalization reference to all the gene counts, the geometric mean of all the housekeeping genes for each sample were calculated and averaged to create a normalization factor. All other gene counts were then multiplied further with the normalization factor to obtain the final normalized gene counts for analysis. The genes AT1R, AT2R, COL1A1, TGFBR2, TGFBR1 and TGFB1 were used for this analysis.

2.11 Statistical analysis

Data is presented as the mean ± SEM when presented as bar graphs or scatter plots. A parametric Student’s t-test was used to compare all baseline differences in protein expression between donors with normal lung function and COPD. All pair-wise comparisons of cells at baseline conditions compared to treatment with TGFβ1, ANG II or GHK were made using paired t-tests. One-way analysis of variance with Tukey post-test was used to compare group data. All statistical tests were performed using GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA), with P values less than 0.05 considered statistically significant.
Chapter 3: Results

3.1 TGFβ1 downregulates AT1R protein expression in healthy ex-smoker-derived parenchymal fibroblasts.

Our first aim was to determine if TGFβ1 signaling was able to regulate the expression of ANG II receptors as previously shown in the literature and if this was dysregulated in COPD parenchymal-derived fibroblasts. As shown in Figure 3.1A, when parenchymal fibroblasts from healthy ex-smokers (white circles), COPD GOLD II (pink circles) and GOLD IV (maroon circles) were treated with recombinant human TGFβ1 (10 ng/ml) for 48 h, we observed a significant decrease in AT1R protein expression by immunoblot (Mean 0.183 ± 0.034 arbitrary units (AU)) compared to untreated cells (Mean 0.254 ± 0.036 AU, P<0.01). As expected, we observed no alterations in the protein expression of AT2R when parenchymal fibroblasts were treated with TGFβ1 compared to baseline (Figure 3.1B). Moreover, we found that the downregulation of AT1R due to TGFβ1 was most prominently observed in fibroblasts from patients with healthy lung function (Figure 3.1C, P<0.05) while the response seemed to be blunted in COPD-derived parenchymal fibroblasts (Figures 3.1D & 3.1E).
Primary parenchymal fibroblasts from healthy ex-smokers (white circles, n=7), COPD GOLD II (pink circles, n=5) and COPD GOLD IV (maroon circles, n=5) were treated with 10 ng/mL recombinant human TGFβ1 for 48 h to determine its effect on (A) AT1R and (B) AT2R protein expression. AT1R protein expression in response to TGFβ1 was plotted separately for (C) healthy ex-smokers, (D) COPD GOLD II and (E) COPD GOLD IV to determine differences in response. * p<0.05 and ** p<0.01 compared to untreated controls by paired t-test.

As other groups have also investigated the effect of TGFβ1 on AT1R expression and previously reported that TGFβ1 can upregulate AT1R expression in healthy non-smoker lung fibroblasts (113), we further examined the published protocol to identify differences with our own work.
The previous investigation used serum-free conditions for both pretreatment and TGFβ1 stimulation of the cells, while our experimental protocol used 10% serum. We therefore conducted a direct comparison of 10% and 1% serum-starvation prior to treatment with TGFβ1 in the corresponding serum concentration in a subset of six healthy ex-smokers. As shown in Figure 3.2A, serum-deprivation with 1% FBS actually augmented the downregulatory effect of TGFβ1 on AT1R protein expression (Figure 3.2A, P<0.05) compared to the 10% FBS treatments (Figure 3.2B), further confirming our findings.

![Figure 3.2](image)

**Figure 3.2** Serum concentration does not reverse the downregulatory effect of TGFβ1 on AT1R protein expression.

To ascertain that the downregulation of AT1R was not an artifact due to the presence of serum in the treatments, parenchymal fibroblasts from ex-smokers with healthy lung function (n=6) were treated for 48 h with or without 10 ng/mL TGFβ1 in (A) 1% or (B) 10% serum. * p<0.05 compared to the untreated control using paired t-test.
3.2 TGFβ1 signaling is preserved in parenchymal fibroblasts derived from healthy ex-smokers and COPD patients.

To determine which aspect of TGFβ1 signaling was regulating AT1R protein expression, we first assessed the basal protein expression of TGFβRII, which is responsible for recruitment and phosphorylation of the accompanying serine/threonine kinase TGFβRI, which consequently phosphorylates intracellular tyrosine kinases for activation of downstream signaling molecules of the TGFβ1 pathway. We found that TGFβRII was not differentially expressed in parenchymal fibroblasts derived from the lungs of either ex-smokers with normal lung function (white circles) or those with COPD (GOLD II: pink circles, GOLD IV: maroon circles) irrespective of disease severity (Figure 3.3). We were unable to optimize staining for TGFβRI immunoblot using commercially available antibodies and therefore its expression could not be assessed.

![Figure 3.3 Basal TGFβRII protein expression in parenchymal fibroblasts does not differ across the different donor groups.](image)

Baseline TGFβRII expression of parenchymal fibroblasts from healthy ex-smokers (white circles, n=9), COPD GOLD II (pink circles, n=5) and COPD GOLD IV (maroon circles, n=5) were measured by immunoblot.
We next explored the basal and TGFβ1-induced activation of the canonical SMAD, and non-canonical ERK MAPK, PI3K and p38 MAPK signaling pathways. We observed that the baseline activity for all four pathways investigated were similar across the different groups (Figure 3.4). When stimulated with TGFβ1, we found elevated phosphorylation of SMAD3 (Figure 3.4A), ERK1/2 (Figure 3.4B), AKT (Figure 3.4C), but not p38 (Figure 3.4D) in parenchymal fibroblasts, and there was no difference in the level of activation across the various donor groups. These data suggest that the differential regulation of AT1R expression in disease cannot be attributed to aberrancies in the phosphorylation of these downstream signaling proteins analyzed.
Figure 3.4 TGFβ1 activates SMAD, ERK MAPK and PI3K signaling in ex-smoker parenchymal-derived fibroblasts.

Parenchymal fibroblasts from healthy ex-smokers (white circles, n=8), COPD GOLD II (pink circles, n=5) and COPD GOLD IV (maroon circles, n=5) were treated with TGFβ1 (10 ng/mL) for 48 h, and activation of (A) SMAD, (B) ERK, (C) PI3K and (D) p38 pathways were assessed by measuring the phosphorylated to total protein ratios of the candidate signaling molecules for each pathway. * indicates p<0.05 and ** p<0.01 using paired t-test.
To determine if TGFβ1-induced AT1R downregulation is due to changes at the transcriptional level, we isolated mRNA following 24 h of TGFβ1 treatment from a sub-group of six healthy ex-smokers for assessment of AT1R mRNA expression by qPCR. As shown in Figure 3.5, the transcript levels of AT1R were extremely low at baseline in parenchymal-derived fibroblasts, and did not alter with TGFβ1 stimulation. These data indicate that TGFβ1-induced downregulation of AT1R protein expression may occur through proteasomal degradation, however studies to assess this process using the proteasome inhibitor MG-132 could not be conducted at this time due to the availability of additional samples.

Figure 3.5 TGFβ1 does not affect AT1R mRNA expression in primary parenchymal fibroblasts.
AT1R mRNA expression was assessed from healthy ex-smoker parenchymal fibroblasts (n=6) treated for 24 h with TGFβ1 (10 ng/mL) in 1% serum.

3.3 ANG II signaling is blunted in parenchymal fibroblasts from COPD patients.

To determine the integrity of ANG II signaling in COPD parenchymal fibroblasts, the baseline expression of AT1R and AT2R was measured by immunoblot. Although we observed a trend for decreased AT1R (Figure 3.6A) and AT2R (Figure 3.6B) protein expression in ex-smokers with
COPD (GOLD II: pink circles, GOLD IV: maroon circles) compared to individuals with healthy lung function (white circles), this did not reach statistical significance.

However, when we assessed AT1R gene expression in human lung tissue we found that COPD GOLD IV lung tissue (Mean 250 ± 72 gene counts, filled bar) had significantly lower AT1R expression than in healthy donor tissue (Mean 640 ± 86 gene counts, open bar) (Figure 3.6C, P<0.0001). In contrast, AT2R gene expression in lung tissues from healthy and COPD GOLD IV patients showed no difference (data not shown).
Figure 3.6 Basal AT1R and AT2R protein expression of parenchymal fibroblasts does not differ with disease, but AT1R gene expression is lower in COPD GOLD IV lung tissue. (A) AT1R baseline expression in fibroblasts measured by immunoblot from ex-smokers with healthy lung function (white circles, n=9) and patients with COPD (GOLD II: pink circles, n=5; GOLD IV: maroon circles, n=5). (B) AT2R baseline expression was also assessed in the same samples by immunoblot. (C) Gene expression of AT1R was measured by NanoString in lung tissues extracted from healthy donors (n=3, open bar) and COPD GOLD IV patients (n=3, filled bar). **** signifies p<0.0001 using a student’s t-test.

We next ascertained if the downstream signaling pathways of ANG II which include the JAK-STAT, p38 MAPK, ERK MAPK and PI3K signaling by phosphorylation of STAT3, were aberrant due to alterations in ANG II receptor expression. As shown in Figure 3.7, we observed p-STAT3 (Figure 3.7A), p-ERK1/2 (Figure 3.7B), p-AKT (Figure 3.7C) and p-38 (Figure 3.7D) activity were not different at baseline or following ANG II (100 nM) stimulation for 48 h in parenchymal-derived fibroblasts in the three donor groups.
Figure 3.7 ANG II does not activate the JAK-STAT, p38 MAPK, ERK MAPK or PI3K signaling in primary parenchymal fibroblasts. Activation of (A) STAT3, (B) p38, (C) ERK1/2 and (D) AKT activities were assessed in healthy ex-smoker (white circles, n=9), COPD GOLD II (pink circles, n=5) and GOLD IV (maroon circles, n=5) parenchymal fibroblasts after stimulation with 100 nM ANG II for 48 h by measuring the phosphorylated to total protein ratios of each signaling molecule.
3.4 ANG II downregulates TGFβ signaling in parenchymal fibroblasts.

Since there is substantial literature for cross-talk between ANG II and TGFβ1, we next sought to determine if ANG II can regulate the TGFβ1 signaling pathway. As shown in Figure 3.8, ANG II downregulated TGFβRII protein expression in parenchymal fibroblasts (P<0.05). When responses were segregated by disease, we found no difference between ex-smokers with normal lung function, GOLD II or GOLD IV COPD patients (data not shown).

![Graph showing TGFβRII and β-tubulin expression](image)

**Figure 3.8 ANG II downregulates TGFβRII expression in parenchymal fibroblasts.** Parenchymal fibroblasts from healthy ex-smokers (n=9), COPD GOLD II (n=5) and GOLD IV (n=5) were treated with ANG II (100 nM, filled bar) for 48 h and compared to untreated samples (white bar) to determine its effect on TGFβRII protein expression. * indicates p<0.05 with a paired t-test.

The addition of ANG II (black bar) to ex-smoker fibroblasts induced a significant increase in TGFβ1 production compared to untreated fibroblasts (white bar) (Figure 3.9A, P<0.05). However, when the responses were divided by disease ANG II only moderately increased the production of TGFβ1 in healthy (gray bar) and COPD (dark purple bar) parenchymal fibroblasts which was not statistically significant (Figure 3.9B), although there was
a trend for TGFβ1 protein levels to be lower from COPD derived fibroblasts (Figure 3.9B). The relevance of our observation of reduced TGFβ1 release by COPD parenchymal fibroblasts was supported by lung tissue gene expression analysis as we found statistically lower expression of TGFβ1 in COPD GOLD IV (Mean 1790 ± 101, filled bar) compared to healthy lung tissue (Mean 2224 ± 171, open bar) (Figure 3.9B, P<0.05).

Figure 3.9 TGFβ1 expression is downregulated in COPD.
To evaluate the effect of ANG II on TGFβ1 production, the levels of TGFβ1 in parenchymal fibroblast culture supernatants measured by ELISA from all groups treated with ANG II (100 nM, black bar) for 48 h were (A) pooled (n=19) and compared to the untreated controls (white bar). These data were then divided (B) by disease into healthy ex-smokers (gray bars, n=9) and COPD (purple bars, n=10). Lung tissue gene expression of (C) TGFβ1 was also assessed by NanoString from healthy controls (n=3, open bar) and COPD GOLD IV donors (n=3, filled bar). * indicates p<0.05 with a student’s t-test.
3.5 Collagen 1 production is strongly induced by TGFβ1 in COPD parenchymal fibroblasts.

TGFβ1 is a potent repair cytokine that is tightly regulated to ensure homeostasis of tissues rather than over-exuberant repair leading to tissue fibrosis. ANG II is one molecule thought to regulate the homeostasis of TGFβ1 and therefore, we wanted to determine if the defective ANG II signaling in COPD fibroblasts results in a fibrotic cell phenotype in the presence of TGFβ1. It is well described in the literature that TGFβ1 is a potent inducer of collagen 1 in mesenchymal cells. Thus, we examined the ability of parenchymal fibroblasts derived from COPD patients to release collagen 1α1 and collagen 1α2 into the cell culture media. We first assessed the gene expression of collagen 1α1 in vivo using lung tissues collected from patients with healthy lung function or COPD GOLD IV. Gene expression of collagen 1α1 was significantly greater in COPD GOLD IV tissues (Mean 972 ± 106) compared to tissues from healthy individuals (Mean 299 ± 39) (Figure 3.10A, P<0.0001). In contrast, we found that the basal production of both collagen 1α1 (Figure 3.10B) and collagen 1α2 (Figure 3.10C) were not different between healthy ex-smokers and COPD fibroblasts. Interestingly, upon the exogenous addition of TGFβ1 we found a significant increase in the production of collagen type 1α2 (Figure 3.10E, P<0.05) in COPD-derived fibroblasts irrespective of severity compared to healthy ex-smokers. Collagen 1α1 followed a similar trend of expression as collagen 1α2 but this was not statistically significant (Figure 3.10D).
Figure 3.10 TGFβ1 augments collagen 1 production in primary parenchymal fibroblasts.
To assess the effect of TGFβ1 on collagen 1 production, fibroblasts from ex-smokers with healthy lung function (white circles, n=9) or with COPD (GOLD II: pink circles, n=5, GOLD IV: maroon circles, n=4) were treated with or without TGFβ1 (10 ng/mL) for 48 h. (A) Collagen 1α1 and (B) collagen 1α2 production were measured in the cell supernatant by immunoblot. To examine differential responses to TGFβ1, the level of TGFβ1-induced (C) collagen 1α1 and (D) collagen 1α2 production was compared between the healthy ex-smokers and COPD donors. * is p<0.05 with a paired t-test.
3.6 The tripeptide GHK-Cu does not induce a similar signaling response as TGFβ1.

Our previous work has found that GHK-Cu, a known factor in skin research to promote wound repair, is able to reverse some of the defective fibroblast phenotype in COPD. We therefore attempted to determine if GHK-Cu could regulate AT1R and collagen 1α1 expression in parenchymal fibroblasts to potentially enhance TGFβ1 signaling *in vivo*. However, while we have shown GHK-Cu induces a gene expression pattern similar to TGFβ1 it did not induce downregulation of AT1R compared to untreated fibroblasts (Figure 3.11A). Similarly, we found that unlike TGFβ1, GHK-Cu does not induce changes in the ability of the fibroblasts to produce collagen 1α1 compared to baseline levels (Figure 3.11B). As shown by these data, there were no differences in expression by disease.
Figure 3.11 GHK-Cu does not regulate AT1R or collagen 1 protein expression.

To determine whether GHK-Cu had a similar response as TGFβ1 in ex-smoker fibroblasts with healthy lung function (white circles, n=8), COPD GOLD II (pink circles, n=5) and COPD GOLD IV (maroon circles, n=5), fibroblasts were treated with or without 100 nM GHK-Cu for 48 h. (A) AT1R protein expression was assessed in cell lysates while (B) collagen 1α1 production was measured in cell supernatants by immunoblot.

As GHK-Cu did not produce a similar fibrotic response as TGFβ1 for AT1R and collagen 1α1 protein expression, we then investigated if GHK-Cu can activate the canonical and non-canonical TGFβ1 signaling pathways. However, as shown in Figure 3.12, GHK-Cu activation of SMAD (Figure 3.12A), ERK MAPK (Figure 3.12B), PI3K (Figure 3.12C) or p38 MAPK (Figure 3.12D) signaling was not statistically different from untreated samples, demonstrating that GHK-Cu does not mimic TGFβ1 in activating the canonical and non-canonical signaling pathways.
Figure 3.12 GHK-Cu does not activate TGFβ1 signaling pathways. Parenchymal fibroblasts from healthy ex-smokers (white circles, n=8), COPD GOLD II (pink circles, n=5) and GOLD IV (maroon circles, n=5) were treated with GHK-Cu (100 nM) or media alone for 48 h. Activation of (A) SMAD3, (B) ERK1/2, (C) AKT and (D) p38 activities were measured with the phosphorylated to total protein ratios of the signaling molecules.
Chapter 4: Discussion

This work demonstrates that TGFβ1-ANG II crosstalk is defective in COPD derived parenchymal fibroblasts. Specifically, we show that TGFβ1 downregulates AT1R expression in parenchymal derived fibroblasts from healthy ex-smokers but not in COPD derived fibroblasts. This is further reflected in our findings that there is significantly lower mRNA expression of AT1R in whole lung tissue from patients with very severe COPD compared to healthy ex-smokers. Furthermore, exogenous TGFβ1 induced increased expression of collagen 1α1 and 1α2 in COPD derived fibroblasts compared to healthy ex-smokers. Increased collagen 1 mRNA was also observed in COPD compared to ex-smoker lung tissue. Interestingly, we found that TGFβ1 production by parenchymal fibroblasts and tissue was reduced in COPD but ANG II was able to augment this production in both healthy ex-smoker and COPD parenchymal fibroblasts. These findings highlight the abnormal responses of COPD parenchymal fibroblasts to TGFβ1 and ANG II mediated signaling, and may elucidate why we observe defective repair of parenchymal tissues in COPD.

TGFβ1-ANG II signaling crosstalk has been a topic of interest in various fibrotic diseases of the kidneys, heart and lungs (94, 95, 110-113). The close relationship between the two pathways has been observed by many investigators to potentiate the activity of TGFβ1 and ANG II signaling through various mechanisms. Here we show that exogenous TGFβ1 induces downregulation of the AT1R in parenchymal fibroblasts from patients who quit smoking, while AT2R expression was not affected. Interestingly, in fibroblasts derived from COPD patients, the TGFβ1-induced downregulation of AT1R was dysregulated, suggesting a defect in the TGFβ1-ANG II crosstalk in COPD-derived parenchymal fibroblasts. In contrast to our findings, a previous report on adult lung fibroblasts derived by explant technique from three cancer
resection and three IPF lung tissues previously demonstrated that TGFβ1 induces upregulation of AT1R protein and mRNA expression through the ERK MAPK signaling pathway (113). This study used predominantly fibroblasts from IPF tissue and cancer resection patients for which smoking status was not defined, which may account for the opposite responses in the two studies as IPF is a fibrotic parenchymal lung disease in comparison to emphysema. There were also additional differences in the cell culture protocols used specifically in comparison to our treatments, which were made in 10% serum conditions as opposed to serum-free conditions. Serum contains a mixture of growth factors and proteases that could influence the cellular response of fibroblasts to TGFβ1; however complete absence of serum in culture medium deprives cells of essential growth factors that are required for normal functions in vivo. Therefore, we tested the effect of serum concentration on the TGFβ1-induced AT1R downregulation in fibroblasts derived from six ex-smokers with normal lung function. Interestingly, we found that in 1% serum, TGFβ1 induced an even more robust downregulation in AT1R protein expression, further supporting our observations.

Since COPD parenchymal fibroblasts were not as responsive to TGFβ1-induced downregulation of AT1R protein expression, we sought to determine the component of the TGFβ1 signaling pathway that was mediating this defective response. Protein expression of TGFβRII, the first receptor to interact with TGFβ1 in the signaling cascade, was not differentially expressed between fibroblasts from healthy ex-smokers and those from COPD. Downstream of the TGFβRII/I receptor complex, we found no differences in the activation of SMAD, ERK MAPK and PI3K signaling pathways that were induced in response to TGFβ1 stimulation between fibroblasts from healthy ex-smokers and patients with COPD, suggesting that the TGFβ1 signaling pathways analyzed were intact in parenchymal lung fibroblasts and do
not contribute to the discrepancy in the response to exogenous TGFβ1. When we assessed mRNA expression of AT1R pre and post TGFβ1 stimulation, we did not observe any changes in mRNA expression. As the turnover of AT1R within the cell has been shown to be 90 min (138), the effect of TGFβ1 on AT1R downregulation could be mediated through a post-translational event such as receptor degradation. Glycosylated AT1R on the cell membrane can be ubiquitinated in preparation for proteasomal degradation (138), and TGFβ1 could be mediating AT1R receptor degradation through this pathway. Due to the availability of donor cells we were unable to test this hypothesis, but in future studies this could be determined through the use of the proteasome inhibitor MG-132 when cells are treated with TGFβ1. In keeping with our findings, in lung tissue from COPD patients we observed a significant decrease in AT1R mRNA compared to tissue from healthy ex-smokers. In contrast with these findings, AT1R protein expression measured by immunohistochemistry has been shown to be significantly greater in lung resection tissues from smokers with mild, moderate and severe COPD (GOLD I-III) compared to healthy smokers and non-smokers (120). The comparisons of these two studies though are difficult as we included only ex-smokers in our study and we are comparing the expression of protein in fibroblasts and mRNA in whole tissue compared to semi-quantitative assessment by histology. Further, the study by Bullock et al., investigated AT1R expression in large and small airways and not the parenchymal tissue again making comparison difficult. Therefore, future studies in additional cohorts will need to be conducted to assess if AT1R is significantly downregulated in COPD and in particular, emphysema.

Although we found TGFβ1-induced SMAD2/3 signaling was intact in COPD parenchymal fibroblasts, canonical SMAD signaling has been previously shown to be dysregulated in COPD. Immunohistochemical staining of lung tissues from patients with mild
COPD (GOLD II) have indicated that epithelial and stromal cells express lower levels of SMAD3 and SMAD7, which was not observed in very severe COPD (GOLD IV) lung tissue (139). Bronchial biopsies from patients with GOLD II status have also been shown to have decreased expression of the inhibitory SMADs, SMAD6 and SMAD7 (140). Despite the identification of the distinct layers of the lung tissue in these studies, stromal cells are composed of various cell types and therefore using immunohistochemistry alone, it is almost impossible to attribute protein expression to distinct cell types without the use of double labeling. Additionally there was poor identification of smoking status in these studies, which has been shown to greatly affect gene expression (32). While immunohistochemistry studies provide valuable “snapshots” of the responses of tissues in disease, unlike in vitro culture it does not allow for the examination of the functionality of signaling pathways of cells in health and disease. This phenomenon has been documented by Zandoort et al. as they found no differential responses in primary parenchymal fibroblasts from ex-smokers with normal lung function compared to COPD, with regards to TGFβ1-mediated upregulation of SMAD7 and downregulation of SMAD3 between the two groups (93).

Given the dysregulation of TGFβ1-induced AT1R expression in COPD parenchymal fibroblasts, we additionally assessed the integrity of ANG II signaling in these cells. Basal protein expression of both AT1R and AT2R in parenchymal fibroblasts was not statistically different with disease however the expression of both receptors was decreased in COPD derived fibroblasts. Comparative gene expression analysis of lung tissues from healthy donors and very severe COPD (GOLD IV) patients showed significantly decreased AT1R expression in disease, indicating that AT1R expression may be downregulated and contribute to defective TGFβ1-ANG
II crosstalk in COPD. In our study, the addition of exogenous ANG II did not activate any of the proposed ANG II signaling pathways that we assessed. Many studies have evaluated the effect of ANG II on lung fibroblasts, but few studies have looked at the activation of ANG II signaling pathways beyond the receptors in the pulmonary system (95, 99, 141). One pathway that has been shown to be activated in human fetal lung fibroblasts in response to ANG II stimulation is the ERK MAPK pathway (142). However, since a cell line was used in this study from a fetal source without exposure to cigarette smoke, it is likely not representative of the primary adult fibroblasts used in our study. However, in response to exogenous ANG II we observed significantly increased production of TGFβ1, as previously described by other studies (95, 99). Therefore, ANG II may signal through unidentified pathways in ex-smoker parenchymal fibroblasts to mediate this response. When analyzed by disease, we observed less ANG II-induced TGFβ1 production in COPD compared to normal derived fibroblasts; however this difference was not statistically significant. Moreover, ANG II downregulated TGFβRII protein expression in the fibroblasts, suggesting that ANG II is functional and possibly activating a different pathway we did not evaluate. In studies examining the ANG II-TGFβ1 crosstalk in human lung fibroblasts, they have shown that ANG II is able to stimulate TGFβ1 signaling by inducing production of TGFβ1 in primary human lung fibroblasts and cell lines via AT1R (95, 99). Likewise, we have shown that ANG II was able to induce an increase in TGFβ1 production, which would most likely augment TGFβ1 signaling activity. The finding that parenchymal fibroblasts from COPD ex-smokers seem to produce less TGFβ1 compared to healthy donors is a novel finding, and further supported by our in vivo TGFβ1 gene expression data where we observe a significantly lower expression of TGFβ1 in tissues from COPD GOLD IV patients compared to healthy controls. In contrast to our work, a previous study by Togo et al. (136),
showed that COPD GOLD II-IV parenchymal fibroblasts from explant cultures produce greater concentrations of TGFβ1 compared to control subjects with normal lung function (136). However, in this study they did not distinguish the patient smoking status which can have a great impact in the observed phenotype of the cultured fibroblasts. In general, the role of TGFβ1 in COPD disease pathogenesis is controversial. Studies have shown increased TGFβ1 production in disease by primary airway, alveolar epithelial cells and monocytes (86, 87, 108, 143), whereas macrophages, which are one of the most elevated inflammatory cells in COPD lungs, and with disease severity, have been shown to have a lower propensity for TGFβ1 production in response to the TLR4 ligand lipopolysaccharide (89). These studies do not negate the fact that in COPD parenchymal lung tissue we and others have reported decreased TGFβ1 mRNA expression which is a reflection on the total tissue state and not just individual cell types. Therefore, subsequent studies with well-defined cell or tissue cohorts are required to truly assess the potential differences in TGFβ1 expression in both airway and parenchymal tissue compartments with disease severity, if we are to truly understand the role of TGFβ1 in COPD.

Paradoxically, ANG II also caused a downregulation in TGFβRII protein expression which would result in reduced TGFβ1 activity. A potential reason for these results may be that activation of AT1R may be causing the production of TGFβ1, while the activation of AT2R known to have antagonist functions, may result in the downregulation of TGFβRII. To our knowledge, this is the first account of a relationship between ANG II and TGFβRII in a human system. In cultured mouse proximal tubular cells, ANG II has been shown to increase mRNA and protein TGFβRII expression, which could promote greater TGFβ1 signaling activation (144), yet in our system, we see the opposite effect. Future studies with AT1R and AT2R inhibitors in the presence of ANG II may elaborate on these observations.
We also show that in parenchymal ex-smoker fibroblasts from healthy donors, TGFβ1 negatively regulates ANG II signaling by downregulating AT1R protein expression. ANG II signaling through AT1R is implicated in the production of TGFβ1 and may be an important inducer of growth factor production, thereby promoting a pro-repair response. Thus, in normal circumstances, when TGFβ1 is already present in the system, activation of its signaling pathway would lead to termination of the signal. This could then be a reasonable explanation for the downregulation of AT1R. However, in emphysematous lesions, AT1R protein expression is already low and therefore exogenous TGFβ1 treatment of cells derived from the region may not experience further downregulation of AT1R expression. The low AT1R protein expression in COPD fibroblasts may also then be implicated in the low production of TGFβ1 we observed as there is limited AT1R for ANG II to induce TGFβ1 production.

With regards to collagen production, TGFβ1 normally induces fibroblasts to produce collagen 1, which we observed in both healthy ex-smoker and COPD parenchymal fibroblasts. We also observed that the induction of collagen 1 was much greater in COPD fibroblasts in response to exogenous TGFβ1. As we have previously described decreased TGFβ1 mRNA in the COPD lung, and a trend for reduced TGFβ1 production by parenchymal fibroblasts, we can only hypothesize that these fibroblasts may be primed to induce an exaggerated collagen production response when TGFβ1 is present within the fibroblasts environment as a compensatory mechanism against emphysematous destruction. The addition of TGFβ1 in the *in vitro* culture system solidifies the *in vivo* mRNA expression data of elevated collagen mRNA in COPD lung tissue. Whether this exaggerated response could be a compensatory mechanism due to the
abnormally low presence of active TGFβ1 in the COPD lung environment is an important line of study for future research.

In a previous study, our group demonstrated that the 127 gene expression profile of severe emphysematous lesions could be reversed in silico using CMap by either addition of TGFβ1 or the tripeptide GHK-Cu (60). Furthermore, GHK-Cu, like TGFβ1, was able to improve the defective functions of collagen 1 remodeling and contraction in GOLD IV COPD derived parenchymal fibroblasts (60). Thus, GHK-Cu has become a candidate molecule as a novel therapeutic for COPD to prevent the progression of emphysema. In models of skin repair it has been established that GHK-Cu can activate wound repair through various functions including recruitment of immune cells to the wound site, enhancing dermal fibroblast and keratinocyte proliferation, and induce collagen synthesis by skin fibroblasts (68-70). We therefore assessed the ability of GHK-Cu to correct the defects in the TGFβ1-ANG II crosstalk in COPD parenchymal fibroblasts, but found that GHK-Cu was unable to enhance AT1R downregulation or collagen 1α1 production. This was confirmed by the inability of GHK-Cu to activate the SMAD, ERK MAPK and PI3K signaling pathways that we observed to be activated in parenchymal fibroblasts in the presence of TGFβ1, suggesting that GHK-Cu may not induce a wound healing response in parenchymal fibroblasts in the same manner as TGFβ1 but rather through transcriptional regulation of genes.

While there are many benefits to the use of primary cells to understand functional signaling pathways in disease there are caveats to the use of in vitro models. Since the parenchymal fibroblasts in this study were obtained from the normal margin of cancer lung resections, the
presence of tumour cells cannot be excluded from our results. However, since we have obtained fibroblasts from all donors in the same manner, this limitation cannot account for the differential phenotypes observed to differences in TGFβ1-ANG II signaling between healthy ex-smoker and COPD derived parenchymal fibroblasts. A second limitation with our in vitro model is that although we have information on whole organ lung function to define COPD severity we do not have any information on the tissue pathology from which the cells were derived. Therefore, we cannot comment on the presence of emphysema disease pathology and if TGFβ1-ANG II signaling is truly associated with the pathological mechanisms of emphysema. Lastly, our model cannot represent the complex interactions present within the lung, especially since we grow our fibroblasts in isolated monolayer cultures which do not replicate the inflammatory or extracellular matrix environment of the COPD lung. Therefore, our observations may not represent the fibroblast environmental responses to TGFβ1 and ANG II in vivo.

Conclusions
In summary, we found a negative relationship between the TGFβ1 and ANG II signaling pathways in ex-smoker parenchymal fibroblasts as shown by the ability of exogenous TGFβ1 to downregulate AT1R protein expression which we propose would promote AT2R signaling to attenuate TGFβ1 production. However, the downregulatory response of TGFβ1 on AT1R protein expression is blunted in disease, and GHK-Cu cannot reverse this defect. Many of our findings in the primary parenchymal fibroblast cultures translated to our in vivo findings of increased mRNA expression of collagen 1, and reduced expression of AT1R and TGFβ1 in lung tissue of severe COPD patients compared to donor controls. Together, our data suggest that the TGFβ1-ANG II crosstalk is defective in COPD and has implications in the disease pathology.
**Future directions**

Further work can be done to elaborate on the mechanism by which TGFβ1 reduces AT1R protein expression including inhibition of the proteasomal degradation pathway to determine if TGFβ1 regulates receptor degradation. TGFβ1 pathway inhibition studies may also provide us with a better understanding as to which specific signaling pathway mediates AT1R downregulation. Our study has also found a paradoxical ANG II response in mediating TGFβ1 signaling in the ex-smoker parenchymal fibroblasts since it induces TGFβ1 production to promote TGFβ1 signaling, but also reduces the expression of TGFβRII which would reduce the signaling activity. ANG II receptor inhibition studies using the AT1R inhibitor losartan and the AT2R inhibitor PD123319 may determine the mechanism of TGFβ1 production and TGFβRII expression regulation mediated by ANG II. Moreover, since the relationship between ANG II and TGFβ1 seems to be cell-specific, it would be of interest to understand the effect of smoking status on the crosstalk between these pathways. With our growing cohort of parenchymal fibroblasts, we can therefore extend our study to include donors of various smoking statuses including non-smokers and current smokers. And finally, more work needs to be done to evaluate the function of GHK-Cu in ex-smoker parenchymal fibroblasts to determine its mechanism of action as a potential therapeutic for emphysema.
References


