Developmental and pathological roles of BMP/follistatin-like 1 in the lung
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CHAPTER 1

GENERAL INTRODUCTION
Preface

The primary aim of this thesis is to explore the functional roles of an endogenous bone morphogenetic protein (BMP) antagonist, Follistatin-like 1 (Fstl1), in lung development and in the pathological processes of chronic obstructive pulmonary disease (COPD). BMP signaling plays active roles in lung development as evident in embryonic and perinatal lethality and lung developmental defect of BMP-deficient mice. It is now apparent that BMP signaling also plays crucial regulatory roles in adult lung homeostasis by modulating inflammation and repair in the lung. However, the involvement of BMP signaling has not been previously explored in the pathophysiology of COPD. We hypothesized that the imbalance between BMP and its antagonist Fstl1 contributes to dysregulation of early postnatal lung development, airway inflammation, and adult airway epithelial cell differentiation similar to that observed in COPD. This thesis sheds light on Fstl1 as a novel mediator of airway epithelial-driven inflammation in the lung and potentially of goblet cell metaplasia and the loss of club cells in COPD epithelium. Therefore, targeting Fstl1 is worth pursuing for the treatment of airway inflammation and epithelial remodeling in COPD. The first part of this chapter provides an overview of COPD pathophysiology, including chronic airway inflammation, airway epithelial remodeling, vascular remodeling, and currently available treatment. The second part of this chapter covers the current understanding of BMP pathway in lung development and chronic respiratory diseases. The third part of this chapter focuses on the latest discoveries on developmental and pathological roles of Fstl1. Finally, the scope of this thesis and the specific aims of our studies are described in the last part of this chapter.

1. Chronic Obstructive Pulmonary Disease (COPD)

The Global Initiative for Chronic Obstructive Lung Diseases (GOLD) guidelines stated COPD as “a common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients”. According to the World Health Organization, COPD will be the third major cause of death worldwide by 2020, surpassing stroke. Cigarette smoking accounts for 85-90% of COPD cases and is the best-known etiological factor for the development of COPD. However, 10-15% of COPD patients are never smokers and only about 15-20% of smokers manifest COPD, suggesting a role for environmental factors and susceptibility-associated (epi)genetic make-up. This has led to the concept that impaired inflammatory responses to cigarettes smoke-induced injury manifest to COPD in susceptible persons. Accumulating evidence from genome-wide (epi)genetic association studies discovered several loci that are strongly associated with COPD susceptibility. The major loci that associated with COPD susceptibility include HHIP (hedgehog interacting protein) and FAM13A (family with sequence similarity 13 member A) on chromosome 4 as well as CHRNA3/5 (cholinergic nicotinic acetylcholine receptor) on chromosome 15. The common symptoms of COPD include breathlessness, chronic and progressive dyspnea, chronic cough, and excessive sputum production. The episodes of acute worsening
of COPD symptoms, the-so-called exacerbations, aggravate primarily in patients with severe disease, and approximately 78% is linked with respiratory infections. The molecular and cellular mechanisms underlying the symptoms and pathobiology of COPD are not completely understood. The current understanding from histopathology and current available treatment of COPD is summarized below.

1.1. The pathology of COPD
The pathology of COPD displays highly heterogeneous phenotypes involving abnormal airway wall architectures, peribronchial fibrosis, destruction of alveolar septa (emphysema), and altered airway epithelial composition, including goblet cell metaplasia and loss of club cells. Major remodeling throughout the respiratory tracts (including proximal, distal and peripheral airways, lung parenchyma and pulmonary vasculature) and changes in the cellular differentiation profile of the airway epithelium collectively manifest in a progressive decline of lung function. The staging of COPD that reflects disease severity is typically determined by spirometry. A new hypothesis addresses the potential involvement of developmental pathways in COPD based on accumulating data from genome-wide association studies and microarray profiling, indicating dysregulation of developmental factors originally known for their roles in embryonic development. BMP signaling is pivotal during lung development, nevertheless, it has been largely unexplored in adult lung as well as the pathogenesis of COPD. BMP signaling is found decreased in the lung of pulmonary fibrosis patients and following bleomycin injury in mice. Accordingly, accumulating evidence indicates a marked increase of the endogenous BMP antagonist Fstl1 in the lung of patients suffer from respiratory diseases. This thus raises the possibility that an imbalance between BMP and its endogenous antagonists may lead to airway inflammation and airway epithelial remodeling in COPD.

1.2. Persistent inflammation of the airways in COPD
Chronic airway inflammation is the primary pathophysiological feature of COPD and is believed to be the major contributor of structural changes in COPD airways. The number of neutrophils and macrophages is positively correlated with COPD severity. Airway inflammation in COPD is associated with infiltration of various innate and adaptive immune cells with prolonged retention time in the respiratory tracts, primarily in peripheral airways (bronchioles), lung parenchyma, and pulmonary arteries. Previously, infiltrating leukocytes were considered to be the key player of airway inflammation and the major factor in COPD development. However, latest discoveries show that the structural cells, such as bronchial epithelial cells, lung fibroblasts, and airway smooth muscle cells also express and secrete molecules that regulates innate airway inflammation and defense mechanisms.
As a part of the normal repair mechanism to injury, airway epithelium of healthy individuals mediate the activation of innate immune responses via pattern recognition receptors (PRRs) to release “danger signals” which subsequently activate Toll-like receptor (TLRs). Activation of TLR receptor promotes release of cytokines to recruit inflammatory cells to the site of injured epithelium in the lung. Resolution of inflammation is responsible for switching “off” the inflammatory response when the toxic insults are successfully cleared out. However, chronic exposure to inhaled toxic compounds, such as cigarette smoke, activates airway epithelial cells to secrete a myriad of pro-inflammatory cytokines and chemokines, including interleukin-6 (IL-6), CXC-chemokine ligand 1 (CXCL1), CXCL8, CC-chemokine ligand 2 (CCL2), C-X-C-Motif ligand 8, IL-1β, tumor necrosis factor (TNF)-α and granulocyte macrophage colony-stimulating factor (GM-CSF). Airway inflammation in COPD is associated with persistent and exaggerated innate and adaptive inflammatory responses involving recruitment of CD8+ T-helper-1 (Th1) cells, neutrophils, monocytes, and lymphocytes. Inflammatory mediators released by bronchial epithelial cells following cigarette smoke-induced injury have a paracrine effect on neighboring mesenchymal cells, such as lung fibroblasts and airway smooth muscle cells. Several studies have shown that these mesenchymal cells are more than just structural cells as they express and release inflammatory mediators, like IL-6, IL-8, TNF-α, which contribute to the neutrophil and monocyte infiltration to the lung.

The number of inflammatory cells, such as macrophages, neutrophils and CD8+ T cells, is increased in sputum and bronchoalveolar lavage (BAL) of COPD patients. Neutrophil chemotactic proteins including leukotriene B4 (LTB4), IL-8, CXCL1 and CXCL8, GRO-a (growth-related oncogene-a), and ENA-78 (epithelial neutrophil-activating protein of 78 kDa) are increased in the sputum and BAL fluid of COPD patients. Macrophages also induce release of LTB4, IL-8, TNF-α, MCP-1, and reactive oxygen species upon cigarette smoke-induced injury. These secreted factors activate and recruit neutrophils to the lung, leading to increased secretion of proteases (neutrophil elastase, proteinase-3, matrix metalloproteinases (MMP)-8, MMP-9, cathepsin G) which is associated with emphysema. In addition, GM-CSF and granulocyte colony-stimulating factor (G-CSF) promote neutrophil survival, proliferation, and differentiation. The transcription factor nuclear factor-κB (NF-κB) is known to be the central regulator of inflammatory protein expression. In addition, E-selectin which mediates neutrophil adhesion to endothelial cells is increased on endothelial cells of bronchial mucosa of COPD patients. Macrophages secrete chemokines interferon-γ inducible protein (IP-10), interferon-inducible T-cell chemoattractant (I-TAC), and monokine-induced by interferon-γ (Mig). The increased numbers of macrophages in COPD patients can be a result of increased recruitment of circulatory monocytes and/or increased cell proliferation and survival. Collectively, accumulation of inflammatory cells in the lung aggravates a chronic inflammatory state and results in airway structural changes, airway obstruction, and respiratory symptoms observed in COPD.
Disruption of epithelial-driven innate immune functions is also strongly affected by impaired airway epithelial structure and composition following epithelial injury which will be described on the next section. Persistent airway inflammation leading to recruitment and activation of neutrophils and macrophages in COPD is illustrated in Figure 1.

**Figure 1. Persistent airway inflammation is a major hallmark of COPD pathogenesis.** The healthy epithelium lining of adult airways is composed of a mixture balance of goblet cells, ciliated cells, basal cells and club cells. Cigarette smoke and inhaled toxic pollutants promotes airway epithelial remodeling, including goblet cell metaplasia, mucus hypersecretion, and loss of secretory club cells. In addition, cigarette smoke and inhaled toxic pollutants induce release of inflammatory mediators from epithelial cells with autocrine and paracrine effects on neighboring mesenchymal cells, such as fibroblasts and airway smooth muscle cells. Pulmonary mesenchymal cells secrete IL-6 and IL-8 that contributes to an infiltration of neutrophils and macrophages to the lungs. The abnormal accumulation of inflammatory cells aggravates airway inflammation in the lungs and leads to the development of COPD. The expression of Fstl1 in COPD compared to non-COPD lungs is investigated in Chapter 3. The role of Fstl1 in inflammatory cytokine release from pulmonary mesenchymal cells is shown in Chapter 5. The effect of BMP4 on adult airway epithelial cells is examined in Chapter 7.
1.3. Remodeling of airway epithelium in COPD

Airway epithelium acts as the first protective barrier between the external environment and the internal lung tissue and the rest of the body. The healthy epithelium lining of adult large cartilaginous airways is made up of a layer of basal cells that are attached to the basal lamina underneath a layer of airway lumen columnar cells with a balanced mixture of goblet cells, ciliated cells and club cells. This epithelial structure is important for the mucociliary clearance function. In trachea, up to 60% of the total cell number are ciliated cells and approximately 20% are mucus secreting goblet cells. The number of ciliated cells falls to approximately 15% by the 5th airway generation. The distal airways predominantly consist of serous and secretory club cells. In the proximal bronchioles, the epithelial cells become more cuboidal and, in addition to ciliated cells, contain secretory club cells. Finally, in the most distal bronchioles, only club cells are identified.

The cellular composition and function of COPD airway epithelium was found to be altered as evident by the loss of tight junctions, basal and goblet cell hyperplasia, and loss of secretory club cells and ciliated cells, resulting in loss of its barrier function and excessive mucus production. Cigarette smoke causes the goblet cell hyperplasia and the loss of ciliated cells, resulting in a reduction of mucociliary clearance and mucus plug formation. In COPD, increased numbers of goblet cells are also found in the small bronchi and bronchioles, where there are normally very few. The cellular events in epithelial repair after injury have been described.

The airway epithelium is more than just a physical barrier as evidenced by the fact that bronchial epithelial cell transcribe and secretes numerous soluble factors, such as cytokines, chemokines and growth factors. Club cells are important in controlling lung inflammation by secreting a number of anti-inflammatory agents, such as secretoglobin (SCGB3A) and uroplakin (UPK3A). Surfactant proteins (SP-A, SP-B, SP-D), also called collectins, are produced by club cells and alveolar epithelial cells and play an important role in airway epithelial defense mechanism. At the bronchiolar level, the club cell secretory protein (CCSP), also called CC10, is able to modulate lung inflammatory and immune responses. However, club cells are reduced in bronchial biopsies from COPD patients and in peripheral and central airways in lung tissue of COPD patients, which could contribute to the aggravated inflammatory process. The air-liquid culture provides a valuable system to mimic a well differentiated epithelial cells in vitro and permits the study of the re-epithelialization after injury. Therefore, this is an excellent tool to study epithelial cell differentiation under various condition. However, there are limited studies addressing the functional roles of BMP signaling in the adult lung epithelial cells which may enlighten the consequence of aberrant BMP signaling in the context of COPD.

1.4. Remodeling of extracellular matrix in COPD

In healthy lung, non-cartilaginous small airways (<2 mm) contribute to only ~25% to the total airway resistance, whereas in COPD lungs, it becomes the major site of increased airway resistance as a result of increased deposition of extracellular matrix, thickening of airway smooth muscle and narrowed airway lumen. In COPD,
infiltrating inflammatory cells release inflammatory mediators that promote production of extracellular matrix from pulmonary mesenchymal cells which contribute to tissue remodeling and respiratory dysfunction.\textsuperscript{30} Multiple studies have reported increased extracellular matrix protein expression, such as fibronectin, collagen I, hyaluronan, and laminin in COPD airway and lung parenchyme.\textsuperscript{67–70} Airway smooth muscle cell hyperplasia and hypertrophy cause thickening of smooth muscle bundles surrounding COPD airways, whereas airway fibroblasts contribute to sub-epithelial fibrosis in the airway wall. The primary roles of pulmonary fibroblasts are tissue repair and remodeling by producing diverse extracellular matrix proteins, such as fibronectin, collagen, and airway smooth muscle actin, whereas airway smooth muscle cells are mainly responsible for airway contraction. Transforming growth factor (TGF)-β1 produced by epithelial cells in the small airways is a key player in tissue remodeling and is found to be markedly increased in COPD. It is associated with increased deposition of extracellular matrix within and surrounding the smooth muscle bundles of COPD, which results in thickened airway walls and increased in stiffness.\textsuperscript{71} An imbalance between the production and degradation of extracellular matrix could possibly explain excessive matrix deposition and may lead to airway fibrosis in COPD in addition to proliferating airway smooth muscle cells induced by cigarette smoking.\textsuperscript{72} In COPD, the number of myofibroblasts (airway fibroblasts with a more contractile phenotype) is increased and contributes to reduction of airway elasticity.\textsuperscript{73} Fibroblasts from individuals with COPD have reduced capacity for tissue repair due to increased prostaglandin E (PGE) and TGF-β1 expression.\textsuperscript{74} The thickness of small airway wall is associated with COPD severity. The obstructed small airways are characterized by abnormal architecture of epithelium and smooth muscle layers and the presence of fibrosis.\textsuperscript{65,75}

1.5. Remodeling of the pulmonary vasculature in COPD
Patients with COPD also frequently suffer from pulmonary arterial hypertension (PAH), one of the common comorbidities of COPD. PAH is characterized by increased arterial pressure in the lungs as a result of narrowing of vessel lumen due to vascular remodeling and increased vasoconstriction.\textsuperscript{76,77} Endothelial cells are important for vascular homeostasis by maintaining vascular tone and remodeling.\textsuperscript{78} Cigarette smoking disrupts endothelial functions of pulmonary arteries in COPD.\textsuperscript{79,80} Endothelial cells are key players in regulation of cell and vessel growth, thus it is believed that endothelial dysfunction initiates vascular remodeling in COPD. Endothelial dysfunction leads to uncontrolled intimal endothelial cell proliferation consequently resulting in thickened pulmonary muscular arteries observed in smokers and patients with mild COPD.\textsuperscript{79,80} Inflamed lung tissue is hypoxic and promotes angiogenesis by increasing growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF).\textsuperscript{77,81} These growth factors are involved in tissue repair and vascular remodeling which eventually contribute to microvascular remodeling observed in COPD. The opposing effects of angiogenic and angiostatic factors such as chemokines (CXC family) that induce or inhibit neovascularization, respectively, determines the angiogenesis process.\textsuperscript{77,82} VEGF and its receptor seem to be important
for endothelial and epithelial cell survival following injury. Numerous studies showed increased expression of VEGF and VEGF receptors (Flk-1 and Flt-1) in pulmonary muscular arteries of patients with mild and moderate COPD and in current smokers without airway obstruction.\textsuperscript{81,83–85} An increase of angiogenic factors are also associated with thickening of the pulmonary arterial walls.\textsuperscript{76,81,83–85} However, VEGF expression is low in total lung homogenates of severe emphysema patients.\textsuperscript{86} It has been proposed that low VEGF may be involved in the emphysema process through cell apoptotic mechanisms, as the lung microvascular endothelial cells and alveolar septal capillary cells needs VEGF for cell survival.\textsuperscript{86,87} Endothelial cell death leads to loss of capillaries may be a major mechanism of emphysema.\textsuperscript{86} Macrophages, CD8+ T lymphocytes, neutrophils, fibroblasts that involves in immune response can promotes angiogenesis in many ways by secreting TNF-α which also is an angiogenic factor. Additionally, the protein expression of endothelin-1 (ET-1) and ET-1 receptor ETA that mediate vascular smooth muscle contraction is increased in PAH.\textsuperscript{88,89}

1.6. Current treatments
Currently, there is no effective cure for COPD. Inhaled corticosteroids (e.g. fluticasone, budesonide), phosphodiesterase 4 inhibitors (roflumilast), short-acting (e.g. albuterol) or long-acting β₂ adrenoreceptor agonists (e.g. salmeterol, formoterol), and long-acting anti-cholinergics (e.g. tiotropium, ipratropium, glycopyrronium bromide, umeclidinium) are used alone or in combination to relieve the symptoms and inflammation.\textsuperscript{90} However, these therapies do not change the course of the disease.\textsuperscript{90} Therefore, understanding the molecular mechanism of COPD is undoubtedly important to identify a novel molecular target that can decelerate the progression of COPD. A better understanding of impaired repair process which contributes to COPD may lead to the identification of new molecular targets and more effective therapy options in COPD treatment.

2. Bone morphogenetic protein (BMP) signaling in the lung
In 1965, Marshall R. Urist discovered osteogenic factors present in bone matrix extracts capable of promoting de novo cartilage formation and bone formation in vivo, namely bone morphogenetic protein (BMP).\textsuperscript{91,92} BMP signaling belongs to the TGF-β superfamily, which is phylogenetically conserved.\textsuperscript{91} Genetic studies in mice demonstrate the importance of BMP signaling in embryonic dorsal-ventral paterning\textsuperscript{93–95} and embryonic lung development.\textsuperscript{3,96,97} It is now evident that BMPs govern diverse cellular effects including stem cell maintenance, migration, differentiation, proliferation, and programmed cell death.\textsuperscript{98} A growing body of evidence indicates the involvement of BMP signaling in normal lung physiology and pathology conditions in adulthood, strongly suggesting that BMP signaling also plays a pivotal role in adult lung homeostasis, including epithelial repair after injury.\textsuperscript{3}
2.1. Players in BMP signaling

2.1.1. BMP ligands

BMPs (which are also referred to as growth and differentiation factors (GDFs)) are secreted glycosylated proteins, with 20 different members identified and characterized. BMP polypeptides consist of an N-terminal secretory signal peptide, a prodomain that ensures proper folding, and a C-terminal mature peptide. Mature BMPs are derived from cleavage of the BMP precursors by serine endoproteases in the trans-Golgi network and subsequently secreted. Homo or heterodimerization of BMP ligands forms an active BMP agonist. Secreted BMPs bind either to the extracellular matrix, extracellular antagonists, co-receptors or to transmembrane receptors. Activation of BMP signal transduction leads to either transcriptional responses (e.g. Smad-dependent signaling) or non-transcriptional responses (e.g. Smad-independent signaling).

2.1.2. BMP receptors and co-receptors

The BMP receptors are composed of two types of transmembrane serine/threonine kinase receptors, type I and type II receptors, which are highly homologous but not redundant, and are capable of activating both Smad-dependent and Smad-independent signaling. The receptors encompass an extracellular ligand binding domain and a cytoplasmic serine/threonine kinase domain. The type I receptor has two additional motifs in its kinase domain, a glycine/serine-rich region prior the GS-box kinase domain and a L45 loop composed of eight amino acids. Type I receptors include activin receptor-like kinase 1 (ALK1), activin receptor type Ia (ActRIa or ALK2), BMP receptor type Ia (BMPRIA or ALK3) and BMP receptor type Ib (BMPRIb or ALK6). These receptors form heteromeric complexes with the type II receptors, including BMP receptor type II (BMPRII) and activin receptors type IIA (ACVRIIA also known as ActRIIA) and activin receptors type IIB (ACVRIIB also known as ActRIIB). The specificity of BMP signaling is tightly controlled by selective extracellular regulators, receptors, co-receptors, and cytosolic receptor-associated proteins to exert specific BMP functions which will be described in more detail below. Activation of BMP signaling is preceded by oligomerization of BMP ligands and type I receptor kinase. Activated type I receptor undergo transphosphorylation of its GS-box via constitutively active type II BMP kinase receptors. After the formation of a heterotetrameric complex, the signal propagates to the nucleus either in intracellular regulator Smad-dependent (canonical) or Smad-independent (non-canonical) manner. Co-receptors that positively regulates BMP signaling are the repulsive guidance molecules (RGM)-a and -c, Dragon (RGMb), the receptor tyrosine kinase (RTK) c-Kit, and the TGF-β1/BMP type III receptors Endoglin and Betaglycan. In contrast, BMP signaling is negatively regulated by the pseudoreceptor BMP and activin membrane-bound protein (BAMBI) and the RTKs Ror2 and TrkC.
2.1.3. Downstream BMP/Smad-dependent signaling
In the canonical pathway, the transduction of extracellular signals to regulation of gene transcription inside the nucleus is mediated by Smad proteins. There are three types of Smad proteins, BMP receptor-regulated Smads (R-Smads), common mediator Smad (co-Smad), and inhibitory Smads (I-Smads). BMP ligands bind to the type I BMP receptors resulting in phosphorylation of R-Smads (Smad1/5/8), followed by trimeric complex formation with co-Smad (Smad4), and translocation of the complex into the nucleus, which subsequently induces transcription of BMP downstream target genes, presumably in collaboration with other co-repressors and co–activators. I-Smads (Smad6 and Smad7) negatively regulate BMP/Smad signaling by preventing phosphorylation of R-Smads, instead targeting R-Smads and type I receptor for ubiquitin–proteasome degradation. Two conserved domains of R-Smads are the N-terminal DNA and protein binding MH1 (mad homology 1) domain and the C-terminal MH2 domain which are connected by a variable proline-rich linker region.

2.1.4. Downstream BMP/Smad-independent signaling
In addition to canonical BMP signaling, BMP ligands activate gene transcription in a Smad-independent manner. BMP ligands can activate the MAPK pathway by recruitment of TAK1, TAB1, and XIAP to type I BMP receptors, which subsequently phosphorylate p38 MAPK or JNK. Additionally, ERK mediates transcription of BMP2-regulated genes. BMP regulates sets of genes which are involved in lung and vascular homeostasis, including epithelial and vascular remodeling. BMP regulated genes, including jagged1, GATA2, and endoglin, have been identified in endothelial cells. BMPs can also activate TGF-β activated kinase 1 (TAK1) leading to p38/JNK/Erk MAP kinase activation. TAK1 mediates phosphorylation of Smad1 at C-terminal serine residues, suggesting it functions as an upstream activating kinase for Smad1/5/8 in BMP signaling.

2.1.5. Fine tuning of BMP signaling
BMP functions are tightly modulated by BMP antagonists, co-receptors, and intracellular regulatory proteins. BMP antagonists function through direct binding with BMP ligands and prevent BMPs from binding to their transmembrane receptors. Pseudo-receptors sequester BMP ligands at the cell surface. Co-receptors and intracellular regulatory proteins interact with the receptors to regulate downstream BMP functioning. The spatiotemporal interplay between BMP and its regulators govern developmental and cellular processes. BMP antagonists are cysteine knot containing motif which form a functional homodimer. BMP antagonists bind selectively to distinct BMP ligands in a context- and concentration-dependent manner to maintain BMP gradients, which have been extensively studied during embryonic lung development. BMP antagonists include Noggin, gremlin, twisted gastrulation (Tsg), and Fstl1. Antagonists are categorized based on their cysteine knot motif: the Chordin/Noggin family contains a ten-membered cysteine ring, Tsg contains a nine-membered cysteine ring and the DAN/Cerberus family contains an eight-membered cysteine ring. Molecular regulation of BMP signaling pathway is illustrated in Figure 2.
2.2. BMP signaling in lung development and adult lung homeostasis

Lung development is orchestrated by precise coordination of several molecular pathways, including BMP signaling. The canonical BMP signaling is important in lung

![BMP signaling diagram]

**Figure 2. Molecular regulation of BMP signaling.** Activation of BMP signaling is initiated by binding of BMP ligands to the type I BMP receptors (ALK1, 2, 3, 6) followed by recruitment of constitutively active type II BMP receptor receptors (BMPRII, ACVRIIA, ACVRIIB) to form a signal-activating complex. In cytoplasm, the signal propagates either via Smad-dependent or Smad-independent manner. In a Smad-dependent signaling, the transduction of BMP signals is mediated by Smad1/5 proteins. Phosphorylated Smad1/5 proteins form a complex with Smad4 protein which subsequently translocate to nucleus and bind to a 52 base-pair BMP response element (BRE) on the promoter region of the target genes. In a non-Smad signaling, BMP ligands can activate the MAPK pathway by recruitment of TAK1 to type I BMP receptors, which subsequently phosphorylate Erk/JNK/p38. Thereby, phosphorylated Erk/JNK/p38 can translocate into the nucleus. Among others, BMP functions are tightly regulated by co-receptors (e.g. Endoglin) and extracellular regulators (e.g. Fstl1).
Fundamental roles of BMP signaling in lung development are evident from BMP mutations associated with congenital lung defects in human and genetic studies in transgenic mice and familial mutation in PAH. Deletion of lung epithelial-specific Smad1 resulted in impaired airway branching morphogenesis, decreased sacculature, disruption of distal lung epithelial cell proliferation and differentiation which contribute to severe postnatal respiratory distress. Wu et al. also revealed that BMP4/Smad1 signaling modulates other molecular pathways including Wnt/β-catenin by expressing Wnt inhibitory factor 1 (Wif1) in the developing fetal mouse lung. Overexpression of BMP4 in the epithelium, on the other hand, leads to smaller lungs and to a marked decrease in epithelial cell proliferation. In adults, lung tissue homeostasis is required for continuous regeneration and subsequent differentiation of stem cells in order to maintain lung architecture and proper functions. BMP signaling maintains an undifferentiated state of the airway and alveolar epithelial progenitors for lung epithelial homeostasis and tissue injury repair.

A tight and precise regulation of pulmonary vascular development is required for normal lung development. Canonical BMP signaling plays key roles during embryonic development of the pulmonary vasculature. Activation of BMPRII promotes pulmonary artery endothelial cell (PAEC) survival, proliferation, and migration. BMPRII is a novel mediator of endothelial nitric-oxide synthase activation.

2.3. BMP signaling in lung diseases
Dysregulation of BMP signaling has been implicated in several lung diseases. Although in adult lung tissue, active canonical BMP signaling is hardly detectable. Reactivation of canonical BMP signaling is observed after lung injury with naphthalene and bleomycin in the same pattern as early lung development in bronchial and alveolar epithelial cells, respectively, presumably as part of repair processes. In addition, BMP ligands expression and BMP/Smad activity are upregulated inflamed bronchial epithelium of allergic asthma. It has been demonstrated that activation of BMP signaling induces expression of its own antagonist, implying a negative feedback loop in the regulation of BMP signaling. The expression of the BMP4 antagonist gremlin was found to be upregulated in patients with idiopathic pulmonary fibrosis (IPH). Additionally, the expression of BAMBI is increased in plasma and lung of COPD patients. The role of BMP in lung cancer is contradictive. In one hand, BMP2 is increased along with activation of Smad1/5 and its downstream target Id1 and promote tumor growth in human lung cancer. On the other hand, low levels of BMP7 in lung cancer tissues are correlated with lymph node metastasis. In vitro, BMP7 negatively regulates cell adhesion and migration of a lung cancer cell line.
2.4. BMP signaling in pulmonary vascular diseases

BMP signaling is important in embryonic vascular development and adult vascular homeostasis. Consequently, dysregulation of BMP signaling has been strongly associated with the pathogenesis of hereditary vascular diseases, including familial PAH. BMP signaling regulates vascular smooth muscle maturation, endothelial cell proliferation and angiogenesis. Endothelial cells are a major player in vascular homeostasis. Endothelial dysfunction, including abnormal proliferation, impaired endothelial mesenchymal communication, decreased endothelial nitric-oxide synthase (eNOS) activity, and loss of bioactive nitric oxide (NO), play a prominent role in the development of PAH. Accumulating studies from genetic association study in hereditary vascular diseases and genetic studies in transgenic mice pinpoint the mutation of BMP components to be strongly associated with PAH. Most importantly, BMP2 and BMP4 failed to stimulate eNOS phosphorylation in PAECs derived from patients carry mutations in the BMPRII gene. This is supported by animal studies of lung endothelial-specific BMPR2 KO mice resulting in spontaneous PAH. Interestingly, impaired BMP signaling was also observed in common forms of PAH, including hypoxic PAH. Mechanistically, the negative regulator of vascular smooth muscle cell proliferation and pro-apoptotic, BMP2 and its downstream non-canonical BMP signaling are upregulated after acute hypoxia exposure. In contrast, prolonged hypoxia exposure results in downregulation of BMPRII expression and inactivation of its downstream signaling in pulmonary vasculature. In addition, the expression of BMPRIb, BMPRII, and Smad4, 5, 6, and 8 was decreased in accordance with reduced levels of active Smad1 and Smad-regulated genes id1 and id3 in lungs of monocrotaline model of PAH. Furthermore, the expression of the BMP antagonist gremlin 1 is increased in small pulmonary vessel walls of hypoxic PAH mouse models. Taken together, these studies demonstrate a central role for BMP and its inhibitors in pulmonary vascular remodeling and the pulmonary vascular resistance in hypoxic PAH.

3. Follistatin-like 1

During lung development and adult homeostasis processes, the functions of BMPs are tightly controlled for proper morphogenesis. Although the expression of BMPs and their receptors is not strictly limited to certain areas, the expression of BMP antagonists is spatiotemporally tightly controlled, indicating that BMP antagonists fine tune the level of BMP functioning and act as molecular switches of BMP signaling. The endogenous antagonist of BMP, Fstl1 (also known as TGF-β-stimulated clone 36 (TSC-36)), was originally identified as a TGF-β inducible gene in a mouse osteoblastic cell line with two isoforms. Fstl1 is a secreted cysteine-rich glycosylated protein of 38 kDa containing an follistatin (FS)-like domain containing 10 conserved cysteine residues and a strong similarity with BM-40/osteonectin/SPARC family. Human Fstl1 is highly conserved with >92% amino acid sequence identity with rat and mouse. The Fstl1 orthologues have been identified and sequenced from human, rat, mouse, avian, xenopus, macaques, and fish. The crystal structure of a truncated form of recombinant Fstl1 containing follistatin-like domain has been resolved. Fstl1 is composed of three distinct domains,
including follistatin N-terminal domain-like (FOLN), extracellular calcium (EC)-binding domain containing two EF-hand motifs, and a von Willebrand factor type C domain (VWC) with cell type specific glycosylation. However, unlike other family members, the calcium-binding domains of Fstl1 are non-functional.  

3.1. Cellular expression of Fstl1
During embryonic development, Fstl1 mRNA is detected in different organs, but the highest expression is found in the lung, predominantly in mesenchymal cells, including vascular and airway smooth muscle cells, but also in endothelial cells and goblet cells of airway epithelium. In adult tissue, Fstl1 has been proposed as a cardiokine, myokine, adipokine, and, osteogenic factor due to its ubiquitous expression and functions in the heart, muscle, bone, skeletal muscle tissue, and trophoblasts. The effects of Fstl1 on its downstream targets and the upstream factors known to regulate Fstl1 expression has been reviewed. In response to divers factors, such as TGF-β1, IL-1β, TNF-α and IL-6, the expression of Fstl1 gene was found to be highly expressed in mesenchymal lineages, including fibroblasts, cardiomyocytes, chondrocytes, adipocytes, and osteocytes. However, recent studies showed that Fstl1 is also expressed by hematopoietic lineages, such as bone marrow-derived osteoclasts and lung alveolar macrophages. IL-13 triggers lung alveolar macrophages to express a significant increase of Fstl1 mRNA, whereas TGF-β and Fstl1 increase Fstl1 expression in lung epithelial cells. Furthermore, Fstl1 triggers inflammatory cells, ranging from monocytes, macrophages, and T-cells to express and release pro-inflammatory cytokines and chemokines, including IL-1β, TNF-α, IL-6, IFN-γ, IL-8, MCP-1, and IP-10. Among others, expression of Fstl1 is negatively regulated by microRNAs (miRs), including miR-198, miR-27a, miR-21, and miR-206. Although Fstl1 is primarily known as a BMP antagonist, current findings suggest that Fstl1 may also regulate various physiological processes by interacting with different receptors, including disco-interacting protein 2 homolog A (DIP2A), toll-like receptor 4 (TLR4), and the sodium-potassium pump.

3.2. Fstl1 signaling in lung and pulmonary vascular development
Fstl1 is expressed in the developing lung. Embryonic knockout of Fstl1 in mice results in an extensive distortion in lung morphology and neonatal lethality due to respiratory failure, demonstrating the functional importance of Fstl1 in lung development. Whole body knockout of Fstl1 exhibits smaller and abnormal lung morphology. Histological analysis demonstrated thickening of alveolar walls and reduction in airspaces and impaired alveolar epithelial differentiation as seen in reduction in mature surfactant protein level. In addition to embryonic lung morphogenesis, the crucial roles of Fstl1 in organogenesis have also been shown in other organs, including brain, heart and dorsal/ventral axis patterning. However, the role of Fstl1 in the development of pulmonary vasculature has not been previously explored.
3.3. Physiological roles of Fstl1
Several studies reported that Fstl1 has diverse biological functions, including regulating cell proliferation, differentiation, migration, invasion, wound healing, tissue repair, cell fate determination, and innate immune responses. The roles of Fstl1 in tissue repair in response to injury have been most widely studied in the cardiovascular system. Fstl1 was found to be increased in models of acute and chronic heart injury, including myocardial infarction, pressure overload-induced hypertrophy, and ischemia/reperfusion injury. Systemic administration of Fstl1 or overexpression of Fstl1 showed to be protective in the cardiovascular system by reducing cell apoptosis and inflammatory responses, and promoting endothelial cell survival and trigger a revascularization process and restore tissue function. Recent studies demonstrated that Fstl1 expression is also induced in response to lung injury. Further investigation is needed to unravel the impact of upregulation of Fstl1 in the lung. Collectively, these data suggest that the injury-induced upregulation of Fstl1 play a clinically relevant role in the modulation of pathological processes. Furthermore, previous studies demonstrate paracrine signaling of Fstl1 in cell and tissue communication. However, the roles of secreted Fstl1 in cell-cell communication in the lung have not been previously studied.

3.4. The pathological roles of Fstl1 in inflammatory diseases
The function of Fstl1 has been most extensively studied in inflammatory diseases and a dual role of Fstl1 in inflammatory responses has been reported. It is clear that Fstl1 modulates inflammatory cytokine expression and release. However, the precise effect of Fstl1 in inflammatory responses, whether as a pro-inflammatory or an anti-inflammatory mediator remain controversial. A great body of evidence identifies Fstl1 as a pro-inflammatory cytokine that is induced by inflammatory cytokines, in turn Fstl1 triggers inflammatory responses from target cells, particularly inflammatory cells. Several studies have shown that Fstl1 is markedly upregulated in inflammatory conditions. Furthermore, silencing of Fstl1 decreases the production of TNF-α, IL-6, and IL-8 from human arterial endothelial cells induced by oxidized low-density lipoprotein. Additionally, high Fstl1 is observed in response to bacterial infection and low degree of inflammation in obese individuals. An anti-inflammatory role for Fstl1 is supported by other studies. Mice treated with recombinant human Fstl1 show a decreased expression of IL-6, MMP3, and MMP9 in animal models of arthritis. Immuno suppressant roles of Fstl1 have been also demonstrated in organ transplantation, and tissue injury. These discrepancies may be explained by the differences in the experimental conditions and different organs. However, the inflammatory roles of Fstl1 in COPD have not been previously explored. Furthermore, Fstl1 has been proposed as a biomarker in several diseases, including systemic-onset juvenile rheumatoid arthritis, osteoarthritis, systemic juvenile idiopathic arthritis, chronic systolic heart failure, acute coronary syndrome, inflammatory responses and oxidative stress.
3.5. The pathological roles of Fstl1 in airway remodeling in the lung
Recent studies showed upregulation of Fstl1 expression in the lungs patients with severe asthma and idiopathic pulmonary fibrosis. Further in vivo studies demonstrated that Fstl1 haploinsufficient mice and specific inactivation of Fstl1 in myeloid cell lineages attenuate airway remodeling in animal models of asthma and lung fibrosis, respectively. Studies from the same group demonstrated that recombinant Fstl1 induces lung macrophages to express pro-remodeling factor MMP9 mRNA and protein through TLR4 receptor activation. Conversely, the other study showed that Fstl1 attenuates MMP9 expression in animal model of arthritis.

3.6. The pathological roles of Fstl1 in airway remodeling in cancer
Fstl1 has been implicated in several cancer, including lung cancer, pancreatic cancer, bone metastasis, ovarian cancer and endometrial carcinogenesis, nasopharyngeal carcinoma, glioblastoma, and prostate cancer. However, the role of Fstl1 in cancer is complex and controversial. On one hand, Fstl1 has been reported as a pro-cancer metastasis. On the other hand, Fstl1 shows an anti-cancer effect. High expression of Fstl1 is associated with poor prognosis of glioblastoma and favors the progression of metastatic prostate cancer. Therefore, Fstl1 has been proposed as therapeutic target for prostate cancer. Targeting Fstl1 prevents bone metastasis by regulating the immune function, suggesting that Fstl1 can mediate cancer cell invasion. Fstl1 is significantly downregulated in metastatic kidney cancer. Fstl1 mRNA is significantly lower in cancerous tissue compared to adjacent normal tissue. A recent study demonstrated an association between Fstl1 polymorphisms and renal cell carcinoma (RCC) risk and prognosis, suggesting that the variant genotype CC of rs1259293 is associated with RCC development by suppressing Fstl1 expression. Fstl1 has been suggested as putative tumor suppressor gene as decreased Fstl1 has been reported, including in kidney cancer, lung cancer, ovarian and endometrial cancer, nasopharyngeal carcinoma, and bone metastasis of prostate cancer. Hypermethylation of the Fstl1 promoter is observed in cancer, leading to cell proliferation and invasion. Re-expression of Fstl1 and Fstl1 treatment negatively regulates lung cancer cell invasion and metastasis and result in growth inhibition of human lung cancer cell lines. Fstl1 transcripts are not detectable in highly aggressive and proliferative lung cancer cells and overexpression of Fstl1 shows an anti-proliferative effect. Tumor suppressant effects of Fstl1 in ovarian and endometrial carcinogenesis has also been suggested. Knockdown of Fstl1 induces apoptosis through a mitotic arrest and caspase-dependent cell death, suggesting that Fstl1 plays important roles in cellular proliferation and apoptosis in lung cancer cells.

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Scope of the thesis

The notion that developmental signaling pathways are reactivated during chronic lung disease attracted scientists to dissect the roles of these pathways related with the pathophysiology of COPD. However, little is known about the involvement of BMP signaling in COPD. BMP/Fstl1 signaling is a key player in embryonic lung morphogenesis, with diverse physiological roles including cellular communication. Recent studies demonstrate that aberrant Fstl1 expression is associated with several pathological lung conditions and inflammatory disorders, underscoring its great importance in adult tissue homeostasis and repair. However, the involvement of Fstl1 in COPD remains unexplored. This thesis will delineate the role of the endogenous BMP antagonist Fstl1 in lung development and shed light on its potential contribution to the pathogenesis of COPD. The specific aims of this thesis are to investigate the role of Fstl1 in pulmonary vasculature development and COPD, particularly in airway epithelial-mesenchymal cell communication and adult tracheal epithelial cell differentiation. To this aim, the developmental roles of Fstl1 were studied in conditional endothelial-specific Fstl1 knockout mice and in vitro study using pulmonary cell lines, including bronchial epithelial cells, lung fibroblasts, and airway smooth muscle cells.

In Chapter 2, the role of endothelial Fstl1 in the postnatal development of the pulmonary vasculature was investigated. The changes in molecular signaling, lung physiology, and morphology of pulmonary vessels were dissected in endothelial-specific Fstl1 knockout mice compared to littermate controls.

Our investigation of Fstl1 expression and BMP/Smad activation in lung tissue of COPD patients is presented in Chapter 3, providing evidences of dysregulation of Fstl1 expression in different parts of COPD lung tissue.

Chapter 4 provides a perspective on the expression levels and contribution of the TGF-β superfamily members, activin-A and its endogenous follistatin antagonist in smoking-induced airway inflammation in COPD both in vitro and in vivo.

The role of Fstl1 in inflammatory cytokine release from lung mesenchymal cells was explored in Chapter 5, which provides evidences on pro-inflammatory roles of Fstl1 1 in coordinating epithelial-mesenchymal communication in inflammatory cytokine release in the lung. We identified epithelial-derived factors in culture supernatants of Fstl1 overexpressing bronchial epithelial cells which mediate Fstl1-conditioned media-induced IL-6 and IL-8 release from lung fibroblasts.

Chapter 6 describes the latest understanding of the contribution of pulmonary mesenchymal cells in modulating airway inflammation in various lung diseases. Furthermore, the effect of BMP4 in differentiation of primary adult tracheobronchial epithelial cells using air-liquid interface culture was studied in Chapter 7. The potential implications of aberrant BMP signaling related with airway epithelial remodeling in COPD lungs was discussed.
References


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