Effect of Semaphorin 3E on Airway Smooth Muscle Cell in Chronic Obstructive Pulmonary Disease (COPD)

By

Duaa Alsubait

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba

In partial fulfillment of the requirements for the degree of

Master of Science

Department of Immunology

University of Manitoba

Winnipeg, Manitoba, Canada

ACKNOWLEDGMENTS

I would like to recognize and thank my supervisor Dr. Abdelilah Soussi Gounni by all means for all the support, guidance, patience, encouragements, and teaching he provided me during my master's journey at his lab. I deeply appreciated all the time he dedicated for me to learn and enlighten my knowledge. He also gave me an opportunity to become an independent researcher.

I would also like to express my sincere gratitude and appreciation to my committee advisors, Dr. Jude Uzonna and Dr. Jiuyong Xie for their support and academic and personal advices. I really appreciate their precious time they offered me for meetings with me and for reviewing my thesis. I am thankful for the valued instructions and comments they provided me during those meetings.

I am also especially grateful to Lianyu U Shan, Hesam Movassagh and Dr. Naresh Redhu for their technical assistance, insightful discussion and friendship they offered me in the lab that made my masters program more ecstatic. I also wish to thank my lab members Nazanin Tatari, Mohammed Ashfaque, and Jyoti Balhara.

Many thanks to the Department of Immunology staff members, Professors and staff alike, for providing an excellent academic and professional environment.

I would also like to acknowledge the Saudi Ministry of Higher Education for supporting me financially.

Dedication

I owe special thanks to my parents, no words can express my appreciation and how grateful I am to them. They have been always behind me all the way and pushed me to be the best that I can be.

ABSTRACT

Introduction: Chronic Obstructive Pulmonary Disease (COPD) is a life-threatening lung disease characterized by airflow limitation, which is poorly reversible and progressive. Current therapeutic strategies for COPD fail to minimize various features of airway remodeling particularly airway smooth muscle (ASM) mass hyperplasia.

Semaphorin-3E (Sema3E) is a member of the semaphorin family proteins that were initially discovered as axon guidance molecule. Previous studies indicated a prominent role of Sema3E in cell migration and proliferation. However, the role of Sema3E in COPD is not known. The objective of this study is to investigate whether Sema3E regulates ASM cell proliferation, a key feature of airway remodeling in COPD patients.

Methods. Human ASM cells were isolated from COPD patients with different severity. Sema3E and PlexinD1 expression was studied using quantitative real-time PCR, flow cytometry and western blotting. Lung tissues from COPD patients were stained for Sema3E expression by immunohistochemistry. Cell proliferation was evaluated using flow cytometry-based EdU incorporation assay and cell count.

Results: HASM cells express Sema3E at mRNA and protein level and in lung tissue obtained from COPD and healthy subjects. PlexinD1, the high affinity receptor for Sema3E is constitutively expressed in HASM cells from COPD compared to healthy subjects. Exogenous treatment with recombinant Sema3E inhibits HASM cell proliferation mediated by platelet-derived growth factor (PDGF) in healthy but not in COPD *in vitro*. Interestingly, HASM cells obtained from COPD patient express p61KDa-Sema3E isoform, which is known to have proliferative function. Furthermore, HASM cells from COPD patient display abundant expression of the endogenous Sema3E binding to its receptor on cell surface.

Conclusion. Collectively, the data suggest that endogenous Sema3E p61kDa, produced by HASMCs, occupy plexinD1 receptor in autocrine manner thus contributing to airway smooth muscle remodeling.

LIST OF TABLES AND FIGUERS

LIST OF ABBREVIATIONS

APC: antigen presenting cell

APC : allophycocyanin

ASM: airway smooth muscle

α-SMA: alpha-smooth muscle actin

BALF: bronchoalveolar lavage fluid

bFGF: basic fibroblast growth factor

BSM: bronchial smooth muscle

CCL2: CC-chemokine ligand 2

CCR2: CC -chemokine receptor 2

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CD40 : cluster of differentiation 40

CD44: cluster of differentiation44

c-DNA: complementary deoxyribonucleic acid

CTL: Control

COPD: chronic obstructive pulmonary disease

CNS: central nervous system

CXCL: CXC chemokine ligand

CXCR3: CXC chemokine receptor 3

DAG: diacyl glycerol

DC: dendritic cell

DMEM: Dulbecco's Modified Eagle Medium

ECM: extracellular matrix

ECP: eosinophilic cationic protein

EDN: eosinophil derived neurotoxin

EDTA: ethylenediamine tetra acetic acid

EdU: 5-ethynyl-2'-deoxyuridine

FES: feline sarcoma oncogene

EGF: epidermal growth factor

EPO: eosinophil peroxidase

ERK: extracellular signal regulated kinase

ERBB2: erb-b2 receptor tyrosine kinase 2

FACS: fluorescence-activated cell sorting

FASL: Fas ligand

FC: fragment crystallizabl

FEV1: forced expiratory volume per second

FVC: forced vital capacity

FYN: FYN proto-oncogene, Src family tyrosine kinase

GAP: GTPase Activating Protein

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

G-CSF: Granulocyte-colony stimulating factor

GM-CSF: granulocyte-macrophage colony-stimulating factor

GOLD: global initiative for chronic obstructive lung disease

GTPases: guanosine triphosphatases

GPCR: G-protein coupled receptor

GRO-α: growth regulated oncogene alpha

GSK-3: glycogen synthase kinase 3

GST1: glutathione-S-transferase

HASMCs: human airway smooth muscle cells

HRP: horseradish peroxidase

ICAM: intracellular adhesion molecule

IFN-γ: interferon gamma

IGF-1: insulin-like growth factor-1

Ig: immunoglobulin

IL-8/CXCL-8: interleukin-8

IL: interleukin

IPT: Immunoglobin-like fold shared by Plexins and Transcription factors domains)

IP3: inositol 3-phosph

ITS: insulin transferrin selenium

JAK: janus activated kinase

JNK: c-Jun NH2-terminal kinase

kDa: kilo Dalton

LT: lymphotoxin

mAb: monoclonal antibodies

MAPK: mitogen activated protein kinase

MFI: mean fluorescence intensity

MMP1: Matrix metalloproteinase-1

MMP12: Matrix metalloproteinase-12

MPO: myeloperoxidase

M-Ras: muscle RAS oncogene

mRNA: messenger ribonucleic aci

MCP-1: monocyte chemotactic protein 1

NE :neutrophil elsatse

NF-κB: nuclear factor kappa B

NLRs: NOD-like receptors

Nrp1: neuropilin-1

PAGE: polyacrylamide gel electrophoresis

PARs: protease-activated receptors

PDGF: platelet derived growth factor

PBS: Phosphate buffered saline

PE: phycoerythrin

PGI2: prostaglandin I2

PGE2: prostaglandinsE2

PGN: peptidoglycan

PI3Ks: phosphatidylinositol 3-kinases

PIP2: phosphatidylinositol bisphosphate

PKC βII: Protein kinase C isoform βII

PLC: phosphoinositide-specific phospholipase C

PLC-γ: phospholipase C gamma

PYK2: pyruvate kinase PYK2

PPC: pro-protein convertases

PSI: plexin-semaphorin-integrin

PVDF: polyvinylidene fluoride

Rac1: ras-related C3 botulinum toxin substrate 1

RANTES: regulated upon activation, normal T cell expressed, and secreted

Rap1: Replication-associated protein

RBD: GTP biding domain

RhoD: ras homolog family member D

Rnd1: Rho family GTPase 1

ROS: reactive oxygen species

R-Ras: related RAS viral (r-ras) oncogene

RTK: receptor tyrosine kinase

RT-PCR: reverse transcription-PCR

S6K1: S6 kinase polypeptide 1

SDS: sodium dodecy1 sulfate

SDS-PAGE: polyacrylamide gels

Sema3E: semaphorin3E

Sema3E-p61: cleaved fragment of sema3E-p87

SEMAs: Semaphorins

SH2: Src homology 2

shRNA :short hairpin RNA

STAT 4: signal transducer and activator of transcription 4

STIM-1: stromal interaction molecule 1

T_C1: cytotoxic T cell

TCR: T cell receptor

TGF-β: tumor growth factor beta

Th2: T helper type 2

Th1: T helper 1 cells

Th17: T helper 17 cells

TLR: toll-like receptor

TM: transmembrane domain

TNF: tumor necrosis factor

Treg: regulatory T cell

Uncl- Sema3E: uncleavable 87 kDa of Sema3E

VCAM: Vascular Cell Adhesion Molecule

VEGF-C: Vascular Endothelial Growth Factor-C

VEGFR2: Vascular Endothelial Growth Factor Receptor-2

WB: Western Blot

WHO: World Health Organization

TABLE OF CONTENTS

LIST OF CONTENT

CHAPTER 1: INTRODUCTION

1.1. Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a global health issue that accounts for a huge economic burden. According to world health organization (WHO), COPD is the third leading cause of death with 3.1 million deaths worldwide in 2012 and predicted to be the third leading cause of death worldwide by 2030 according to Global initiative for obstructive lung disease (GOLD). The morbidity cost including medical prescriptions, consultations, visits to the emergency room and hospitalization are significant. Canadian thoracic society estimated total cost of hospitalization at $2 billion a year with more than 714,000 Canadians suffer from COPD and more than 256,000 COPD patients require hospitalization a year[1].

Smoking is the primary cause of the disease. Other risk factors for COPD include exposure to indoor air pollution such as biomass fuel used for cooking and heating, and occupational dusts such as vapors, irritants, and fumes [2]. According to WHO, the disease now affect men and women almost equally in industrial countries. Furthermore, the prevalence among women and nonsmoker subjects is rising due to biomass fuels used by women for cooking and the burning of wood especially in parts of the Middle East, Africa and Asia.

The Global initiative for Chronic Obstructive Lung Disease GOLD defines COPD as a disease state characterized by the presence of airflow obstruction due to chronic bronchitis or emphysema; the airflow obstruction is generally progressive and partially reversible and may be accompanied by airflow hyperactivity.

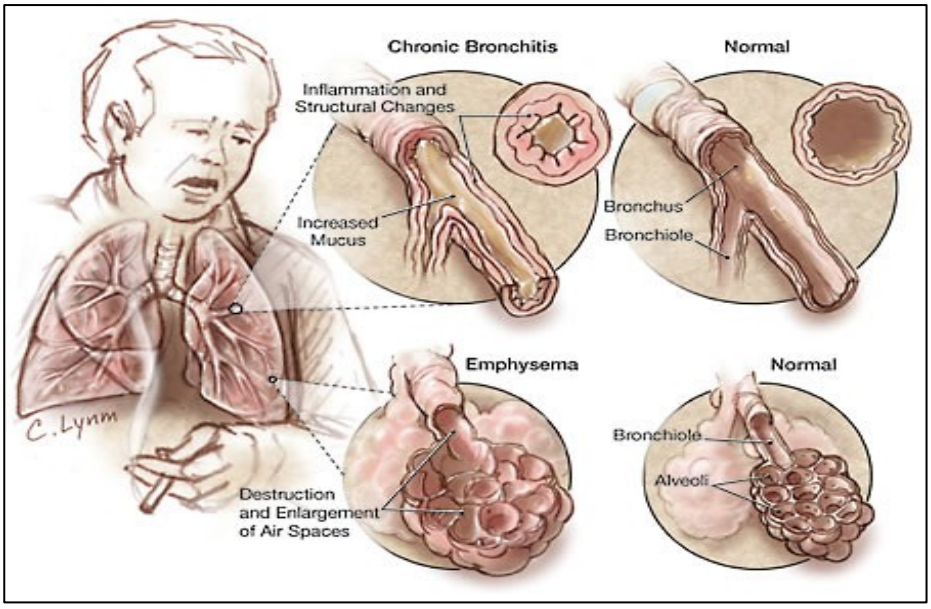


Figure 1. The airways of COPD patient. Airflow limitation is due to chronic bronchitis or emphysema in smoker's airways. (Doctor Tipster. (n.d.). 2015)

There are four stages of the disease diagnosed by the pulmonary function test or spirometric test and the symptoms noticed on the patients[3, 4]. The spirometery tests the reduction in forced vital capacity (FVC) due to air trapping, rather than a change in forced expiratory volume on one second (FEV1) [4].

GOLD classified the severity of COPD using a post-bronchodilator forced expiratory volume in one second / forced vital capacity (FEV1/FVC) ratio <0.70 to define irreversible airflow obstruction into mild COPD with (FEV1 ≥ 80%), Moderate (50% ≤ FEV1 < 80%), severe (30% ≤ FEV1 < 50%), and very severe (FEV1 < 30%).

Clinically, many medications for COPD are available but the effectiveness is partial. They aim to improve quality of life and slow down the progression of the disease [5]. Clinicians prescribe drugs to COPD patients to maintain control of symptoms and prevent COPD exacerbation. The treatment and therapy used to control COPD include antibiotics for bacterial infection, inhaled

bronchodilators, corticosteroids, aerosol therapy, pulmonary rehabilitation, oxygen therapy for hypoxic patients and surgical option for those with very severe COPD[5]. COPD is a complex disease that results from the interaction between environment and genetic factors [6, 7]. The most significant environmental risk factor of COPD is cigarette smoking[8]. However, smoking account for only 15% for the etiophathogenesis of COPD especially in high-income countries, whereas, in low-income countries, most of COPD patients are non-smokers particularly women and elderly[8, 9]. This suggests that other factors contribute to the development of COPD.

Among the factors involved in COPD, genetic risk factors are considered a key contributor to the development of COPD [10, 11]. Alpha1-antitrypsin deficiency is the proven genetic risk factor in COPD [7, 12, 13]. Furthermore, polymorphisms in matrix metalloproteinase genes MMP1, MMP9 and MMP12 may account for the development of COPD[14]. Similarly, several genes have been reported to be significantly associated with COPD phenotypes. These include SERPINE2 and CXCL_8/IL-8 both located on chromosome 2, in addition to mormicrosomal epoxide hydrolase, glutathione-S-transferase GST1 and TGF-ß situated on chromosomes 12 and 19, respectively [8, 15].

1.2. Mechanisms of COPD

There are many functional changes occur inside the lungs as a result of the disease process. Once an irritant is inhaled, the epithelium attempts repetitively to protect itself and repair the injury induced by this noxious agent [16]. This further leads to marked structural changes to the epithelium with thickening and squamous metaplasia accompanied by an enhanced mesenchymal response at some sites and alveolar destruction at others [17]. Narrowing and reduction of the small airways and lung parenchyma are the predominant features of COPD

[18]. These, result in the development of small-airway obstruction and emphysema, which is associated with progressive airflow limitation [18].

Once an irritant is inhaled by individual, it causes neutrophils, T-lymphocytes, and other inflammatory cells to accumulate in the airways[19]. These events contribute to airway remodeling characterized by modification and structural changes at the cellular and tissue level. In COPD, the inner wall area of the large airways is thicker and the peripheral airways remodeled[20]. The abnormality in the airway wall in COPD is manifested by an increase in airway fibroblast, smooth muscle mass, mucous, and increase in glandular size, in addition to other alterations of the bronchial vasculature and nerves [21].

1.3. Pathophysiological processes in the lung of COPD patients

The respiratory system is divided into upper and lower respiratory tract and each part has its own function in the body. Circulating mucus produced by goblet cells at the upper airways moistens and clear trapped particulate. The lower airways consist of the tracheobronchial tree, which starts with trachea and branches off to end with alveoli. The tracheobronchial tree branches into primary, lobar and segmental bronchus and into smaller terminal and respiratory bronchioles. In COPD the peripheral airways are the major site of obstruction specifically in the smaller conducting airways that include bronchi and bronchioles[22]. The COPD airways narrow to less than 2mm in diameter[23]. This obstruction causes the reduced forced expiratory flow that defines COPD. The inhaled tobacco also leads to the destruction of alveolar support of the peripheral airways in COPD. This causes a decrease in the elastic force available to push the airflow out of the lung[22]. Mucus hypersecretion accounts for the occlusion of the airways lumen in the disease[24]. Collectively, these explain the decline in FEV1 observed in COPD.

Neutrophils, macrophages, $CD8^+$ T-lymphocytes, and B cells infiltrate and accumulate in the lung of COPD patients[25]. The activation of these cells in response to tobacco smoke causes the release of inflammatory mediators, which aid in the pathogenesis of COPD. The mechanisms for ongoing pulmonary inflammation and damage in COPD is due to increase in oxidative stress, disturbance in the protease–antiprotease balance with an elevated production of proteases and/or decreased levels of antiproteases, increased programmed cell death and profibrotic conditions in the small airways[26-28].

In COPD, the repair environment in the airways produces a number of changes in cytokine and growth factor expression. In response to cytokines the extracellular matrix remodeled and contribute to fibroblast, smooth muscle cells and other mesenchymal phenotypic and functional changes [29]. Gradually, this process causes structural and physiological lung changes and damages that get progressively worse.

1.4. Cellular component involve in COPD

In the pathogenesis of COPD, neutrophils, macrophages, and CD8+ T-cells are considered as key effector cells[30]. After cigarette smoke exposure, resident alveolar macrophages are activated. Neutrophils are rapidly recruited in response to macrophage and epithelial cell-derived chemokines and leukotrienes. Macrophages, CD8+ and CD4+ T-lymphocytes accumulate within days to weeks and continue to accumulate with time[19]. The abnormal accumulation of inflammatory cells persists throughout the disease process, even after smoke cessation[31].

1.4.1. T and B Lymphocytes

Heavy cigarette smoke exposure stimulates humoral and cellular components of the adaptive

immune response. In COPD, a type 1 immune response is initiated[32].

Dendritic cells take up the antigens in the small airways and parenchyma, where the lung tissue suffers the most injury by cigarette smoke. Dendritic cell in COPD triggers a Th1 and a cytotoxic CD8+ T-cell response [33]. Increased number of apoptotic cells in the lungs of smokers with COPD correlate with the numbers of CD8+ cytotoxic T-cells in the alveolar wall. Furthermore, an increase number of structural lung cells undergoing apoptosis in emphysematous lungs support the idea that CD8+ T-cells are inducing apoptosis of endothelial and epithelial cells in emphysema[34].

The number of CD4+ T-cells expressing activated STAT4 correlated with the degree of airflow obstruction, which leads to the commitment of Th 1 lineage. Th1 and Th17 CD4+ T-cells are also found in lung of smokers suffering from COPD in a smaller numbers compared to CD8+ T-cells[35]. Th1 cells secrete interferon-γ that plays a role in activating Th17 cells to enhance anti-microbial effect. The latter release cytokines to clear infection by inducing the epithelial cells to produce antimicrobial peptides, chemokines, and granulocyte growth factors G-CSF and GM-CSF which in turn promote the accumulation of neutrophil at the site of injury[36, 37]. Many studies showed the decrease number of T regulatory cells (Tregs) in COPD especially at the site of small airways [38, 39]. Lower levels of IL-10 predict the presence of fewer numbers of Tregs, a subset of CD4+ T cells in the lungs of COPD[40].

B-cell aggregates are present in COPD lungs. Cigarette smoke extract upregulate B-cell-derived CXCL13 that is required for B-cell migration toward COPD lung. CXCL13 induce lung B cells to upregulate the membrane-bound lymphotoxin. lymphotoxin (LT) further establish a positive feedback loop and promote CXCL13 production in the lung of COPD[41]. Monoclonal B-cells

in the lymphoid follicles proliferate in response to specif lung antigens seen in COPD because no bacterial or viral products were seen in the follicles. Recent findings demonstrated that in patients with COPD, IgG autoantibodies with avidity for epithelial and endothelial cells are dominant [42, 43].

1.4.2. Dendritic cells (DCs)

Innate and adaptive immune responses are linked by DCs that have been shown to be one of the key cells in the pathogenesis of COPD. There is evidence in the literature that cigarette smoking is associated with an expansion of dendritic cell numbers n in the lower respiratory tract and a marked increase in the number of mature cells in the lung parenchyma[33]. Langerin+ DC (CD207) and CD1a (markers of Langerhans cells) increase significantly in patients with COPD, compared to non-smokers and healthy-smokers. This accumulation of Langerhans-type DC in COPD increased with disease severity [44-46]. Specifically, in the small airways and alveoli of smokers and COPD patients the number of myeloid DC with Langerhans-type cell markers (CD207, CD1a, and Birbeck granules) accumulates in huge numbers[46]. Experimentally, mouse exposed chronically to high doses of cigarette smoke manifest pulmonary inflammation and emphysema associated with increase in number of myeloid DCs in BALF fluid and lungs[47]. Cigarette smoke extract exposed interstitial type DC impairs their maturation and instruct T-cell to differentiate toward a Th2 phenotype [48]. Besides their role in antigen presentation, DCs are also capable of producing substantial amounts of proteinases particularly MMP12, which may account for the imbalance of proteinase–antiproteinase in COPD leading to emphysema as shown in mouse upon cigarette smoke exposure [49].

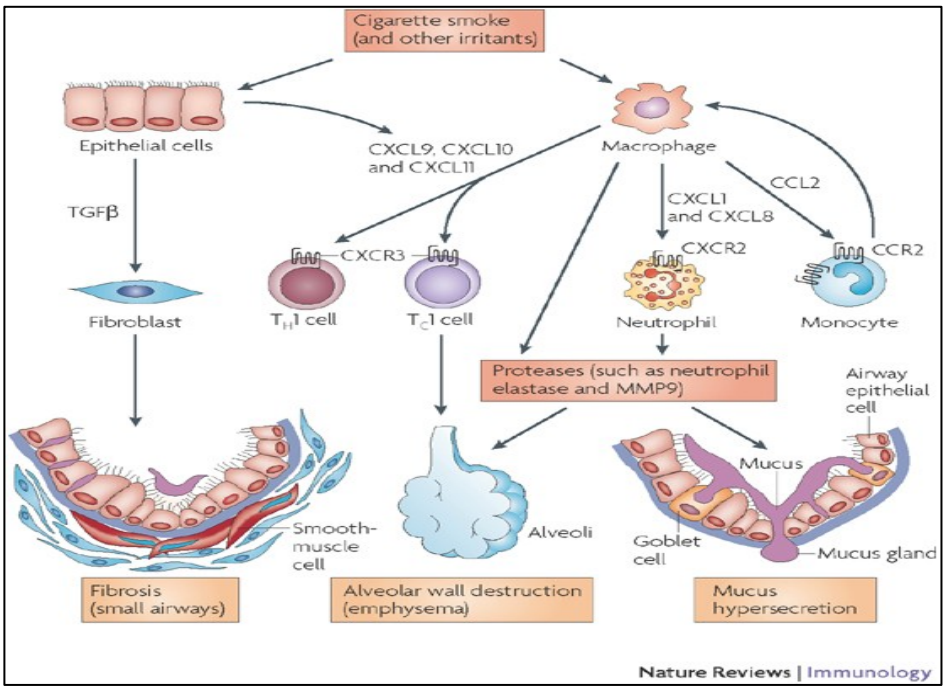


Figure 2. Inflammatory and immune cells involved in chronic obstructive pulmonary disease (COPD). Inhaled cigarette smoke and other irritants activate epithelial cells and macrophages to release several chemotactic factors that attract inflammatory cells to the lungs, including CC-chemokine ligand 2 (CCL2), which acts on CC-chemokine receptor 2 (CCR2) to attract monocytes, CXC-chemokine ligand 1 (CXCL1) and CXCL8, which act on CCR2 to attract neutrophils and monocytes (which differentiate into macrophages in the lungs) and CXCL9, CXCL10 and CXCL11, which act on CXCR3 to attract T helper 1 (T_H1) cells and type 1 cytotoxic T (T_C1) cells. These inflammatory cells together with macrophages and epithelial cells release proteases, such as matrix metalloproteinase 9 (MMP9), which cause elastin degradation and emphysema. Neutrophil elastase also causes mucus hypersecretion. Epithelial cells and macrophages also release transforming growth factor-β (TGFβ), which stimulates fibroblast proliferation, resulting in fibrosis in the small airways. (Barnes et al., 2008)

1.4.3. Macrophages and Monocytes

In COPD, airways of humans and experimental animals show a marked increase and predominance in the number of activated macrophages in the lung parenchyma[50, 51]. Macrophages are also play an important role in initiating the neutrophilic inflammatory response by releasing neutrophil chemotactic factors in COPD patients[52, 53]. Furthermore, pulmonary macrophages contribute to the proteolytic process by producing and secreting potent proteinases. Lung macrophages produce matrix metalloproteinases (MMPs); the cysteine (thiol) proteinases, and cathepsins B, H, L, and S[54-56]. They also mediate lung inflammation in COPD by markedly reduced their capacity to ingest apoptotic cells [57]. Indeed, lung macrophages showed decreased expression of PKC βII, which is required for apoptotic cell uptake[58].

1.4.4. Neutrophils

Neutrophils are one of the primary effector inflammatory cells in COPD and its proteinases are responsible for the main pathological features, especially neutrophil elastase (NE)[51, 59]. Neutrophilic inflammation in COPD related to subsequent decline of lung function. Many studies showed an increased numbers of neutrophils in sputum and bronchoalveolar lavage fluid (BALF) of COPD patients compared to non-COPD smokers[60]. Neutrophils number increases correlate with the severity of the disease and the degree of airflow obstruction [61, 62].

In smoker's sputum, elevated levels of IL-8 correlate with increase number of neutrophil and with percent-predicted FEV1 [64]. In COPD Pulmonary macrophages through releasing of neutrophil chemotactic factors recruit neutrophils to the respiratory tract therefore playing an important role in proteolytic process by releasing proteases [25]. These proteases including

NE, aid in the pathogenesis of COPD particularly in severe emphysema. Thus, correlate with a deficiency of alpha 1 antitrypsin (α1-AT) in lungs of COPD patients. Proteases disrupt the lungs by enhancing the release of mucus, degrading elastin, fibronectin, and collagen. Moreover, these proteases can also decrease the activity of immunoglobulins and activate components of the complement cascade in lungs of smokers [65-69].

1.4.5. Eosinophil

Eosinophil infiltration in the airways correlates with airway obstruction. Airway eosinophilia observed in patients with COPD during acute exacerbations and with stable disease. However, no differences have been observed in eosinophil numbers in sputum taken during a stable disease period compared with those taken during an acute exacerbation in the same patients [70-72]. In bronchial biopsies of patients with COPD a higher numbers of eosinophils have been found compared to healthy controls [77]. Interestingly, eosinophilia in COPD is linked to an upregulation of RANTES (regulated on activation, normal T cell expressed and secreted) but not IL-5[73, 74]. In addition, there is upregulation in eotaxin- 1/CCL11 and CCR3 with increased number of eosinophil in COPD [75]. In acute exacerbations of the disease, increased level of TNF is noticed which induce production of RANTES [76].

Eosinophils are activated and recruited in COPD by IL-6 and IL-8, which are highly found in the sputum [72]. Moreover, bronchoalveolar lavage fluid of COPD patient there is increase eosinophil and ECP levels in induced sputum associated with exacerbations [77]. In addition to ECP, EPO levels are raised in the sputum of patients with COPD [78]. It has been reported that during exacerbations of COPD, eosinophil infiltration of the airway walls is associated with increased amounts of ECP in bronchoalveolar lavage fluid and induced sputum [25, 76].

Eosinophil have a role in antiviral host defense in COPD with viral infections by releasing ECP and EDN acting as ribonucleases [80]. Therefore, to identify patients who will benefit from corticosteroid therapy, sputum eosinophilia is used as predictive marker [79].

1.5. Cytokines and chemokines involved in COPD

The expression of cytokines and chemokines is higher in COPD patients compared to asymptomatic smokers. In sputum of patients with COPD, an increased level of IL-6, IL-1β, TNF, CXCL8/IL-8, and CCL2/MCP-1 has been observed. Moreover, many proinflammatory and antinflammatory cytokines are released by alveolar macrophages in COPD patients. In BAL fluid of chronic smokers, levels of TNF-α, IL-1 β, IL-6, CXCL8/IL-8, a CCL2/MCP-1 were increased compared to non-smokers. Higher expression of CCL2/MCP-1, TGF- β 1 and CXCL8/IL-8 mRNA and protein has been observed in bronchiolar epithelial in macrophages of smokers with COPD compared with non-COPD smokers [81-83].

1.5.1. Interleukin 8 (IL-8 or CXCL8) is mainly produced in the airways of COPD from Leukocytes, monocytes, T-cells, neutrophils, natural killer cells and airway epithelial cells and the levels correlate with degree of the airflow obstruction and progression of emphysema [19] [84]. Furthermore, the level of sputum CXCL8 correlate also with neutrophil activation markers MPO and NE [85]. In COPD, CXCL8 production is influence by many factors including proinflammatory cytokines such as, IL-1β and TNF, bacteria and bacterial products, viruses, and oxidative stress [86-89].

1.5.2. Tumor necrosis factor alpha (TNF) is one of the most important proadhesive cytokines in COPD along with interleukin-1β (IL-1β)[90]. The main source for TNF in COPD is macrophages and other cells like activated monocytes, T-cells, mast cells, epithelial cells, and probably smooth muscle cells [91]. It has been observed that in serum and bronchoalveolar lavage samples from patients with COPD higher levels of TNF have been measured with further increases during exacerbations of the disease [83]. Genetic polymorphism in TNF causes the overexpression of this cytokine in early COPD development or rapid progression [92]. In line with this, overexpression of TNF in mice model is associated with the development of emphysema [93]. TNF appears responsible for emphysema of COPD and 70% of the tissue destruction following cigarette smoke exposure in mouse models of COPD, probably by enhancing neutrophil migration into the lung [94].

1.5.3. Interleukin-1 beta (IL-1β) is also produced primarily by macrophages, neutrophils and epithelial cells in COPD. In COPD patients increased levels of IL-1β in the sputum is observed in patients with increased disease exacerbations [40, 83]. Furthermore, IL-1β production by epithelial cells from COPD patients is enhanced *in vitro* following cigarette smoke exposure compared with controls [95]. There is some evidence that overexpression of IL-1β can be due to genetic polymorphisms [96]. Over expression of IL-1β in animal studies using inducible IL-1β systems and complex knockout mice have an enhanced mucin production. In smokers with emphysema, IL-1β induces MMP-8 and MMP-9 levels significantly in bronchoalveolar lavage (BAL) fluid in COPD. *In vitro,* cultured human airway smooth cells stimulated with TNF-α and IL-1β induce MMP-9 expression and cell migration in smokers [97].

1.6. Proteases and antiproteases

In health, proteinase activity is controlled by anti-proteinases. In COPD the production of proteolytic enzymes, such as neutrophil elastase, and several matrix metalloproteinases (MMPs), is increased in the lungs and there may be a reduction in the levels of antiproteinases, such as alpha 1 antitrypsin [28]. This imbalance between the proteinases and antiproteinases play a huge role in disease pathogenesis.

1.6.1. Alpha1-antitrypsin (AAT) deficiency (protease inhibitor (PI) type Z)

AAT is an acute phase protein that provides the major defense against neutrophil elastase [98, 99]. AAT deficiency is mostly caused by Z allele (ZZ-AT) homozygous (PI ZZ) at the SERPINA1 locus [100]. This deficiency is accompanied with rapid decline in lung function and is associated with the development of early onset COPD [101, 102]. Moreover, smokers with AAT Z isoform deficient tend to develop more severe pulmonary impairment at an earlier age than non-smokers [101]. Many studies linked the increased prevalence of emphysema with an individual who has extremely low levels of AAT Z isoform [103].

1.6.2. Matrix metalloproteinases (MMP's)

Matrix Metalloproteinases comprise of a family of more than 20 related proteolytic enzymes believed to be essential for development, tissue remodeling and repair in inflammation [8]. Both neutrophils and macrophages produce large amounts of MMPs and their inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs)[104, 105]. Abnormal expression of MMPs has been linked to pulmonary emphysema by its destructive processes [106]. These enzymes degrade collagen, inactivate AAT and activate tumor necrosis factor-α (TNF-α)[107]. The polymorphism of MMP-9 is a genetic factor that develops smoking-induced pulmonary emphysema [108]. In cigarette smoker individuals MMP-9 and MMP-12 account

for most of the macrophage-derived elastase activity [109].

1.6.3. Oxidative stress /reactive oxygen species ROS

Cigarette smoke is one of the exogenous oxidants exposed to the lungs; although there are endogenous oxidants that are continuously affect the lungs by metabolic reactions for example during activation of phagocytes. Tobacco smoke contents of 10^4 oxidants per puff induce reactive oxygen or nitrogen species[109]. Mutations in genes encoding these enzymes or their pathways disturb the balance between oxidant/antioxidant[110]. The oxidants burden has wide effects in the lungs that include remodeling of extracellular matrix (ECM) and vasculature, inactivation of antiproteases, mucus secretion, apoptosis, and reduced cell proliferation[111]. Oxidants in COPD patients damage components of the lung extracellular matrix (Elastin and collagen). Furthermore, the activation of transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) induce levels of ROS in initiating inflammatory responses in the lungs[32].

1.7. Airway remodeling

1.7.1. Epithelial cells

Airway epithelial cells are the first line of defense against environmental agents such as cigarette smoke. Epithelial cells express innate immune system receptors (TLRs, NLRs, PARs),which recognize an irritant (cigarette smoke) and initiate downstream signaling that modifies cellular behavior and clearance by mucus[113]. Epithelial cells produce a balance of anti-inflammatory and pro-inflammatory molecules to regulate the inflammation and prevent inflammatory diseases [114]. Epithelial cells release anti-inflammatory and immunosuppressive

metabolites and express cell surface molecules. These include: cytokines (IL-10, TGF-β), protease inhibitors (SLPI, SERPINA1 (α1-antitrypsin), SERPINB1, TIMP-1), and inhibitory arachidonic acid metabolites (PGE2, PGI2, lipoxin A4), B7-H1, B7-DC, IL-13RA2 and FASL [114, 115]. Moreover, to maintain the balance they also induce pro-inflammatory cytokines and chemokines that selectively attract inflammatory cells like neutrophils, monocytes, eosinophils, and basophils to eradicate pathogens; and DCs, T-cells, and B-cells to initiate adaptive immunity and transport of secretory immunoglobulin [114, 115][116]. Gene polymorphisms in epithelial cells are strongly associated with the development of lung disease [117].

Another feature of remodeling is depicted by airway epithelial squamous metaplasia and sub-epithelial fibrosis. It is well established that submucosal glands at the surface of epithelium in the airways are a significant source of fluid and mucin. In COPD mucous hypersecretion-increase mucin cell number and mucin gene expression, and decreases in mucus clearance, are correlated with the degree of physiologic impairment in epithelial cells [118]. Furthermore, cytokines such as, IL-1βand TGF-β induces pathologic epithelial mesenchymal interactions leading to the development of airway epithelial squamous metaplasia and sub-epithelial fibrosis [119, 120].

1.7.2. Fibroblast / Myofibroblasts

Fibroblasts experience changes in number and phenotype in COPD and play a central role in the production and homeostasis of the ECM components [121]. In COPD the increased number of myofibroblasts indicates that they are hyperproliferative and resistant to apoptosis [127]. TGF-β is considered as pro-fibrotic mediators *in vitro* and one of the most potent cytokines in

airway remodeling in COPD [122]. Activation of fibroblasts by TGF-β produced by inflammatory cells, and/or by fibroblasts themselves induces pulmonary fibrosis, which can also be induced *in vivo* by over-expression of TGF-β in the lungs [123] [121].

Fibroblasts also contribute to proteases/antiproteases imbalance by producing matrix metalloproteinase (MMPs) and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs)[125]. Metalloproteinase MMPs potentially promote the fibrotic and inflammatory milieu by activating growth factors and chemokines in the lung [126].

1.7.3. Airway smooth muscle (ASM) cell

In COPD as inflammation continues, the airways constrict, becoming excessively narrow and swollen, which manifest the symptoms of bronchitis in the patients. This causes the reduction in forced expiratory flow that defines COPD due to destruction of alveolar support of the peripheral airways [128]. The structural narrowing of the airway lumen is mediated by a remodeling process including fibrosis and mesnchymal cells hyperplasia [29]. Airway smooth muscle cell hyperplasia and hypertrophy are central to the manifestation of COPD pathogenesis [129]. The smooth muscle bundle is composed of elongated, spindle-shaped cells, which are bound together in irregular branching fasciculi. Smooth muscle fibers are generally shorter than skeletal muscle fibers and contain centrally located single nuclei. It surrounds the entire airway below the level of the main bronchus, in a roughly circular orientation. The peripheral airway is where the smooth muscle cells remodeled compared with the central airways in COPD, reaching a maximum in the membranous bronchioles [130, 131].

1.8. Airway smooth muscle (ASM) cells role in COPD

A cross section from COPD patient show increased smooth muscle of segmental and subsegmental airways by: 1.07 mm^2 and 0.5 mm^2, compared with 0.5 mm^2 and 0.19 mm^2 in control cases. They also stain for nuclei and mean number of smooth muscle nuclei in sections of segmental airways was 1,793 in the bronchitis compared with 717 in the controls. This study by HOSSAIN and HEARD is consistent with hyperplasia as well as hypertrophy phenomenon in COPD patients. In COPD, ASM cells play a central role in the modulation of structural and functional responses of the airway [132]. ASM cells produce pro- and anti-inflammatory mediators that affect cell proliferation, migration, and apoptosis these include, IL-13, IL-1β, IL-5, IL-6, IL-8, IL-17, PDGF, and TGF-β. IL-6 cytokine produced by ASM cells following stimulation by IL-1β or TNF can induce ASM hyperplasia and modulate immune cell function [133].

1.8.1. Bronchoconstriction of the airway as a result of ASM cells hyperplasia and hypertrophy

In COPD the constriction of the airways in the lungs due to the tightening of surrounding smooth muscle, manifests symptoms in patients like coughing, wheezing, and shortness of breath. The inflammation persists in the airways of COPD patients via the influx of cytokines and chemokines by both inflammatory as well as structural cells [40]. These inflammatory modulators stimulate ASM proliferation directly or indirectly as a result of T-cell-ASM interaction mediated by cell surface expression of various cell adhesion molecules CAM proteins such as ICAM-1, CD40, and CD44. Changes in ASM phenotype or number associated with bronchoconstriction contribute to the increased ASM contractility [134].

1.8.2. Mechanisms of hyperplasia

In airways of smokers, the increase in ASM cell mass, which called hyperplasia, is one of the most important components of the airway wall remodeling process [135]. This increases in ASM cell number could occur through different mechanisms: increased rates of division, decreased rates of apoptosis, or through migration of mesenchymal cells to the ASM bundles [136].

1.8.3. ASM proliferation

ASM has multifunctional capacity, although classically it has been of interest because of its contractile response linked to bronchoconstriction [136]. Differentiated smooth muscle cells are phenotypically plastic and capable of proliferating and increasing in size, migrating and produceing of extracellular matrix and inflammatory mediators [137-139]. Several factors control ASM cell proliferation. However, the mechanisms that regulate ASM cell growth remain unclear[140]. Polypeptide growth factors, G-protein coupled receptor (GPCR) agonists and pro-inflammatory cytokines drive ASM cells proliferation [141]. Mitogens that induce ASM proliferation by activating receptors with intrinsic tyrosine kinase activity (RTK) include: basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and insulin [142-144]. TNF, IL-6 and IL1β, which can affect ASM cells by an autocrine loop, exert their effects on gene regulation through cell surface glycoprotein complexes through Src family proteins, MAPK and JAK/STAT cascades [145-148]. These factors can also stimulate ASM cells contraction [140]. In pulmonary diseases, acetylcholine and cysteinyl leukotrienes a contractile agonists acting via G-protein coupled receptors (GPCRs) induce ASM thickening [149]. Evidence also suggests that exposure of ASM to cytokines or growth factors not only affect proliferation but also alter contractility and calcium homeostasis and induce SMC

hypertrophy[150]. All together, these factors activate PLC, which increase cytosolic calcium. Activated PLC hydrolyzes phosphatidylinositol bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 and DAG activate other cytosolic tyrosine kinases, serine and threonine kinases. These events induce pleiotropic effects including the activation of proto-oncogenes, which are a family of cellular genes (c-onc), involved in controlling normal cellular growth and differentiation [151, 152].

In lung diseases, RTK growth factors (EGF, PDGF, and IGF-1) have been implicated in disease pathogenesis by inducing of ASM cells. In addition, combinations of these growth factors (e.g. EGF + PDGF, insulin + PDGF) produce a synergistic ASM cells proliferative responses in the airways [153]. In vivo, PDGF stimulation of murine, ASM induces PI3K or S6K1, which causes the mitogenic activity on the cells [133]. Moreover, in human ASM cells PDGF, EGF, and thrombin, induce PI3K activity that in turn transduces their signals by activating distinct pathways through RTKs and GPCR. These data showed that PI3K involved in mitogenic signaling in the airways [136, 154].

In many cell types, upon serum or growth factors stimulation, PI3K has been showed to be a key mediator of S6K1 activation, which is a critical step for mitogen-induced cell cycle progression through the G_1 phase [155]. In human ASM cells EGF and thrombin induced activation of S6K1 appears to be PI3K dependent [156]. A recent study demonstrated that GPCR activation by inflammatory and contractile agents could synergize with RTK activation to augment human ASM growth [157]. Other factors that Enhance human ASM cells growth and synthetic functions are the activation of PI3K or the transcription factor NF-κB in ASM cells [158-161].

1.9. Semaphorin (Sema)

Semaphorins are large family of proteins that were first discovered and associated with axon guidance molecules crucial for the development of the nervous system [162]. They have an ability to provide repulsive or attractive cues for migrating cells and growing neurites, i.e. axons and dendrites [163]. They exert functions on regulating axonal pathfinding, fasciculation, and branching and target selection [164].

Semaphorins are classified into eight classes, based on sequence similarities and their structural forms [167]. Semaphorins found in invertebrate species are grouped into classes 1, 2 and 5, Classes 3-7 contains vertebrate semaphorins (except for Sema-5c, which is found in invertebrates only); and Class V contains semaphorins found in the genomes of certain DNA viruses. SEMAs can be secreted (classes 2 and 3), cross the plasma membrane (classes 1, 4, 5, and 6), or be linked to a glycosylphosphatidylinositol-bound membrane protein (class 7)[167]. All semaphorins classes share a conserved sequence of approximately 500 amino acids in their extra cellular domain, allowing them to exert their effects through their receptors [168]. Semaphorins contain a large amino-terminal sema domain that is required for downstream signaling [169]. A conserved stretch of amino-acid residues near the carboxyl-terminal of the sema domain bears homology to the N-terminal of β-integrins and is designated as the PSI domain [167].

Recent studies have show the involvement of Semaphroins in different body organs and systems such as immune, respiratory, and cardiovascular systems, and in pathological disorders, including cancer [170-173]. Semas are regulators of vascular patterning, tissue morphogenesis, and tumor formation and play important roles in the mature immune system [165, 166]. Semaphorins can also influence other cellular processes, such as cell division, differentiation

and survival [173-175]. In cancer, Sema4D, Sema5A and Sema6A promote angiogenesis [176, 177]. In Inflammatory and immune diseases Sema4A is expressed by dendritic cells and required for antigen-specific T cell priming and helper T cell differentiation [178]. Furthermore, in mouse model of contact hypersensitivity, Sema7A knockout mice have reduced immune response, which leads to exacerbated EAE pathology and enhanced delayed-type hypersensitivity responses [178].

1.9.1. Semaphorin receptors

Most semaphorins bind directly to plexins, but various other membrane-associated proteins can also act as receptors or co-receptors like neuropilins[179]. While most class 3 (secreted) semaphorins exert their axon guidance effect through a receptor complex that consists of a Npn ligand-binding subunit and a Plexin signal-transducing element [180].

1.9.1.1. Neuropilin: Two single-pass transmembrane receptors or neuropilins have been

Identified in vertebrates: neuropilin 1 (Nrp1) and Nrp2 [179]. The binding of class 3 semaphorins to plexins is facilitated by neuropilins [181]. Moreovere, neuropilin can act as a core receptor for semaphorins with binding domain located in the a1/a2 domains for instance; Nrp1 serves as a Sema4A receptor in the immune system [182]. The extracellular portion of Nrps contains two repeat complement-binding (CUB) domains (a1 and a2 domains), two coagulation factor-like domains (b1 and b2 domains) and a juxta-membrane meprin/A5/mu-phosphatase (MAM) homology domain (c domain)[183].

1.9.1.2. Plexins: are large 200-kDa transmembrane proteins have been identified in vertebrates

(plexins A1-A4, B1-B3, C1, D1) and two in invertebrates (Plexin A and Plexin B)[184].

Plexins act as substrates for kinases such as (Fes) and Src. Classes 4–7 (membrane-bound) Semaphorins bind directly to and activate Plexin receptors, independently of the presence or absence of Nrp co-receptors [185].

Class 5 and class 6 Semaphorins signal through class A plexins directly whereas, class A plexins activated by secreted class 3 Semaphorins requires neuropilins as co-receptors to stabilize the semaphorin–plexin interaction except sema3E[179]. Class 4 and Class 5 Semaphorins signal through class B plexins, whereas, plexinC1 activated by Sema7A [179]. PlexinD1 serves as class 3 semaphorins receptor in a neuropilin-dependent manner and can bind precisely Sema3E and Sema4A freely of neuropilin[178].

The plexin extracellular region contains several different motifs and domains, including a divergent sema, PSI, and Ig-plexin-transcription (IPT)/glycine-proline (G-P)-rich domains [181]. In addition to the intracellular or cytosolic region contains R-Ras/M-Ras GAP domain, which is separated into two segments by a Rho GTPase-binding domain (RBD). The GAP domains interact with the R-Ras/M-Ras family of small G-proteins, while the RBD interacts with another family of small G proteins that includes Rnd1/Rac1/RhoD. Upon Sema binding, the cytosolic GTPase-activating protein (GAP) domain of plexins is activated and switches off R-Ras and Rap1 small GTPases, which are key stimulators of integrin-dependent cell adhesion to the ECM [181].

Rap1-GTP, via RIAM, activates talin, which in turn binds the integrin subunit cytotail and conformationally activates integrins[186]. R-Ras controls active integrin traffic and Rac GTPase activation [187]. Other receptors mainly for membrane bound Semaphorins are the oncogenic receptor tyrosine kinases, MET and RON [188].

These receptors play a role in cancer metastasis and progression [189]. The extracellular domain of plexins shares sequence similarities with MET and RON receptor tyrosine kinases [181]. MET, ERBB2, VEGFR2, FYN, FES, PYK2 and SRC are cytoplasmic or receptor tyrosine kinases (RTKs) that interact with plexins which lead to the functional activation of the kinase and ultimately to the initiation of distinctive intracellular signaling cascades [190].

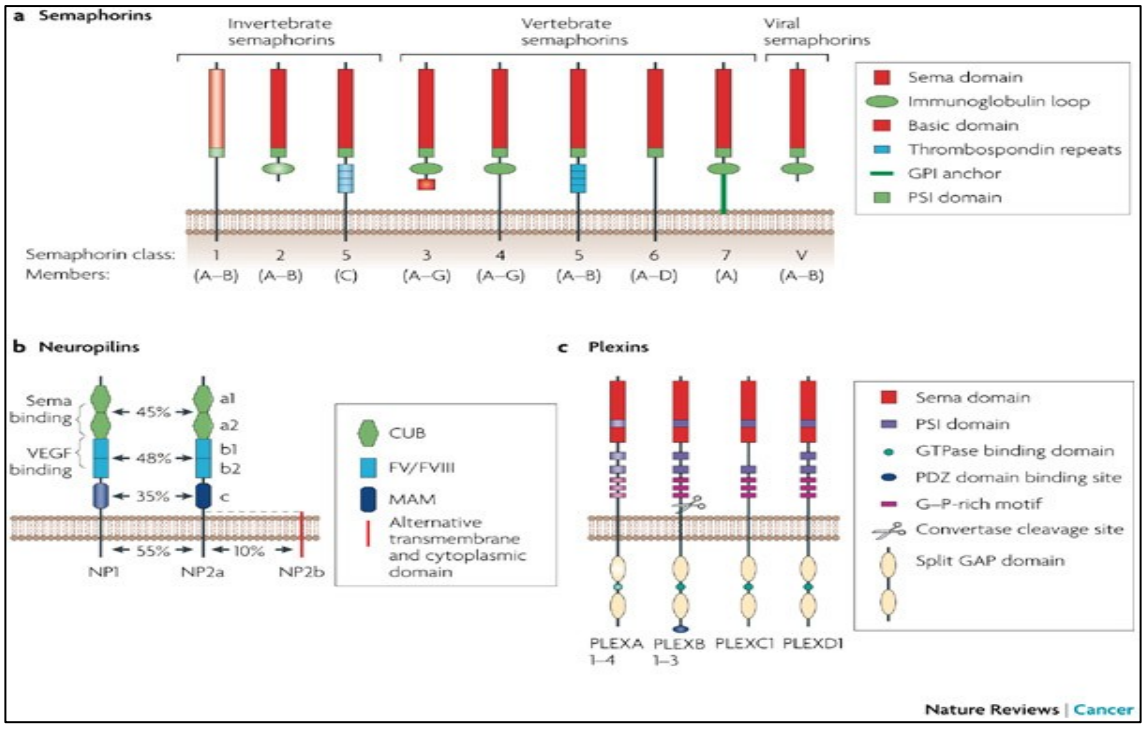


Figure 3**. The structural features of the various classes of semaphorins and the structural features of their neuropilin and plexin receptors.** (Gera et al., 2008)

1.9.2. Class 3 semaphorins

Seven Sema3 (Sema3A–G) are the only secreted vertebrate semaphorins [191]. SEMA3s signal through a receptor complex composed of neuropilin (Nrp) 1 or 2 and type A or D plexin, which act as the ligand-binding and signal-transducing subunit, respectively [192]. Nrps are expressed in both the nerves and the endothelium that serve as Sema3 and VEGF (Vascular Endothelial 2/Flk1/Kdr), respectively [192]. Many studies showed that both plexin-A1 and Nrp1

but neither protein alone, are required to mediate the collapsing effect of Sema3A, which induces collapse of the growth cone via a dramatic rearrangement of filamentous actin (F-actin) and endocytosis of the growth cone plasma membrane [193].

Sema3 is distinguished by the presence of a conserved basic domain at their C-termini [194]. Secreted Sema3 contain (from the *N*- to *C-terminus*) a seven-blade β-propeller sema domain, a plexin–semaphorin–integrin (PSI) domain, an immunoglobulin (Ig) domain and a short basic domain [173]. For class 3 semaphorin function a proteolytic cleavage by furin-like proteases at a C-terminal pro-peptide at RXXXR consensus sites is required [181]. They Initially characterized by their growth cone collapsing properties in the chick dorsal root ganglion, modulating sensory connectivity from the dorsal root, and preventing the ingrowth of axons ventral [168]. Studies have shown that class 3 Semas recognized as key players in immune, cardiovascular, bone metabolism and neurological system [170, 195, 196]. For example Sema3A were first identified for its ability to induce the collapse and paralysis of axonal growth cones from sensory neurons in vitro, also regulates bone through its modulation of sensory innervation during development, and work as immunosuppressive molecule by reducing levels of pro-inflammatory cytokines, such as IFNγ and IL-17, and increasing levels of the anti-inflammatory cytokine IL-10 [197-199]. Another example is SemaE that showed to decrease adipose tissue inflammation and improved insulin resistance in obese mice [200]. Class 3 Semaphorins seem to be important players in the tumor microenvironment along with Sema3A and Sema3F, which has an anti-metastatic effect on cancer cells [173, 178]. Class 3 Semas including Sema3E Sema3A, Sema3B Sema3D and Sema3F induce an anti- angiogenic effect on cells as well [201]. In addition, in several mouse xenograft models Sema3F showed a reduction in cancer cells angiogenesis, tumour growth and

metastasis [178]. Paracrine Sema3 signaling via PlexinD1 guides the anatomical patterning of specific subsets of angiogenic vessels [192].

1.9.2.1 Semaphorin3E

Sema3E is synthesized as a full length 85- to 90-kDa protein binds with high affinity to plexinD[202]. Sema3E gene is located on chromosome 7 [202]. In vivo, Sema3E binds to the intersomitic regions of mouse embryos, where plexin-D1 is expressed [203]. Sema3E cleaved at furin-sensitive sites to generate biologically active isoform of Sema3E p61. Sema3E-p61 is the main species of endogenous Sema3E [204]. This cleavage by furin is exclusively process bioactive form in Sema3E but not in others [202]. Sema3E is well studied in cancer and it is upregulated in metastatic cancer cells [204]. In human colon cancer, liver metastasis, and melanoma progression Sema3E and PlexinD1 are found to be highly expressed [205]. Functionally, Casazza et al. have discovered that Sema3E promoted invasiveness of tumor cells and inhibited tumor growth. This metastatic potential was dependent on PlexinD1 expression but was independent of Nrp expression [206]. Furthermore, it promotes epithelial–mesenchymal transition (EMT) in ovarian cancer [207]. Sema3E mediated repulsion or attraction of axonal guidance neuronal system [208].

1.9.2.2. p61 is the biologically active species of Sema3E

Sema3E-p61 is cleaved by furin, which belongs to pro-protein convertases (PPC) family protien. PPC constitute a family of nine calcium-dependent serine endoproteases. PPCs cleavage site is after the consensus motif RXK/RR [209]. All class-3 semaphorins preserve is a KRRXRR cleavage site and in case of sema3E the cleavage generates aNH_2-terminal

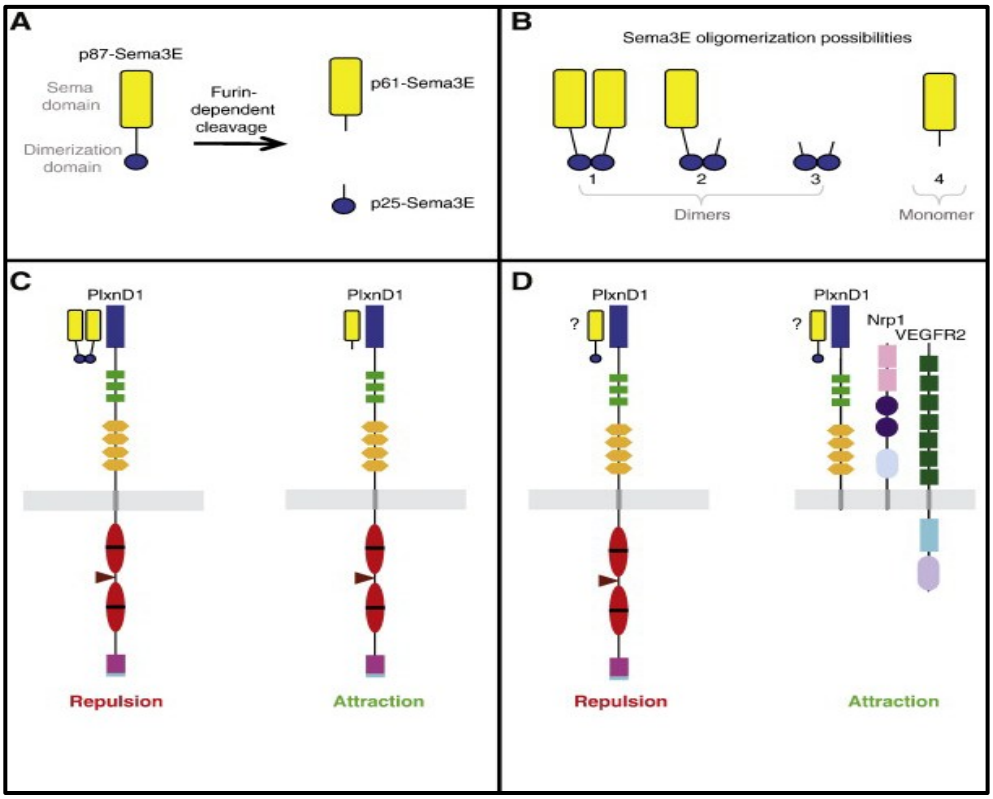


Figure 4. Sema3E p87 KDa cleave into smaller fragment f p61 and p25 KDa by furin - like proprotein convertases (PPC). Sema3/PlxnD1 signaling is capable of promoting attraction and repulsion in both the vascular and nervous systems. The full-length Sema3E (p87-Sema3E) is cleaved by a furin-like endoprotease to yield two products. The longer one contains the Sema domain (p61-Sema3E) while the shorter one contains the dimerization domain (p25-Sema3E)(**A)**. Each isoform perform different function in different context for example full length (p87-Sema3E) in the vascular system dimers induces repulsion via plexinD1 (left)(**B)**. However, the p61-Sema3E monomer containing the Sema domain can function as an attractant in some endothelial lines expressing PlxnD1 (right)**(C**). In the nervous system, Sema3E acts as a repellant when signaling through PlxnD1 alone (left) **(D)**. However, in neurons expressing also Nrp1 and the VEGF receptor VEGFR2, binding of Sema3E elicits an attractive/growth-promoting axonal response (right)(**D**). (Gay et al., 2011)

fragment of ∼60 kDa. PPCs is upregulated in cancer environment with it proteolytic ability can activate protumorigenic factors such as insulin like growth factor-1 and its receptor, transforming growth factor-β; VEGF-C; and metalloproteases such as MT1- MMP. This contributes to by the induction of tumor invasiveness and tumor metastasis [210].

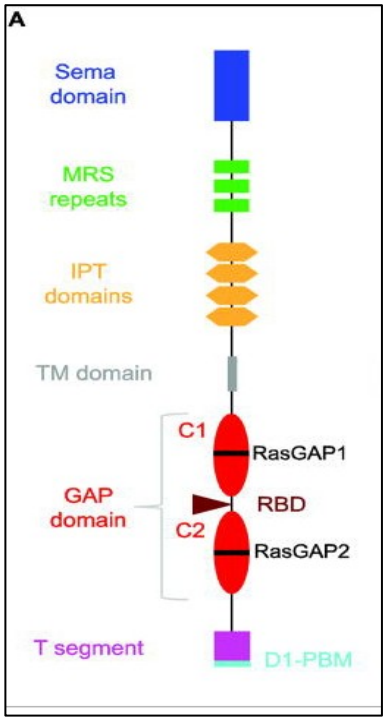
Sema3E-p61 a cleaved from of Sema3E is known to have a prometastatic in cancer and attraction activity in vascular and nervous systems [203, 206]. In endothelial cells monomer p61-Sema3E binding to PlexinD1 function as an attractant molecule [192] .The effect of Sema3E-p61 in cancer is by the transactivation of the oncogenic tyrosine kinase ErbB2 that associates with the Sema3E receptor PlexinD1 [178]. Whereas, the binding of mutated, uncleavable variant of Sema3E (Uncl-Sema3E) to PlexinD1 does not promote metastatics preading [206]. Moreover the dimerization of full-length Sema3E, in the vascular system induces repulsion via PlexinD1 [178]. Taken together, these data indicate that different isoforms of Sema3E play different role in different cells and organs.

1.9.2.3. Sema3E binds PlexinD1 but is Nrp independent

PlexinD1 is a member of plexins family and the only receptor found in vertebrates a key player in vascular, neuronal and immune system development implicated the etiology of congenital defects and cancer [192, 203, 211, 212]. It is expressed in many embryonic tissues in particular in endothelial cells of the developing vasculature [213]. As mentioned earlier in plexins, plexinD1 share all similar domains and motifs generally with other Plexins. The stretch of ectodomain of plexins sized (~860–1400 aa) consists of sema domain, three cysteine-rich motifs MRS repeats, and four IPT domains. The sema domain composed of ~500 aa, and constitute the first amino-terminal.

Topologically, the sema domain is similar to integrins and which displays a seven-bladed *Beta*-propeller structure.

The second is comprised of three cysteine-rich motifs called MET-Related Sequences (MRS repeats or PSI-Plexins, Semas and Integrins- domains) consisting of ~50 aa long each. PlexinD1's is distinctive among all plexins in the third MRS it has six, rather than eight, cysteines. The function of the MRS repeats is unknown. Finally, the last stretch of the PlexinD1 ectodomain contains four IPT domains (Immunoglobin-like fold shared by Plexins and Transcription factors domains; also found in MET family members) that contain glycine and proline rich repeats [179, 192].



igure 5. PlexinD1 structure. PlexinD1 contains a Sema domain (blue), three MRS (MET-Related Sequence) repeats (green), four IPT (Immunoglobin-like fold shared by Plexins and Transcription factors) domains (orange) and the transmembrane domain (brown). The cytosolic tail of PlxnD1 is also known as the Sex and Plexins (SP) domain. It contains a split GAP (GTPase Activating Protein) domain with two highly conserved C regions (C1 and C2; red). Each C region contains a Ras GAP motif (RasGAP1 and RasGAP2; black), each of which includes conserved arginine residues. A Rho GTPase-binding domain (RBD, beige) is located between the C1 and C2 regions. Finally, a short C-terminal region follows the GAP domain. the terminal (T) segment (pink). The T segment of PlxnD1 ends in a short PDZ- binding motif (D1-PBM; aqua) that physically associates with GIPC1. (Gay et al., 2011)

1.9.2.4. Sema3E/plexinD1network

Sema3E/PlexinD1 signaling plays many roles in body organs and systems particularly, the development of the cardiovascular, skeletal and nervous systems. Sema3E-plexinD1 signaling modulates angiogenic pathfinding by restricting this network restrict the migration of neuronal growth cones navigation pathways [210]. Moreover, knockout mouse embryos lacking either plexinD1 or Sema3E activity show dramatic defects in segmental Arteries development, such as premature and ectopic sprouting, aberrant pathfinding accompanied by the formation of abnormally long filopodia and improper branching [188]. In vascular and nervous systems Sema3E/plexinD1 interacting induce attraction and repulsion [188]. Sema3E/plexinD1 grid in mediates a neuronal system repulsive activity. However, it showed the reverse function in neurons when Sema3E facilitate its binding to plexinD1 by Nrp1 and the VEGF receptor VEGFR2. This attractive response requires only the extracellular domains of PlexinD1 and Nrp1 and VEGFR2's intracellular tail is required as the signal transducing subunit [210].

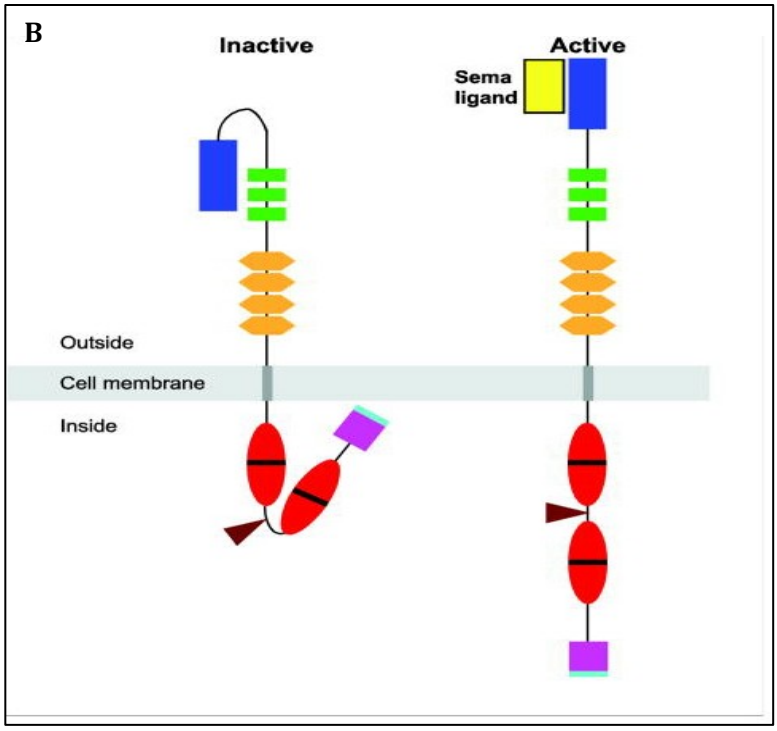


Figure 6. Sema3E signal through PlexinD1. Activation model of PlexinD1. In the absence of its Sema ligands, PlexinD1 is in a conformationally inactive folded state, in which the Sema domain contacts the rest of the extracellular portion and the GAP domain is non-functional. Upon Sema binding PlexinD1 undergoes conformational changes that activate its GAP domain and likely enable additional perotein-protein interactions **(B).** (Gay et al., 2011)

As much as this network of sema3E/plexinD1 play a huge role in health it also mediate many diseases. CHARGE syndrome caused by impaired Sema3E-PlexinD1 signaling [188]. Furthermore, in cancer cells reducing the level of Sema3E or PlexinD1 impair their metastasis without affecting tumor growth [211]. Whereas, in colon cancer patients, high levels of Sema3E and PlexinD1 are associated with prometastatic and invasiveness of cancer cells [200].

1.9.2.5. Sema3E/PlexinD1 signaling

PlexinD1 intracellular tail contains two highly conserved intracellular domains known as the SEX-PLEXIN domain and SEMA/PLEXIN domains signal in to the cells [214]. SEMA/PLEXIN domains of plexinD1 include two C regions RasGAP domain plexinD1. Each of RasGAP domains includes a short motif of (GTPase)-*A*ctivating *P*roteins (GAPs) and monomeric GTPases of the R-Ras subfamily.

A monomeric Rho GTPase-Binding Domain (RBD) is sandwiched between the C regions. PlexinD1 acts as a RasGAP to antagonize both integrin-mediated cell extracellular matrix (ECM) adhesion and PI3K a modulator of cell survival, growth and migration signaling. Rho family GTPase 2 (Rnd2) is required for the activation of the RasGAP activity of plexinD1. P61-Sema3E requires both plexinD1 and Rnd2 to exert its inhibition of tumor vascularization [192]. Upon Sema3E-PlexinD1 stimulation in pre-existing PlexinD1-Rnd2/RLG complexes undergoes an Rnd2/RLG-dependent intracellular conformation change that translates the concentration and distribution of extracellular Sema3E cues into an intracellular gradient of distinct PlexinD1 activities [192].

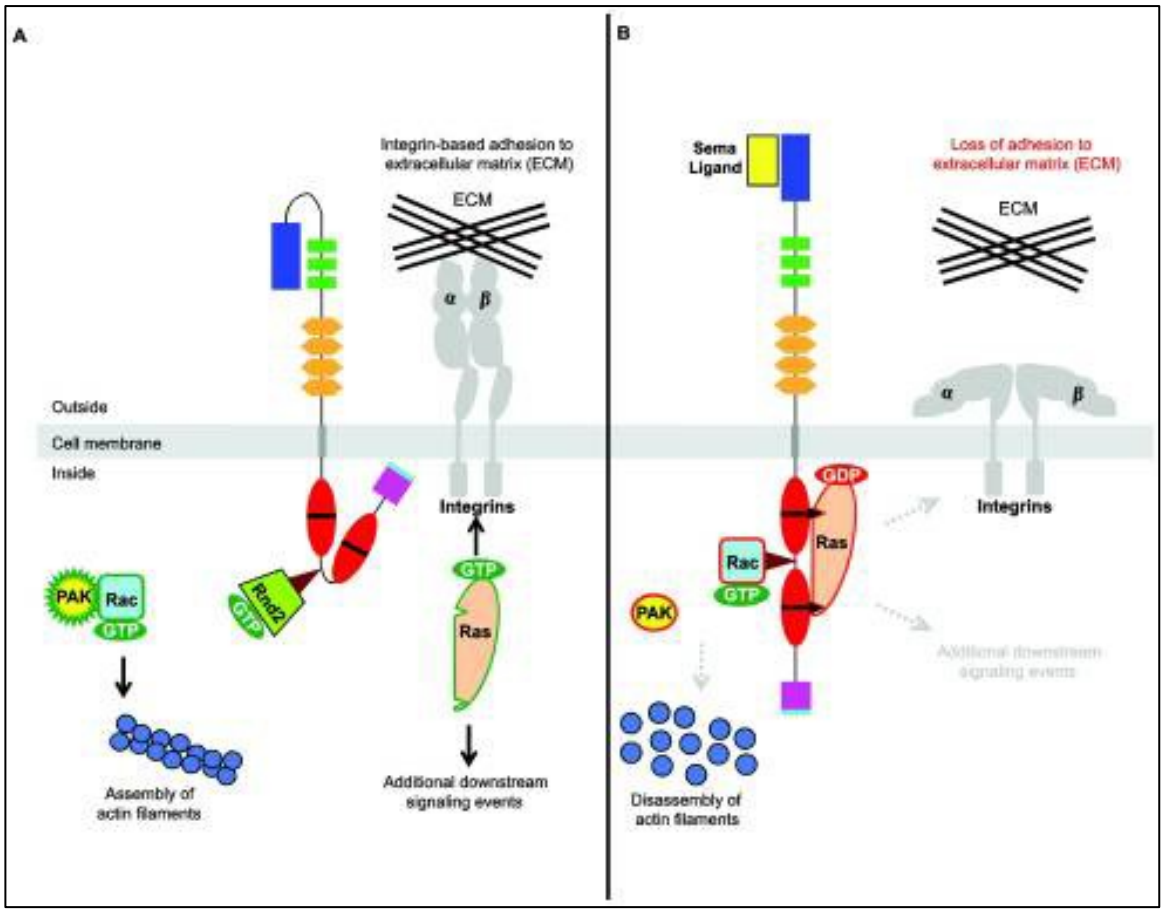


Figure 6. A general model of Sema3/PlexinD1-mediated repulsion. In the absence of ligand-mediated activation PlexinD1 is in a conformation that enables its association with GTP- bound Rnd2 but prevents its interaction with active, GTP-bound Rac and R-Ras. Thus, GTP- bound Rac is able to bind to PAK (p21-activated kinase) to stimulate the assembly of actin filaments to support cell migration while active GTP-bound Ras promotes integrin-mediated adhesion to the extracellular matrix (ECM) and mediates additional downstream signaling events(**A)**. Upon binding of its Sema3 ligand, PleixnD1 undergoes a conformational change and binds the active forms of both Rac and R-Ras GTPases. By sequestering Rac, PlexinD1 leads to the inactivation of PAK and the collapse of the actin-based cytoskeleton leading to retraction and/or turning responses **(B)**. PlexinD1 inactivates R-Ras GTPases by either enhancing GTP hydrolysis (as shown) or by sequestering them resulting in the loss of integrin-based adhesion to the ECM and likely reducing as well other R-Ras mediated signaling events (Ito et al., 2006; Oinuma et al., 2004a; Oinuma et al., 2006; Rohm et al., 2000; Sakurai et al., 2010; Uesugi et al., 2008). (Gay et al., 2011)

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1. ETHICS STATEMENT

All the experimental procedures were approved by the Human Research Ethics Board of the University of Manitoba, Winnipeg, Manitoba, Canada. Written informed consent for ASM harvesting was obtained from all patients.

2.1. Reagents

2.2.1. *Chemicals*

Recombinant human Semaphorin3E, and platelet-derived growth factor-BB (PDGF-BB) were purchased from R&D Systems. Furin inhibitor, cathepsins G, neutrophil elastase were purchased from Calbiochem®. Fetal bovine serum (FBS), and sodium pyruvate were purchased from HyClone (Logan, UT, USA). 100X L-glutamine, Dulbecco's modified Eagles medium (DMEM), Ham's F-12, trypsin-EDTA, (penicillin streptomycin) were purchased from Invitrogen Canada Inc.(Burlington, ON, Canada). Alkaline phosphatase-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa). Unless stated otherwise, all other reagents were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada)

2.2.2. *Antibodies*

Monoclonal mouse IgG1anti human Semaphorin3E antibody, allophycocyanin (APC)-conjugated mouse anti-human plexinD1 monoclonal antibody, isotype control mouse IgG2b , goat anti-human plexinD1 affinity purified polyclonal antibody, polyclonal goat IgG

human/mouse semaphorin 3E antibody, phycoerythrin (PF)-conjugated mouse monoclonal anti human neuropilin-1, and the isotype control mouse IgG2A , were purchased from R&D Systems. Sema3E goat unlabeled and APC-conjugated isotype control antibodies were obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada) and eBiosciences (San Diego, Calif) respectively.

2.2. PREPARATION OF HUMAN AIRWAY SMOOTH MUSCLE CELLS

Two sources of human airway cells, namely bronchial smooth muscle (BSM) and human telomerase reverse transcriptase (hTERT) immortalized bronchial smooth muscle cells were prepared as we previously described [215]. In this study, these cells collectively will be referred to Human airway smooth muscle cells (HASMC for human ASM). These cells were obtained from macroscopically healthy segments of second to fourth generation main bronchus of patients suffered from lung adenocarcinoma and undergoing surgery (Dr. H Unruh, Section of Thoracic Surgery, University of Manitoba, Canada) [215].

To extend the life span of these cells, primary low-passage cultures of BSM were infected with a retrovirus vector encoding the (hTERT) gene. A plasmid (pGRN145) containing hTERT cDNA expression vector was a gift from Geron (Menlo Park, CA)[215]. The hTERT expression cassette was cloned into PLXIN (Clontech), and replication-incompetent moloney murine leukemia virus retrovirus was generated in HEK293 retroviral packaging cells.

Primary and first-passage cultures of human airway smooth muscle cells were infected with the hTERT retrovirus and selected with 100 mg/ml G418 for 1wk. Expression of hTERT was verified in immortalized cells by RT-PCR using telomerase-specific primers. Immortalized

cells were passaged (4:1 dilution) up to 60 times with no evidence of senescence [216, 217]. The expression of human telomerase reverse transcriptase (hTERT) is known to extend the life span of endothelial cells, fibroblasts, and smooth muscle cells [216-218]. HASMCs grow in cell culture to 70% confluence, the cells take the spindle-shaped morphology and a hill- and-valley pattern and retain smooth muscle-specific actin, SM22, and calponin protein expression and mobilize intracellular Ca2+ in response to acetylcholine, a physiologically relevant contractile agonist [215]. In all the experiments, primary ASM cells were utilized at passages 2-5, and hTERT cells at passages 10-20.

2.3.1. *Culture of HASMCs*

Low passage HASMCs were grown on uncoated plastic dishes in complete Dulbecco modified Eagle medium (supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% fetal bovine serum). Unless otherwise mentioned, cells were grown to a subconfluent (~60%-70%) condition and serum starved to synchronize growth-arrest at G_0/G_1 phase for 2 days in Ham's F12 supplemented with 1× ITS (5 μg/mL insulin, 5 μg/mL transferrin, and 5ng/mL selenium), 100 μg/mL streptomycin, and 100 U/mL penicillin and before each experiment.

2.3.2. *Cell Stimulation*

Cells were then stimulated in fresh FBS-free Ham's F12 medium containing human recombinant sema3E 10ng/ml, PDGF-BB 10ng/ml, furin inhibitor 80umol/L working concentration or vehicle (medium alone) for time periods specific to each experiment.

2.4. RNA ISOLATION AND RT-PCR

48 hour serum-deprived confluent ASM cell cultures were harvested, and total cellular RNA was extracted using TRIzol® method (Invitrogen Canada Inc., Burlington, ON). Briefly, 5-10 $X10^6$ cells were homogenized/lysed in 1ml TRIzol ® reagent on ice; organicaqueous phase separation was performed by adding 0.2 ml chloroform per 1ml TRIzol ® and shaking vigorously at room temperature (RT) and centrifugation at 12000 g for 15 minutes at 4°C. The aqueous phase was separated into a fresh R-Nase-free tube and 0.5 ml isopropyl alcohol per 1ml TRIzol ® was added before incubation at RT for 10 minutes and centrifugation at 12000 xg for 10 minutes. Supernatant was discarded; the RNA pellet was washed with 1ml of 75% alcohol per 1 ml of TRIzol® vortexed once, and centrifuged at 7500 xg for 5 minutes at 4^oC. Supernatant was discarded; the RNA pellet was air-dried and resuspended in 30-50 µl of RNase-free water. Total RNA was with NanoDrop (Thermo Scientific, Wilmington, DE, USA) instrument.

Reverse transcription was performed by using 2 µg of total RNA in a first-strand cDNA synthesis reaction with High Capacity cDNA Reverse transcriptase kit as recommended by the supplier (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers were synthesized on the basis of the entirecoding region of human genes *SEMA3E, PLXIND1, NRP1* or *GAPDH*. The RT-PCR was conducted in a thermal cycler "Mastercycler" (Eppendorf Canada, Mississauga, ON). Each cycle included denaturation (94°C, 1min) annealing (primer-specific temperature °C, 1min) and extension (72°C, 1min30s). The initial denaturation period was 5 min, and the final extension was 10 min.

Oligonucleotide primers were synthesized on the basis of the entire coding region of human SEMA3E as follows: Forward primer 5'- AAAGCATCCCCAACAAACTG-3' and Reverse primer 5'- CTGGCTCGAGACCCTTACTG-3'and for human PLXIND1 forward primer 5'-

TGGATGTCGCAGCTTACTTG-3' and reverse primer 5'- CCCCAACCCACAGTTCTCTA-3' for human Nrp-1 primers were synthesized forward primer 5'- TATTCCCAGAACTCTGCCC-3' and reverse primer 5'- TGTCATCCACAGCAATCCCA-3' human housekeeping gene, glyceraldhyde-3-phosphate dehydrogenase (GAPDH) are forward primer 5'-AGCAATGCCTCCTGCACCACCAAC-3' and reverse primer 5'-CCGGAGGGGCCATCCACAGTCT-3' were manufactured from Invitrogen. Real-time levels of RNA were assessed by quantitative PCR using ABI 7500 Real-Time PCR System and analyzed by 7500 System SDS software version 1.3.1(Applied Biosystems, Foster City, CA, USA). Product specificity was determined by melting curve analysis. Calculation of the relative amount of each cDNA species was determined by delta-delta cycle threshold (ddCt) method. The amplification of target genes was normalized to respective GAPDH levels.

2.5. WESTERN BLOTTING

Protein concentrations were quantified by bicinchoninic acid assay (BCA) for determining the total concentration of protein in a solution. For immunoblotting we loaded 8ug of lysates from each sample was separated on 7% SDS polyacrylamide gel and electro-transferred onto PVDF membrane (Amersham Pharmacia, ON). The membrane was blocked for 2 hours at room temprature with 5% skim milk, after that incubated overnight at 4 °C with polyclonal goat anti human semaphorin 3E antibody (R&D systems) as primary antibody, followed by the secondary antibody (rabbit anti goat). All antibodies were diluted in 5% skim milk primary antibody dilution factor (1:2000) and the secondary (1:5000). All the blots were developed by enhanced chemiluminescence as recommended by the supplier (Amersham Pharmacia, ON). A band non-specific to sema3E in the same gel was used as loading control.

2.5.1. *Preparation of cell supernatants*

The sups were collected and centrifuged at 1200rpm for 10 min at 4°C to remove cellular debris. Then the sample was concentrated using the concentrated using Amicon ultra .5 ml centrifugal filter. This devise allowed eliminating all material less than 10kDa (10k device), the sups were concentrated 14 folds leading to 35ul of final volume.

2.5.2. *Digestion of recombinant sema3E*

Recombinant sema3e (R3E) was digested using serine proteases and NE: in brief 10ng of R3E in F12/ITS medium was supplemented with50ug of neutrophil elastase or cathapsin G and incubated in 37°C water bath for 30 min (total volume 15ul), 3ul of loading buffer dye was then added to the mix and denatured at 100 °C for 10 min

2.5.3. *Preparation of cell lysate*

HASM cells were cultured and serum starved for 48 hours then lysed on ice for 10 min by adding the 100ul of M-PER lysis buffer (Thermo Scinitific) supplemented with a cocktail of protease inhibitors contains: AEBSF, Bestatin, E-64, Pepstatin A, Phosphoramidon, Leupeptin, Aprotinin with addition of more proteases inhibitor PMSF and Sodium orthovanadate (Sigma-Aldrich) and centrifuged for 10 min to collect protein lysate and discard the nuclei pellet.

2.6. EdU cell proliferation assay

Serum-deprived HASMCs were stimulated with the aforementioned concentrations of Sema3E with or without PDGF. Click-iT EdU flow cytometric assay kit (Invitrogen) was used to further investigate the Sema3E effect on HASMC proliferation. Briefly, EdU reagent was added at a 10-

μmol/L concentration 40 hours after stimulation, and cells were harvested 8 hours later. Cells were collected, fixed with 4% PAF for 30 min in room temperature, and then incubated with saponin-based permeabilization buffer provided with the kit. Click-iT reaction cocktail containing copper sulfate and fluorescent dye azide (Alexa Fluor 488) was freshly prepared and added to the samples. Cells were washed by saponin buffer (1ml) and recollected by centrifugation at room temperature at 1200 rpm for 5 min. EdU incorporation into DNA was assessed with flow cytometry. Analysis was done using flow jo single cell analysis software (FLOW JO, LLC data analysis software). Data was presented as percentage of EDU positive cells

2.7. Flow cytometric analysis of receptor expression

Primary cultured confluent HASMCs were detached with Versene (0.02% EDTA; Lonza, Walkersville, Md) without trypsinization. The single cell suspensions (1×10^5 cells) were separately incubated with APC-conjugated mouse anti-human plexinD1 or isotype control Ab (IgG2b subclass) for 30 minutes at 4°C. The cells were washed twice with flow buffer, resuspended in PBS containing 0.1% BSA, and analyzed on FACScan (BD Biosciences, San Jose, Calif). FACS analysis was performed by FlowJo software (Tree Star, Ashland, Ore).

2.8. Cell fractionation assay

Half million HASM cells from COPD with different severity were cultured in 110mm petri dishes until reaching 75 % confluence. Cells were then serum deprived for 48 h then harvested using fractionation buffer which contains; 1 x solution of 250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA. Cells scraped from plates immediately

and placed in 1.5 ml eppendorf tube. And passed lysate through a 25 G needle 10 times using a 1 ml syringe and left on ice for 20 min then centrifuge for 5min for 4°C at 720 G (3000 rpm) to get the nuclear pellet. Nuclear pellet then washed once by adding 500 µl of fractionation buffer again. The supernatants taken from the first centrifugation constitute the cytosolic and membrane fractions. This fraction was centrifuge again at 8000 rpm (10,000 G) to obtain the cytosolic fraction. We then concentrate the supernatant by centrifuging through the filter unit.

2.9. Immunohistochemistry

Bronchial biopsies were obtained from COPD patients characterized clinically according to GOLD criteria see table 1. Briefly, formalin-fixed tissues were paraffin embedded, and 5-µm-thick sections were prepared, deparaffinized in xylene, and rehydrated through graded concentrations of alcohol to water and then boiled with microwave for 10 min in sodium citrate buffer (pH 6.0). Sections were washed and then incubated with blocking solution (1% BSA and 0.1% cold fish skin gelatin in TBS) for 60 min at room temperature. Monoclonal mouse IgG_1anti human semaphorin 3E, mouse anti-human plexinD1 monoclonal antibodies, (both at 10 µg/ml) were added, and sections were incubated overnight at 4°C. Slides were then washed twice with TBS, followed by incubation for 1 hour at room temperature with biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were then washed extensively with PBS and incubated with streptavidin-alkaline phosphatase for 30 min at room temperature. After washing with TBS, the slides were developed using Fast Red and counterstained with Mayer's hematoxylin. Positive cells were stained red after development with Fast Red (Sigma-Aldrich Canada). Isotype-matched control mAb was used for negative control.

2.10. Statistical Analysis

Statistical analysis was performed by using GraphPad Prism Software Version 3.02 for Windows (GraphPad Software Software, San Diego, CA, USA). Unpaired t test, Mann-Whitney test were used and differences were considered to be statistically significant at $*P < .05$, $**P \leq .01$, and $***P \leq .001$.

Table 1

Clinical characteristics of bronchial sections isolated from COPD patients

Patient (GOLD Classification)	Age	Gender	Smoking status	FEV1%	FEV1/FVC%
Non-COPD	76	Female	Quit 5 years ago	87%	74%
Non-COPD	81	Male	Quit 20 years ago	100%	84%
Non-COPD	80	Male	Quit 40 years ago	114%	74%
Non-COPD	80	Female	Never smoke	104%	80%
Mild COPD	60	Female	Quit one week	90%	69%
Mild COPD	73	Male	Smoker	88%	68%
Mild COPD	76	Female	Smoker	83%	67%
Moderate COPD	75	Male	Smoker	71%	71%

Table 2

Clinical profile of COPD patients used for bronchial HASMC isolation

Subjects/patients	GOLD classification	Age	Gender	Smoking Status	FEV1 %	FEV/FVC%
Subject 1	Non-COPD	78	Female	None	113%	73%
Subject 2	Non-COPD	64	Male	Quit 30 years ago	103%	75%
Subject 3	Non-COPD	65	Female	Quit 8 years ago	87%	76%
Subject 4	Non-COPD	63	Female	Quit 25 years ago	108%	74%
Subject 5	Non-COPD	57	Female	Quit 10 years ago	108%	84%
Subject 6	Non-COPD	63	Male	Quit 15 years ago	72%	75%
Subject 7	Non-COPD	56	Female	Quit 4 years ago	111%	93%
Subject 8	Non-COPD	65	Male	Quit 21 years ago	86%	72%
Subject 9	Non-COPD	67	Male	Smoker	86%	81%
Patient 1	Mild-COPD	65	Male	Quit 21 months ago	92%	70%
Patient 2	Mild-COPD	73	Male	Smoker	88%	68%
Patient 3	Mild-COPD	78	Male	Quit 30 months ago	83%	55%

Patient 4	Moderate-COPD	79	Male	Quit 38 months ago	78%	68%
Patient 5	Moderate-COPD	76	Male	Smoker	65%	65%
Patient 6	Moderate-COPD	62	Male	Smoker	72%	58%
Patient 7	Severe-COPD	59	Male	Smoker	49%	52%
Patient 8	Severe-COPD	67	Male	Smoker	44%	37%
Patient 9	Severe-COPD	69	Male	Smoker	47%	50%

CHAPTER 3

3.1. STUDY RATIONALE

COPD is the third leading cause of death according to world health organization. It represents a significant cause of morbidity as well, with a huge economic burden. Among those with a smoking history the prevalence of COPD in Canada in different provinces was estimated to be between19% and 21% of aged 40 or older. Clinically, symptoms are controlled in COPD patients but the main cause left untreated. One of the main features of the disease is the airway remodeling or structural changes. Airway smooth muscle (ASM) cells contribute to the remodeling when they increase in mass causing flow obstruction in the lungs. Numerous studies have clearly shown that ASM cells are capable of releasing and responding to inflammatory mediators. Therefore, determining the factors that can modulate cells proliferation, which is linked to the degree of airflow limitation, is an essential process in resolving the disease. Although airway wall thickness by ASM cells in COPD took the spotlight for a while, the current therapies on airway wall remodeling are unknown.

Sema3E was shown to inhibit endothelial cells proliferation via mechanisms that involve R-Ras and Arf6 activation. Very recently, study from our lab has shown that Sema3E inhibits ASM cells proliferation mediated by a growth factor PDGF-B in the context of asthma (Movassagh H et al, JACI, 2014). Taking into account that COPD and asthma share pathological and physiological characteristics, we decided to investigate the effect of Seama3E on ASM from COPD patients.

3.2. Global hypothesis

Our hypothesis is that Sema3E inhibits human airway smooth muscle cell proliferation in *COPD.*

3.3. AIMS

The overall goal of this study is to investigate the effect of a recombinant Semaphorin 3E on airway smooth muscle proliferation mediated by growth factor PDGF-BB. To achieve this goal we have to investigate three aims; first, investigate the expression of plexinD1 receptor in HASM cells from COPD in vitro; and in vivo in COPD lung sections. Second, evaluate the effect of Sema3E on HASM cells proliferation. Third, evaluate the expression of Sema3E in HASM cells from COPD patients at mRNA and protein levels.

CHAPTER 4: RESULTS

4.1. HASMCs from COPD patient displayed enhanced mRNA expression of sema3E receptor, PlexinD1 compared to cells isolated from healthy subjects.

Previous studies have shown that plexinD1is expressed in many organs including heart, brain, lung, kidney, and testis [211]. Semaphorin 3E (Sema3E) binds plexinD1 with high affinity in vascular system [219]. Moreover, our laboratory has recently demonstrated an enhanced expression of PlexinD1 on human airway smooth muscle cells from healthy subjects compared to allergic asthmatic patients *in vitro* [220]. To find out if Sema3E can also affect human airway smooth muscle cells (HASMCs) function in COPD, we first determined the expression of plexinD1 at mRNA and protein level in human airway smooth muscle cells obtained from COPD in comparison to those from healthy subjects. Nine HASMCs from COPD and healthy subjects were cultured in complete medium (DMEM, 10% FBS) for

24hrs. The clinical characteristics of COPD and healthy subjects are described in Table 1. mRNA was then purified using Trizol method and equal amount of mRNA was reverse transcribed using Superscript reverse transcriptase enzyme. There after, the complementary DNA (cDNA) was subjected to amplification using Taq polymerase in the presence of Syber green dye and specific oligonucleotides for *PLEXND1* or *GAPDH* gene respectively as described in the realtime PCR procedure (materials and methods). GAPDH is a housekeeping gene that served as our reference gene. Human Universal Reference Total cDNA (Clontech, Calif) was used as a positive control in all RT-PCR experiments, and no cDNA served as negative control. As depicted in figure (8), mRNA for plexinD1 was highly expressed in primary HASMCs from nine COPD patients compared to normal subjects ($P<0.05$, unpaired t test).

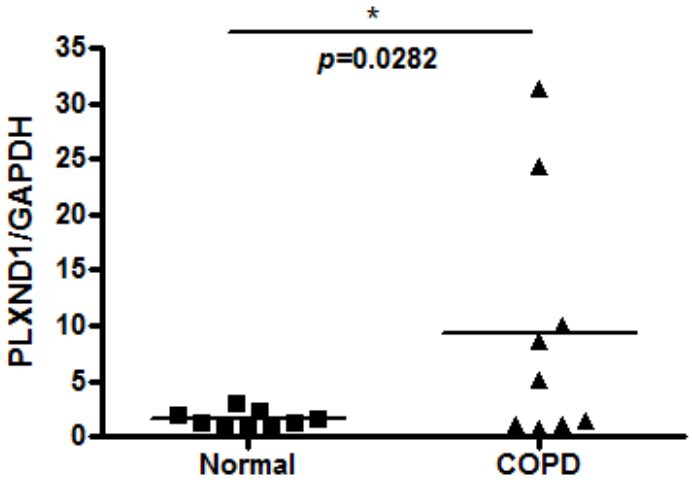
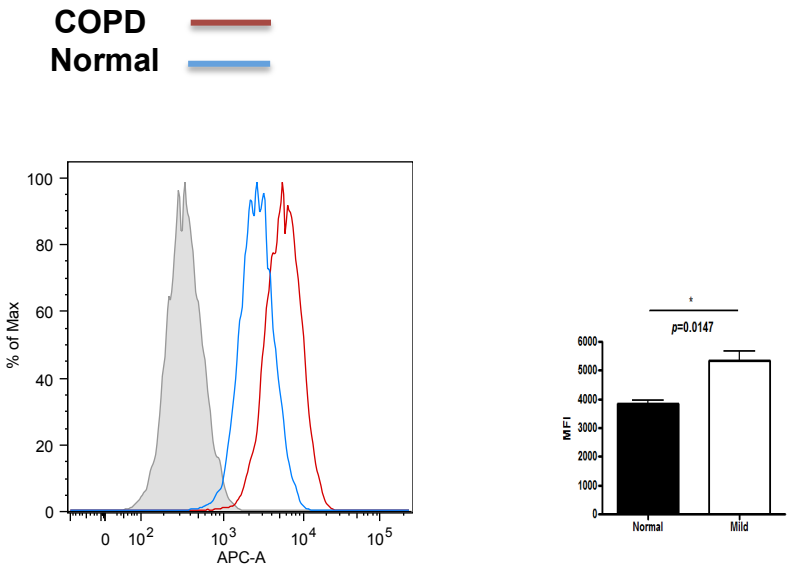


Figure 8. mRNA expression of plexinD1 on HASMCs obtained from COPD patients and normal donors. Expression of plexinD1 on primary HASMCs was examined by Real time-PCR with the use of specific primers. RNA level studied was performed on 9 different HASMCs under the same conditions. *GAPDH*, glyceraldehyde phosphate dehydrogenase gene; *PLXND1*, plexinD1 gene, $P<0.05$ unpaired t-test

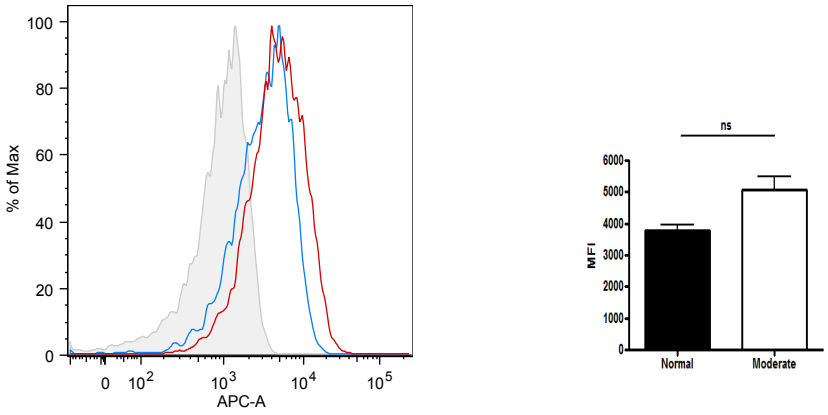
4.2. HASMCs from COPD patient displayed surface expression of PlexinD1

To detect the surface expression of plexinD1 in HASMCs flow cytometry was performed. Cells were first detached using EDTA, incubated with with FC blocker for 30 min at 4°C (obstruct non-specific antibody binding) and stained with specific mAb directed against human plexinD1conjugated with Allophycocyanin (APC) for 2hrs at 4°C with gentle rotation. As showed in Figure (9), as for cells isolated from normal subjects, plexinD1 surface expression was also detected on HASMCs from COPD patients (mild, moderate and severe). Although the data is very preliminary due to the limited subjects performed, it is noteworthy that the more severe the disease is, the less plexinD1 surface receptor is expressed on HASMCs.

A


COPD
Normal
% of Max
APC-A
*
p=0.0147
MFI
Normal
Mild

B


% of Max
APC-A
ns
MFI
Normal
Moderate

C

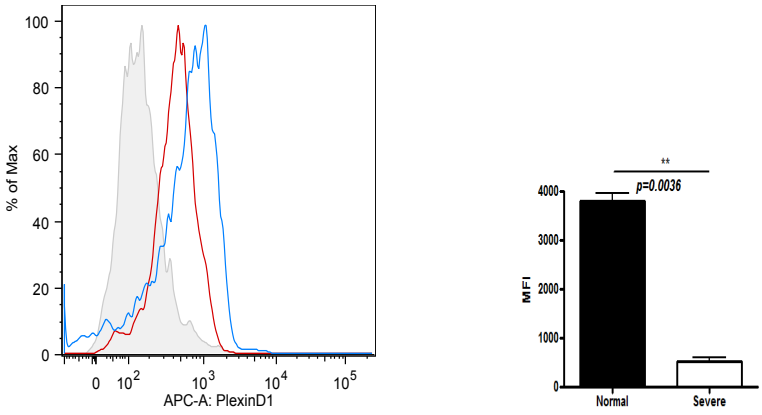


Figure 9. Surface protein expression of PlexinD1 on HASMCs compare between COPD patients and normal donors. Protein expression studies were performed by flow cytometry on HASMCs from COPD patients (mild, moderate and severe) (n=2) and normal subjects (n=2) under the same conditions. HASMCs were incubated with anti human plexinD1 (mouse IgG_1) APC conjugated. The isotype control mouse IgG_{2b} showed no immunoreactivity. The MFI was calculated by subtracting the isotype control. The graph represents the MFI average for each disease severity compared to normal. Data represents the mean ±SD of three separate experiments. Mann-Whitney *U* test was performed to analyze the differences between the samples. **P*<0.05, ***P*<0.01 compared to unstimulated control. *APC*, Allophycocyanin

4.3. PlexinD1 is expressed ex vivo in lung sections of COPD patients

Furthermore, an *ex vivo* study confirmed PlexinD1 expression in COPD bronchial sections. Table 2, summarized the clinical characteristics of COPD patients undergo bronchoscopy. Using immunohistochmistry, we showed specific plexinD1 immunoreactivity in ASM bundle within the lung of normal and COPD subjects (figure (10) a and c; red staining). No immunoreactivity could be detected with the isotype control (figure (10) b and d).

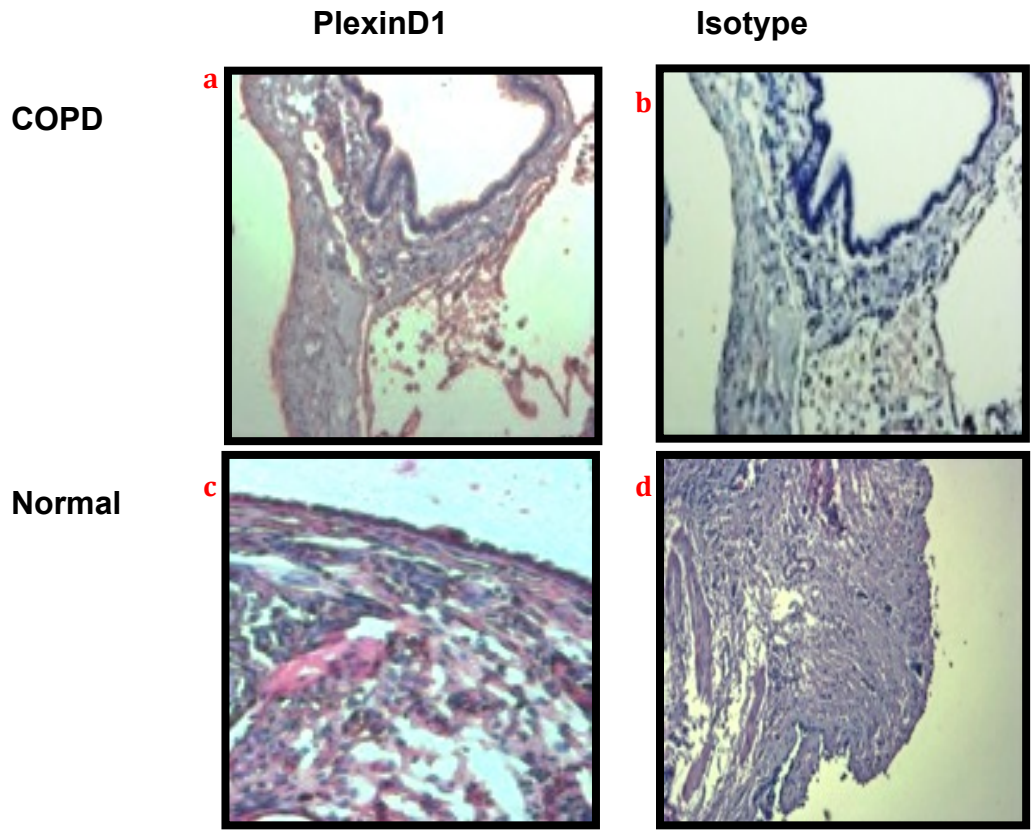


Figure 10. **Immunohistochemical analysis of plexinD1 expression on HASMCs**. Bronchial sections of mild COPD patient and normal subject were stained with goat anti human PlexinD1 antibody followed by biotin conjugated rabbit anti goat and streptavidin alkaline phosphatase and visualized by Fast red. Magnification at ×100 ×200 and ×400 (n = 5) No immunoreactivity could be observed with goat IgG primary antibody used as the isotype control (b and d)

4.4. Recombinant Sema3E inhibits PDGF-induced HASMCs proliferation in normal subjects but not in COPD

The effect of Sema3E on proliferation has been studied within the endothelial cells [202, 204, 221]. These studies demonstrated an inhibitory effect of Sema3E on endothelial cells proliferation [204]. Specifically, Sema3E worked as an anti-angiogenic agonist by inhibiting cell growth and tube formation and by suppressing the vascular endothelial growth factor (VEGF) signaling pathway [222]. More recently, our laboratory showed that Sema3E was able to inhibit growth factor (PDGF and EFG) induced proliferation and migration of primary bronchial HASMCs isolated from allergic asthmatic patients and healthy donors [223].

Asthma and COPD are hypothesized as different expressions of a single disease. According to Global Initiative for Chronic Obstructive Lung Disease GOLD, the two diseases share some similarity particularly clinical symptoms (e.g. obstruction) and or modes of treatment (e.g. steroids) to certain extent. However, asthma symptoms are reversible except the severe form compared to COPD[224]. Along with these facts that COPD and asthma share pathological similarities, we sought to investigate the role of Sema3E on HASMCs isolated from COPD. Our hypothesis is that Sema3E inhibit PDGF induced proliferation of HASMCs obtained from COPD patients. The aim of this part was to investigate the role of recombinant Sema3E on PDGF induced proliferation in HASMCs from COPD patients and healthy donors.

Serum deprived HASMCs isolated from COPD cells were treated with 10ng/mL of recombinant Sema3E in the presence or absence of PDGF-BB (10 ng/mL) for 40 hrs. A fluorescent-labeled thymidine analogue, 5-ethynyl-2′-deoxyuridine was added at 16 hrs and cells harvested at 24hrs from stimulation. Then, incorporation of a fluorescent-labeled thymidine analogue, 5-ethynyl-2′-

deoxyuridine (EdU), into newly synthesized DNA was measured as an indicator of proliferation by flow cytometry.

As showed in figure (11), Sema3E inhibited PDGF-induced proliferation in human telomerase reverse transcriptase (Htert) –immortalized bronchial HASMCs (Figure 11a). However, there was no effect of Sema3E on HASMCs cells isolated from COPD patients mild (Figure11b), moderate (Figure 11c) or severe (Figure 11d) (see Table 2 for clinical characteristics of COPD subjects in whom their HASMC proliferation was studied).

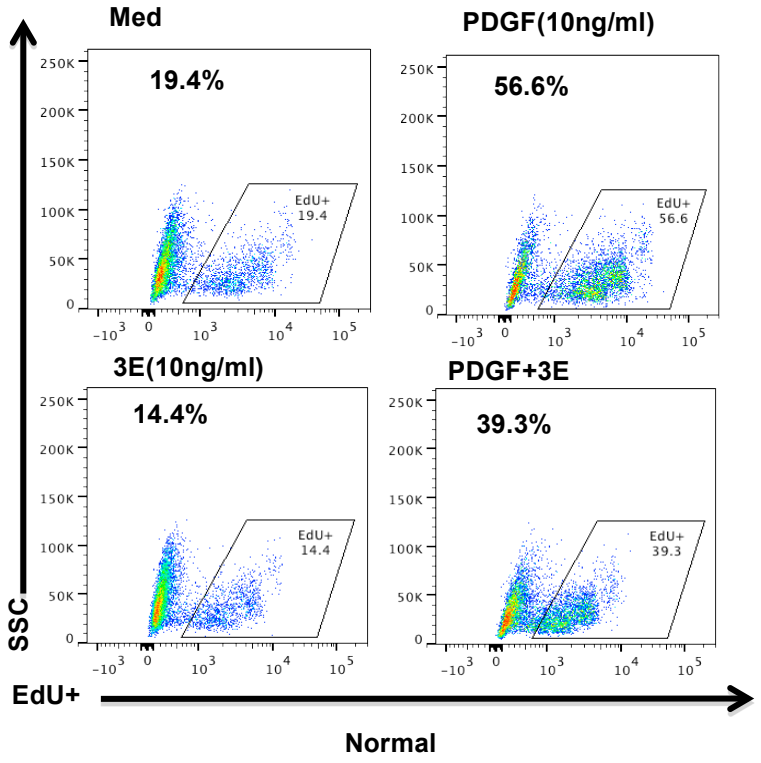
Figure 11e shows the statistical analysis in which Sema3E inhibited HASMCs proliferation mediated by PDGF in non-COPD significantly with P value ($P < .05$, Mann-Whitney U test) compared to COPD cells.

The possible mechanisms to explain our new findings that contradict the observation in cells form allergic asthmatic are numerous. However, we would like to highlight two possibilities that may account for our result First, HASMCs from COPD patients may produce endogenous Sema3E excessively which by autocrine pathway occupy plexinD1 receptors. This event prevents the exogenous Sema3E from binding and exerting its effect on HASMCs from COPD. Second, previous studies have showed that Sema3E can be cleaved by furin protease *in vivo* leading to P61 and 25kDa fragments[190]. The P61 isoform have antagonistic function in contrast to the un-cleaved from P87 isoform [204]. Furthermore, HASM cells from COPD cells produces like other inflammatory cell recruited to COPD lung, matrix degrading proteinases like cathepsin G, L, furin and MMPs (1-8-9-12)[64]. These proteases may cleave the native form (p-87) to p-61, which has been shown to have a pro-proliferative effect when binding to its receptor plexinD1 on cell surface [204, 225]. Figure (12) illustrates a schematic representation of these

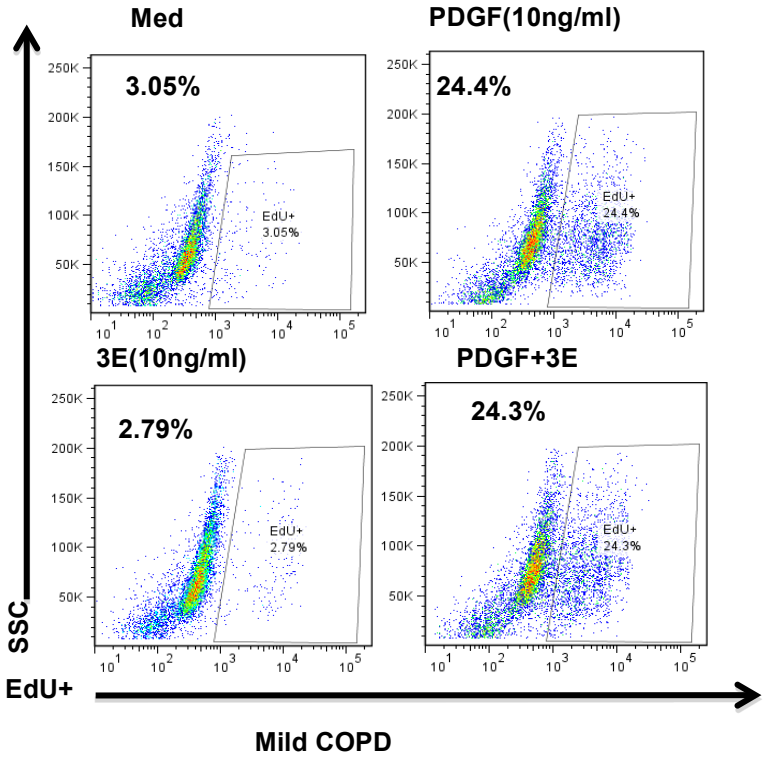
possible mechanisms.

We further investigated these possibilities by evaluating Sema3E isoforms produced by HASMCs isolated form COPD patients. In particular, we investigated the mRNA expression of Sema3E by HASMCs, the presence of surface bound Sema3E on HASMCs obtained from COPD and finally the nature of isoforms expressed and released in the supernatants of these cells.

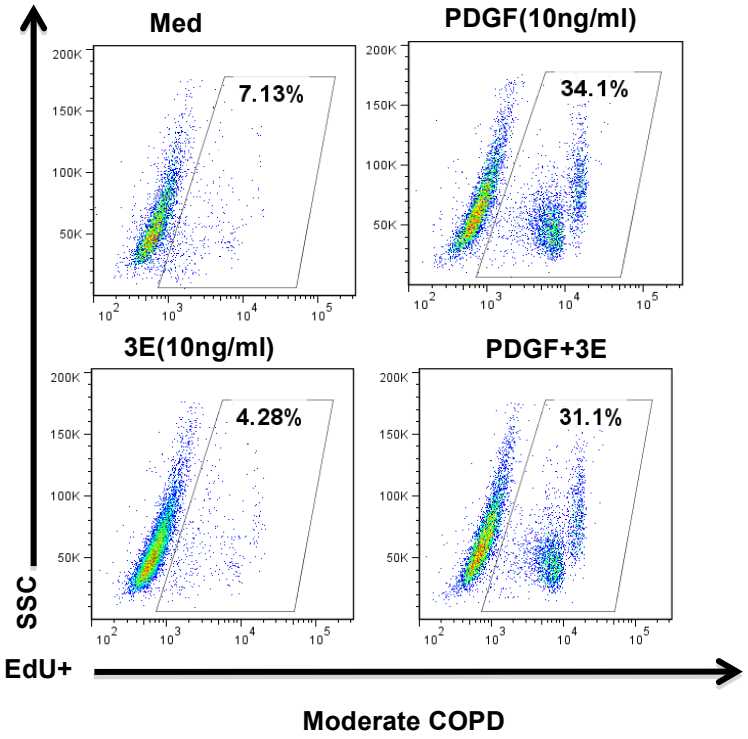
A


Med
19.4%
EdU+
19.4
PDGF(10ng/ml)
56.6%
EdU+
56.6
3E(10ng/ml)
14.4%
EdU+
14.4
PDGF+3E
39.3%
EdU+
39.3
SSC
EdU+
Normal

B

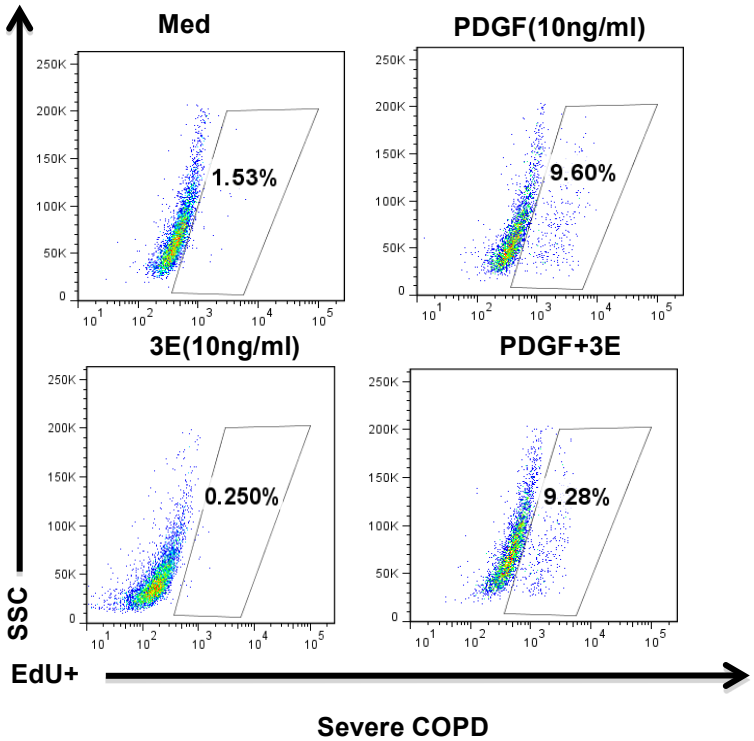

Med
3.05%
EdU+
3.05%
PDGF(10ng/ml)
24.4%
EdU+
24.4%
3E(10ng/ml)
2.79%
EdU+
2.79%
PDGF+3E
24.3%
EdU+
24.3%
SSC
EdU+
Mild COPD

C



Moderate COPD

D



Severe COPD

E

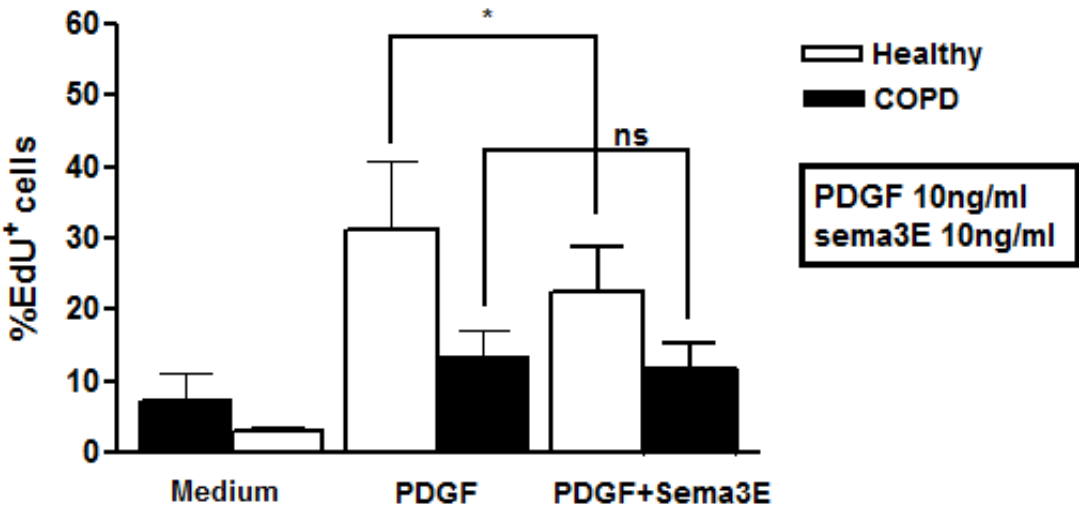


Figure 11. Recombinant Sema3E showed no effect on PDGF mediated HASMCs proliferation from COPD patients compared to healthy donors. Basal and PDGF-mediated proliferation of immortalized bronchial, primary tracheal normal HASMCs (A), Also primary bronchial HASMCs from mild (B) moderate (C) severe (D) COPD patients were studied by EdU incorporation assay. The results were quantified and statistically (E). The graphs are based on at least 3 independent proliferation experiments. *SSC*, Side scatter. $*P < .05$,ns (non-significant)

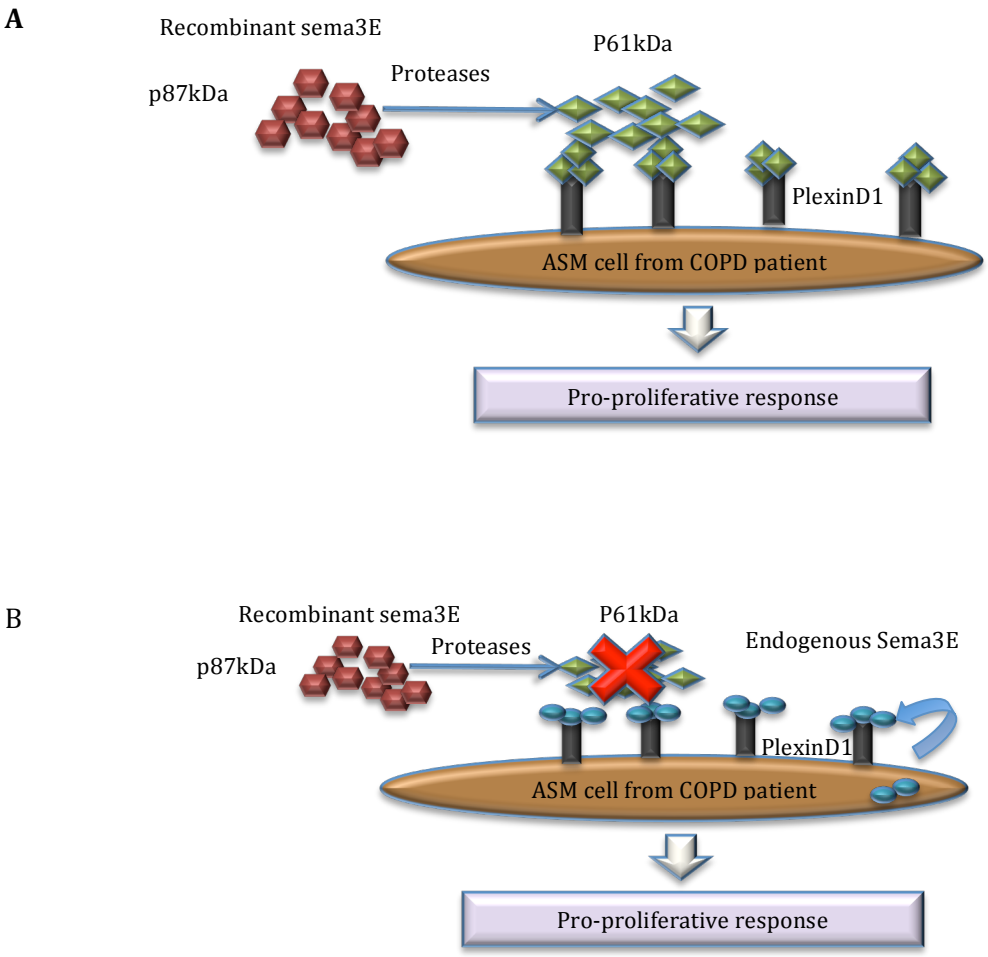


Figure 12. Schematic representation of the nonresponsive recombinant Sema3E (R3E) (P-87 kDa) on HASMCs from COPD patient. Adding R3E to HASMC may subject to proteolytic cleavage in COPD rich environment of proteinases. Thus, cleave the R3E from P-87kDa to P-61kDa which bind to cell surface receptor and exert its function as pro-proliferative isoform **A**. The other possibility is autocrine loop that the endogenous Sema3E (P-61kDa) isoform released from the cell occupy the cell surface receptor preventing the exogenous from binding to the cell **B**.

4.5. Sema3E is expressed in human ASM cells from normal and COPD subjects in vitro and within ASM cells bundle in lung section

As indicated above, we further investigated if Sema3E is produced constitutivily by HASMCs in COPD. mRNA level of sema3E in HASMCs isolated from normal and COPD cells was investigated using Realtime-PCR. Serum fed HASMCs was harvested at 24hrs and mRNA isolated as described in materials and methods Human Universal Reference Total cDNA (Clontech, Calif) was used as a positive control in all experiments, and no cDNA was served as negative control tubes. Sema3E mRNA level was not significantly expressed between COPD patients with different severity compared to normal subjects (Figure 13).

The expression of Sema3E in *ex-vivo was* also investigated by performing immunohistochemistry on HASMs from lung sections. Lung sections specimens from healthy controls (n=5) and COPD patients (n=5) were stained using mAb anti-mouse Sema3E. Positive Sema3E immunoreactivity was detected within ASM cells bundle in COPD lung section (Figure 14).

Collectively, this data suggests that HASMCs from COPD patients were non responsive to recombinant Sema3E treatment due to the endogenous expression of Sema3E. This later may occupy the cell surface receptor, PlexinD1, and prevents the effect of recombinant Sema3E on cell proliferation.

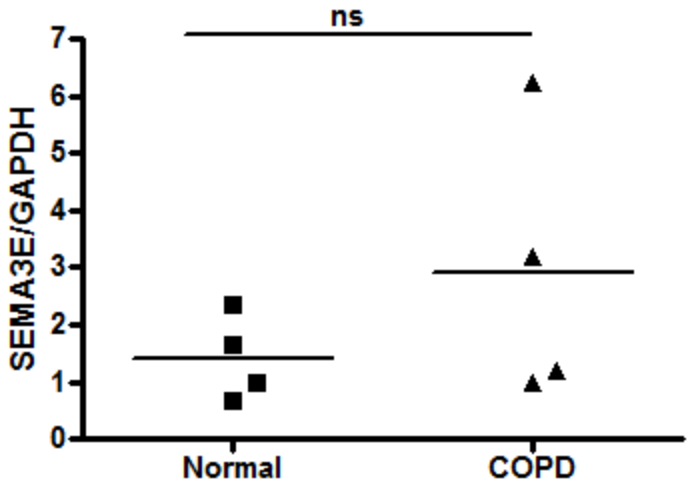


Figure 13. HASMCs obtained from COPD and normal subjects express Sema3E at mRNA level. RNA was isolated using TRIzol method and Reverse Transcriptase to synthesize cDNA. RT-PCR amplification was done with Sema3E specific primers. GAPDH was used as internal control. *GAPDH*, glyceraldehyde phosphate dehydrogenase gene; *SEMA3E,* Sema3E gene

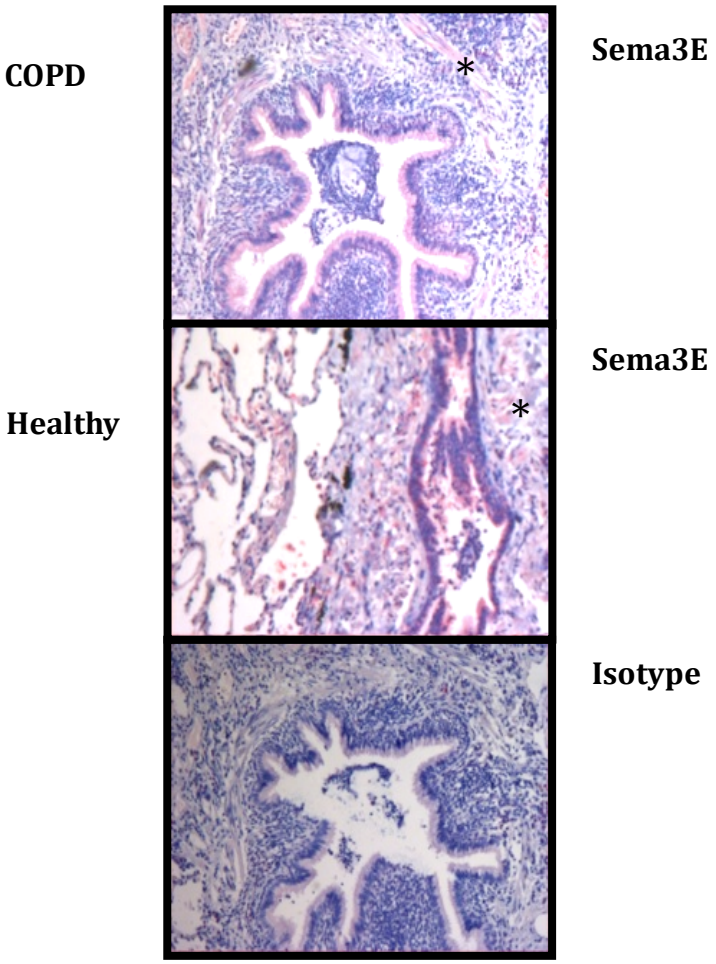


Figure 14. HASMCs express Sema3E. Lung sections from COPD patients and normal subjects were stained with specific rabbit polyclonal anti human Sema3E. Sections were then incubated with biotin labeled secondary antibody (Swine anti Rabbit) followed by streptavidin alkaline phosphatase and revealed by fast red. Sections were counterstained with hematoxylin. Isotype control rabbit staining show no immunoreactivity.* smooth muscle cells

4.6. HASMCs from COPD patients release Sema3E-truncated isoform that bind plexinD1 receptor

As we previously described, Sema3E is secreted protein [199]. In order to investigate whether Sema3E released binds to the cell surface receptor expressed in HASMCs in autocrine manner we performed flow cytometry to assess Sema3E surface expression by HASMCs. Forty eight hours serum starved HASMCs from COPD patients (mild and moderate) were incubated with APC labeled anti-human Sema3E mouse IgG_1 antibody (R&D) or isotype control for 30 min after blocking the non-specific FC receptor mediated binding of antibody with FC blocker for 30min on ice. As showed in figure 15, HASMCs from COPD displayed cell surface expression of Sema3E compared to isotype control.

This observation suggests that, in COPD, HASMCs release higher amount of Sema3E protein that may bind to the receptor on cell surface (Figure 15). Taken together these results suggest that the receptor is occupied with the endogenous Sema3E. We further confirmed the release and constitutive expression of Sema3E in HASMCs from COPD patients (Figure 16). Supernatants and protein lysate were collected from serum deprived (48 hours) primary HASMCs from COPD patients. Total proteins were further fractioned using ultracentrifugation to separate cell cytosol from the membrane proteins. HASMCs lysate from COPD patients expressed both the full-length p87-Sema3E and p61-Sema3E isoforms (Figure 16A) whereas in the supernatants p-61-Sema3E is the dominant isoform (Figure 16B). These results combined with cell bound Sema3E detected by FACS (Figure 15) suggest an autocrine loop where the endogenous Sema3E released in the supernatants bind to the receptor on cell surface. This in turn, prevents the recombinant Sema3E p87 kDa to exert its inhibitory role on HASMCs obtained from COPD cells.

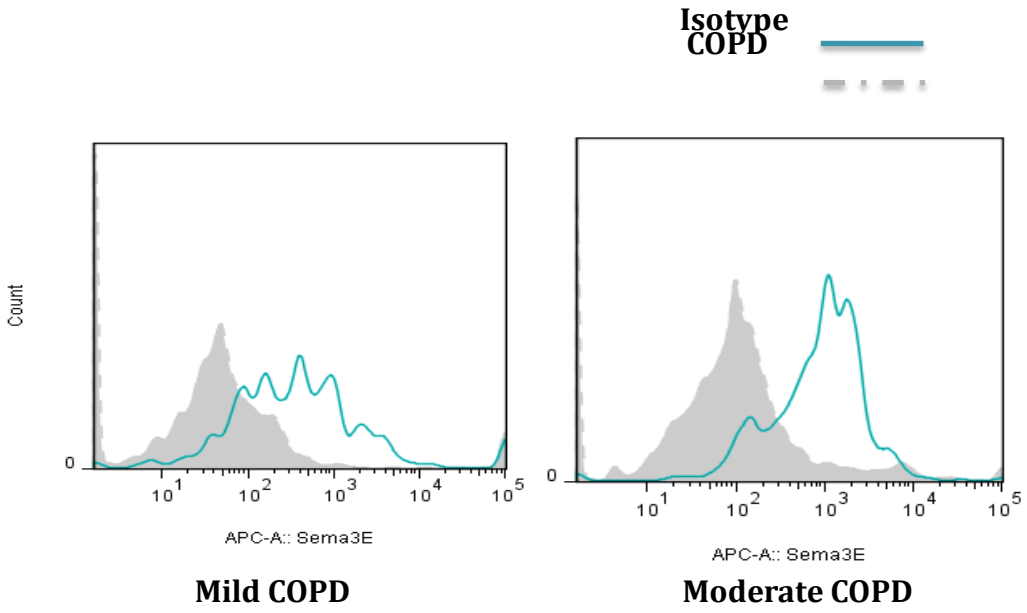


Figure 15. Surface expression of Sema3E on HASMCs from mild and moderate COPD patients. Extracellular expression of Sema3E protein analyzed by FACS. Cells were incubated with mouse IgG_1 Sema3E antibody APC labeled (R&D) for 30 min on ice. The control tube cells labeled with isotype mouse IgG_{2b} showed no immunoreactivitiy.

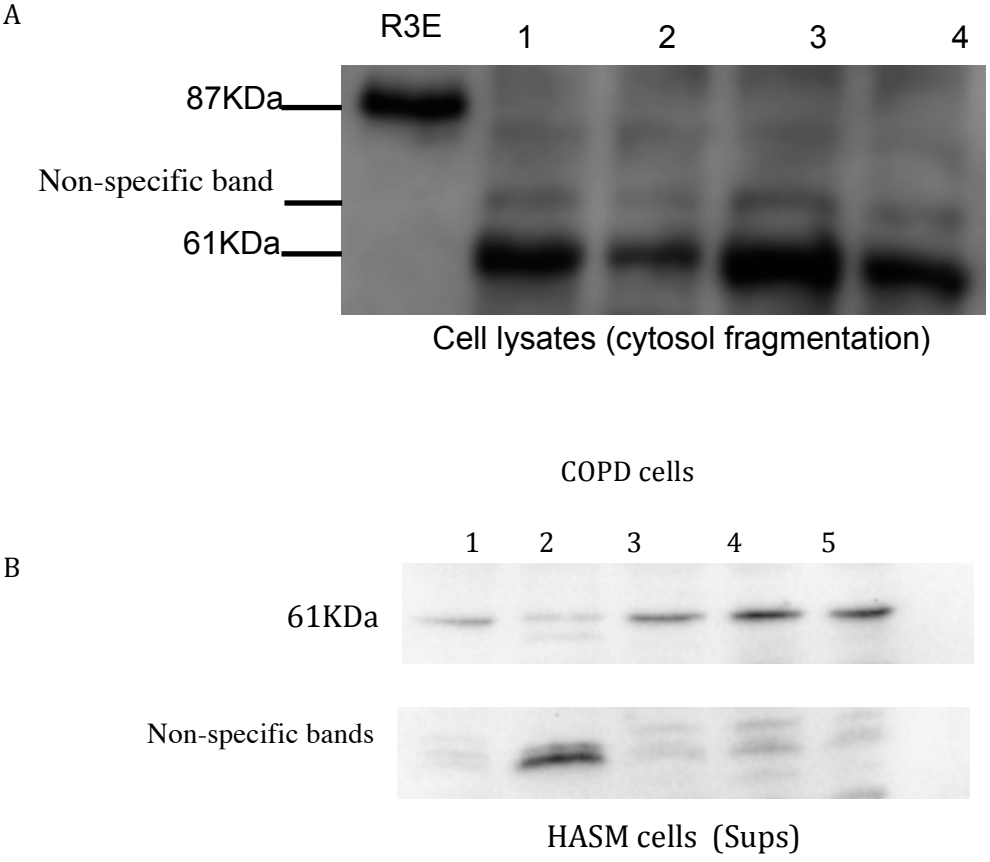


Figure 16. Sema3E isoform released from HASMCs in COPD patients with different disease severity. Sema3E basal protein expression was evaluated by western blotting. HASMCs were cultured until 70 % confluency. (A) Protein lysates were collected and fractionated to separate the cytoplasmic and nuclei fraction using ultracentrifugation and analyzed by WB. (B) Supernatants were collected. Both sups and lysates were concentrated 14X using ultra .5 centrifugal Filter Devise (10K cut off). 8ug of protein were resolved on polyacrylamide gels (SDS-PAGE) and were transfer onto PVDF membranes. After blocking with 5% non-fat milk, membrane were incubated with primary anti-Sema3E (goat Ig) overnight at 4˚C, followed by rabbit anti goat 2° antibody for 1 hour at room temperature.

4.7. Sema3E can be degraded by neutrophil derived protease (elastase and cathepsin G)

Sema3E is synthesized as a full-length precursor molecule of approximately 87kDa (p87-Sema3E), which is then subjected to proteolytic maturation by furin proprotein-convertases (PPCs) into p61kDa isoform [202]. P61-Sema3E is well studied in cancer cells as pro-metastatic molecule[204]. As shown in the previous figure, HASMCs from COPD patients expressed p61-Sema3E as the dominant isoform concomitant with the previous findings in supernatants and cell lysates (figure 16 A and B). Studies show that cathepsin G and neutrophil elastase are the main inflammatory cell matrix degrading proteinases in the lung [226]. In order to test if these proteases contribute to Sema3E cleavage, we digested recombinant Sema3E with cathepsin G and neutrophil elastase for 30 min at 37°C and assessed the presence of different isoforms by western blotting. The proteases cleaved recombinant Sema3E p87kDa to smaller fragment p61kDa (Figure 17).

Since COPD provide a rich environment for many proteases including furin [15, 64]. It is controversial that these proteinases will contentiously degrade the full-length Sema3E to the pro-proliferative isoform [225]. Therefore, our data suggest that the effect of sema3E on HASMCs in COPD is mediated by p61-Sema3E.

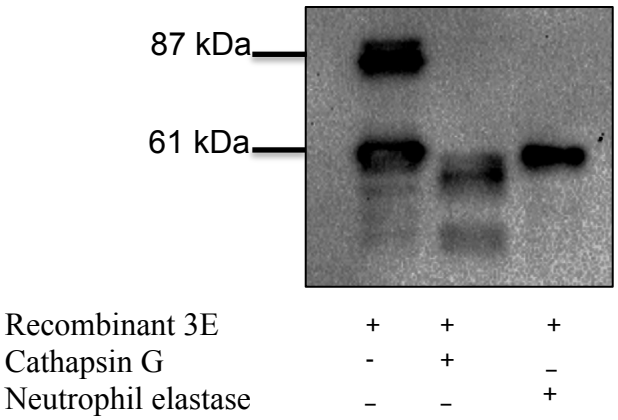


Figure 17. Recombinant Sema3E P-87KDa cleaved into P-61KDa in presence of proteases. Recombinant Sema3E was treated with proteases (neutrophil elastase and cathapsin G) in 37° water path for 30 minutes and then evaluated by western blotting.

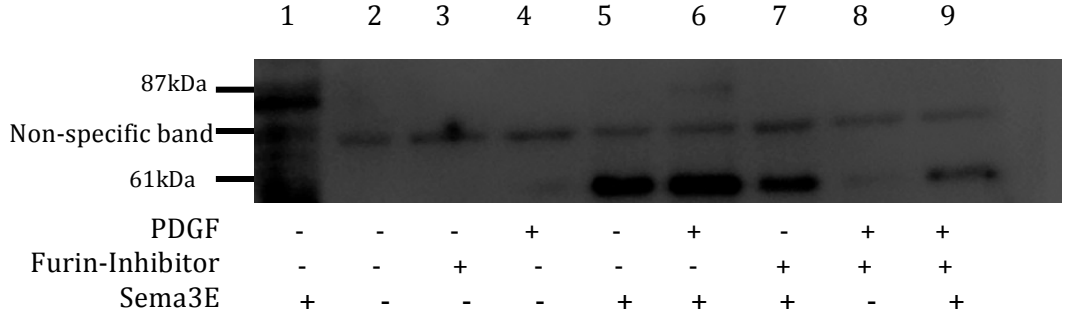
4.8. Effect of furin Inhibitor on Sema3E isoforms expressed by HASMCs from COPD patients

We further examined the effect of furin inhibitor on Sema3E isoforms released by HASMCs from COPD and healthy patients. HASMCs were serum starved for 48 hours and incubated with fruin inhibitor (80 umol/ml) for 4 hours in conditioned medium in presence or absence of PDGF. The immunoblotting analysis of HASMCs supernatants from healthy donors and COPD patients indicates that the furin inhibitor concentration used in this assay was not able to block the cleavage of recombinant or endogenous Sema3E. As such, the lanes where exogenous Sema3E was added displayed a p61kDa Sema3E band. In COPD patients, furin inhibitor prevented parts of cleavage of endogenous p87kDa to p61kDa (Figure 18B lane 3). Also, accumulation of endogenous p61kDa is lost in the absence of furin inhibitor (Figure 18B lane 2 compared to lane 3). This data suggest that p61kDa has other cleavage sites that lead to the production of small fragment as previously shown in cancer[225].

Taken together, our data suggest that in COPD airway smooth muscle cells express high-level proteases activity that may account for the cleavage of endogenous Sema3E to p61kDa known to have a pro-proliferative function.

A

Normal



B

COPD

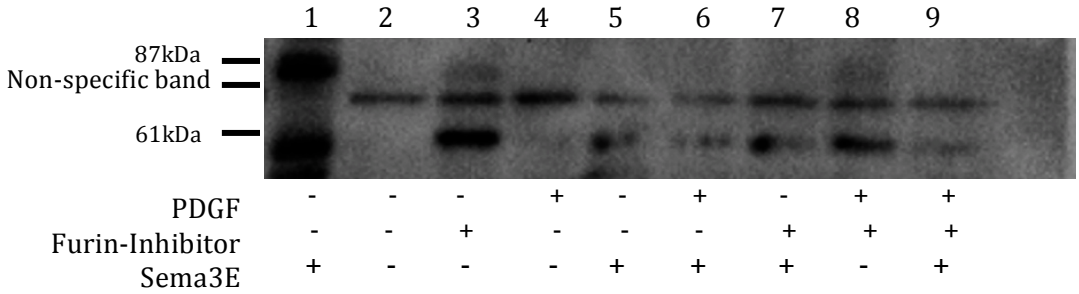


Figure 18. HASMCs were treated with PDGF and Sema3E in presence or absence of furin-Inhibitor. HASMCs were cultured in 37˚C until 50% confluency then serum starved for 48 hours Ham's F12 supplemented with 1× ITS in 12 well plate. Cells were stimulated with Sema3E and PDGF and co-stimulated in presence and absence of Furin-Inhibitor. Supernatant were collected and concentrated to 14X using ultra-10K devise. Centrifugal Filter. 8ug of protein were resolved on polyacrylamide gels (SDS-PAGE) and were transfer onto PVDF membranes. After blocking with 5% non-fat milk, membrane was incubated with primary anti-Sema3E (goat Ig) overnight at 4˚C, followed by rabbit anti goat antibody for 1 hour at room temperature.

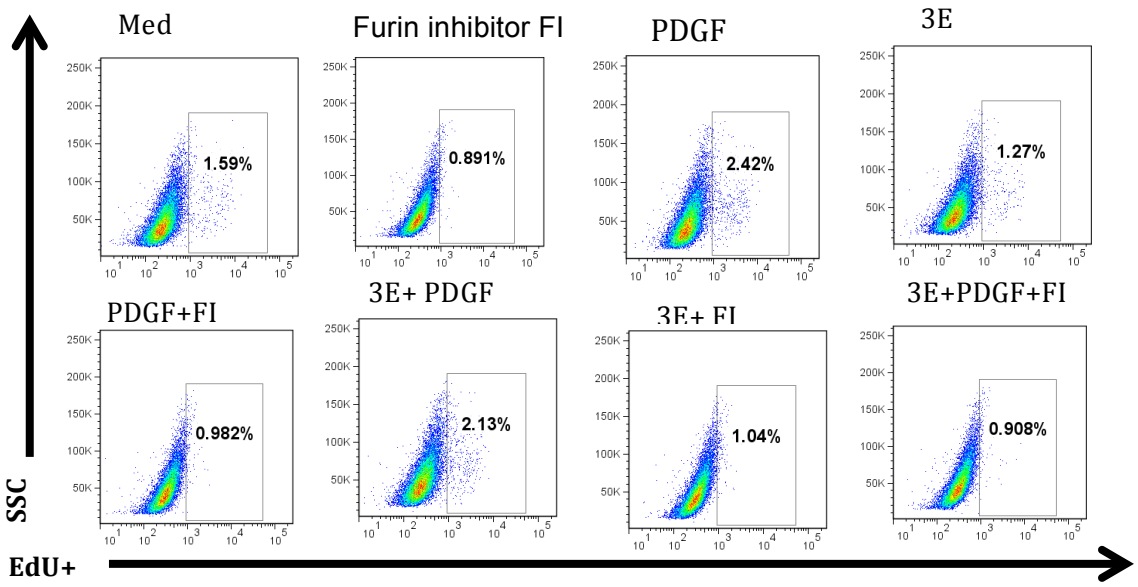
4.9. The effect of furin-inhibitor on PDGF and or Sema3E-mediated proliferation of HASMCs

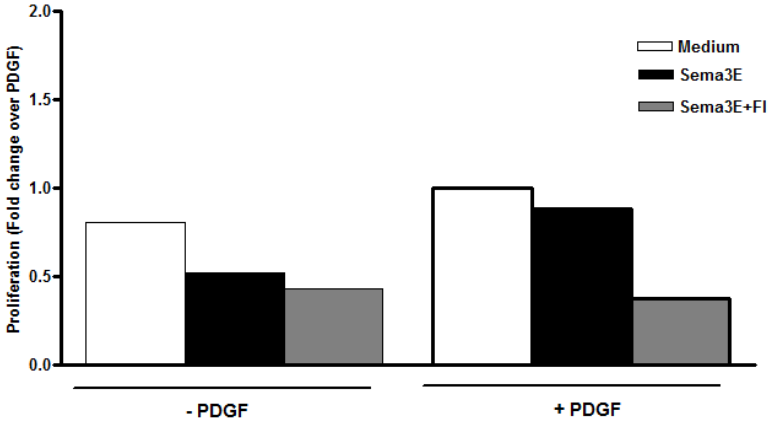
Additionally, we evaluated the function of the truncated Sema3E isoform compared to the full length by stimulating the cells with PDGF and Sema3E in the presence of absence of furin-inhibitor. HASMCs from normal subject and COPD patient were serum starved for 48 hours and stimulated with Sema3E (10ng/mL) and PDGF (10ng/mL) in presence or absence of furin inhibitor. EdU (5-ethynyl-2'-deoxyuridine) incorporation into DNA of replicating cells was assessed as surrogate marker of proliferation. As shown in figure (19) A and B, furin inhibitor decreases HASMCs proliferation in both normal and COPD at the baseline. It also affects PDGF induced HASMC proliferation in both cell types. This suggest that furin inhibitor affects proliferation and cell growth as has been shown previously [227].

In COPD patients, exogenous Sema3E enhances PDGF induced proliferation that was reduced in presence of furin inhibitor (Figure 19B). Taken together our data suggest as demonstrated in pervious studies that furin inhibitor has wider effect on inhibiting cell proliferation[228, 229].

A

Normal


Med
Furin inhibitor FI
PDGF
3E
1.59%
0.891%
2.42%
1.27%
PDGF+FI
3E+ PDGF
3E+ FI
3E+PDGF+FI
0.982%
2.13%
1.04%
0.908%
SSC
EdU+


Proliferation (Fold change over PDGF)
2.0
1.5
1.0
0.5
0.0
Medium
Sema3E
Sema3E+FI
- PDGF
+ PDGF

B

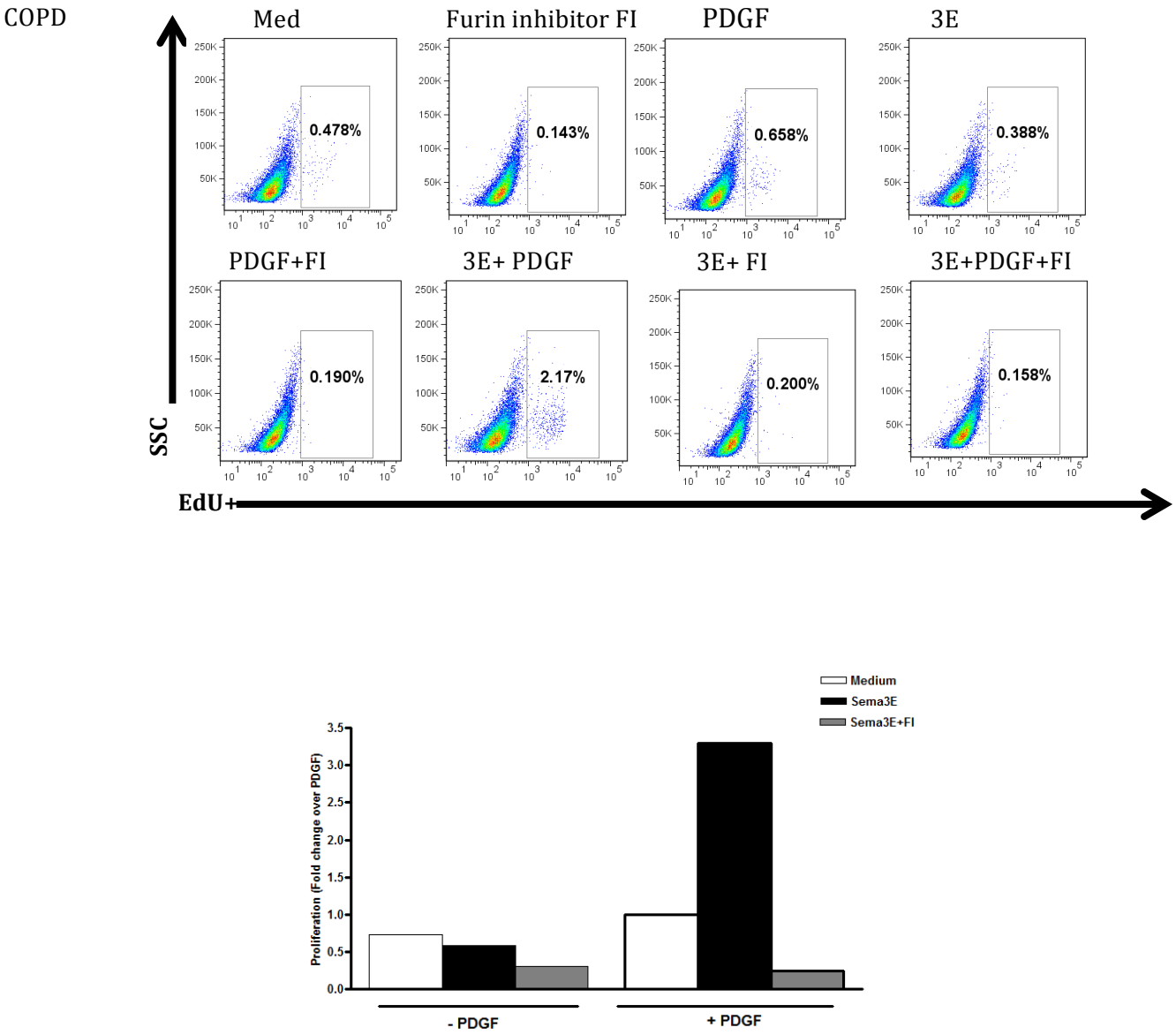


Figure 19.Furin inhibitor affect HASMCs proliferation. HASMCs were cultured in 37˚C until 50% confluency then serum starved for 48 hours in Ham's F12 supplemented with 1× ITS in 12 well plate. Cells were stimulated with sema3E and PDGF and co-stimulated in presence and absence of Furin-Inhibitor and studied by EdU incorporation assay 48 hours after stimulation. *SSC*, Side scattered, *FI,* furin inhibitor, *3E*, Sema3E

4.10. COPD cells express Sema3E co-receptor, Neuropilin

Although Sema3E signals mainly through plexinD1, neuropilin can act as a co-receptor particularly in endothelial cells [177]. In neuronal system, Sema3E have a dual function signaling through plexinD1/neuropilin complexes or through neuropilin specifically [201, 222]. Signaling through neuropilin alone mediates an attractive function in neuronal system whereas signaling through plexinD1/neuropilin reverses this function[206]. In this study, we predicted that in HASMCs from COPD, Sema3E could mediate its function via neuropilin binding on cell surface. Hence, we evaluated the protein surface expression of neuropilin by flow cytometry. Semi-confluent (50- 60% confluence) serum starved primary HASMCs from COPD patients and healthy subjects were detached using EDTA and incubated with PE conjugated mouse anti-human neuropilin mAb or isotype control for 2hrs with gentle agitation at 4°C. As shown in figure (20)A, neuropilin surface expression was detected on HASMCs from one mild COPD.

RNA preparations from primary serum-deprived HASMC from COPD and normal subjects were first analyzed by RT-PCR. As shown in figure (20)B HASMCs from COPD patients express neuropilin at mRNA level. GAPDH products were of similar intensity between all samples, suggesting equality of the RNA preparations with considering patients clinical variations (see table 1). Taken together, our data suggest that the binding of Sema3E to neuropilin may play a role in non-responsiveness of HASMCs to the recombinant Sema3E in COPD.

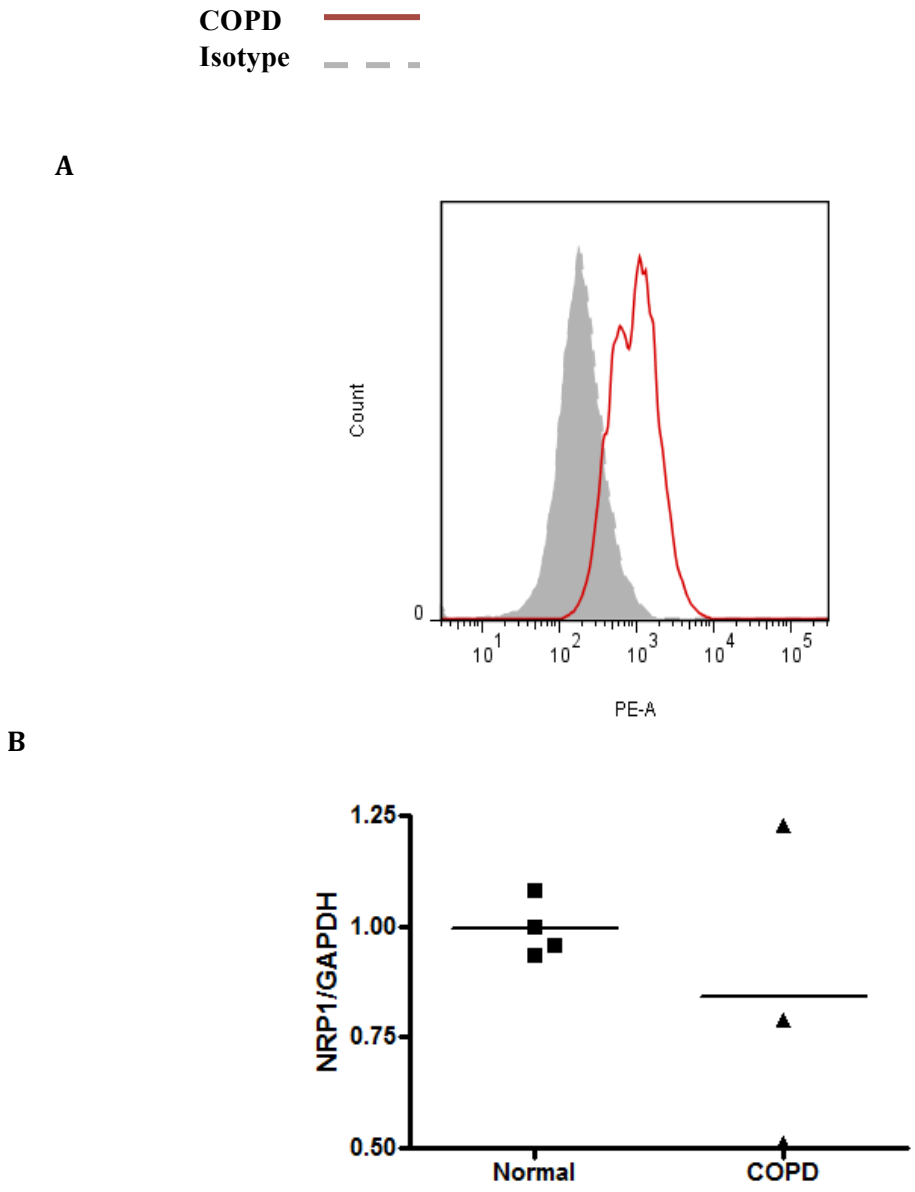


Figure 20. HASMCs from COPD patient express neuropilin at mRNA and protein level. Neuropilin surface expression detected by flow cytometry, cells were incubated with the cells with PF-conjugated mouse monoclonal anti-human neuropilin-1. Neuropilin expression on primary HASMCs was examined by RT-PCR with the use of specific primers. RNA level studied was performed on at least 3 different HASMCs under the same conditions. *GAPDH*, glyceraldehyde phosphate dehydrogenase gene; *NRP1*, neuropilin gen, PE

CHAPTER 5: DISCUSSION

5.1. THE ROLE OF SEMA3E IN HASMCs IN PROLIFERATION IN AIRWAY PULMONARY DISEASES

Previous studies have demonstrated that smooth muscle cells contribute to airway wall remodeling by increasing either in mass or size[230]. This eventually leads to clinical symptoms especially airflow obstruction in both asthmatic and COPD patients[231]. Airways obstruction in pulmonary diseases noticed in bronchial biopsies from asthma and COPD patients caused by the thickening of the airway walls [232]. In pulmonary diseases, the inflammation is believed to triggered by the influx of inflammatory cells T cells (Th1, Th2, Th17, Treg, and other unknown subtypes) along with APCs, macrophages, neutrophil, eosinophils, mast cells and their cytokines (IL-4, IL-5, and IL-13, IL-8, INF-beta, INF-gamma, TNF-alpha, IL-10 and many more) into lungs[233]. Nevertheless, the structural changes in airway wall including ASM cell hyperplasia or hypertrophy and other cell types fully define the airflow obstruction in lung diseases.

In vitro studies showed that in asthma and COPD, ASM cells proliferate significantly in response to mitogens [234, 235]. Moreover, in COPD, ASM cells hyperplasia has also been shown as one feature of airway remodeling that can play a role in augmenting the disease severity and pathology. However, there are other factors and cells that also drive the remodeling in pulmonary disease such as epithelial cells fragility and thickening, mucus hypersecretion, increased vascularity, fibrosis, squamous metaplasia, and increased deposition of collagen[236]. Yet, none of the existing therapeutic approaches is developed to target ASM cells hyperplesia in COPD.

Previous report showed that PDGF-BB induced ASM cells proliferation is inhibited significantly

in response to Sema3E in cells from asthmatic patients suggesting that Sema3E could have a potential therapeutic benefit in controlling ASM growth in obstructive diseases such as asthma and COPD [223]. Sema3E was initially studied as chemorepellent in nervous system[201]. Moreover, Sema3E is involved in cell proliferation in cancer. In cancer, Sema3E already proved to be capable of targeting cancer progression or invasiveness [202, 223, 237]. In the present study, we showed that Sema3E does not exert any inhibitory effect on PDGF-mediated proliferation of HASMCs from COPD patients. In order to understand this discrepancy, we first investigated the receptor expression in HASMCs from COPD patients.

Many studies showed that Sema3E mediates its effect through its receptor plexinD1[201, 210, 238]. Sema3E/plexinD1 network has been show to play a key role in proliferation and migration of cancer cell and ASM cells independently of other co-receptors mediating VEGFR2, and Nrp1 in particular[202, 223]. In HASMCs, Sema3E signaling through plexinD1 is critical for cell proliferation and migration, suggesting that Sema3E and its receptor are involved in the regulation of ASM remodeling in chronic airway diseases such as asthma[223]. In contrast, the present study showed that HASMCs from mild and moderate COPD cells expressed higher or similar amount of plexinD1 compared to normal cells; whereas, the expression level is low in HASMCs from severe COPD patients.

Although this data of limited value since it did interrogate only 2 patients from each severity, it is tempting to suggest that the decreased expression in HASMCs from severe COPD patients may be due to the binding of endogenous Sema3E to plexinD1 receptor.

Studies showed that ASM cells produce and secret many proinflammatory mediators including cytokines. In this study, we showed for the first time that ASM cells from COPD and healthy

donors express Sema3E protein in vitro, and ex vivo within lung section of COPD patients and normal subjects. Moreover, Sema3E mRNA level and the protein level were higher in HASMCs from COPD compared to healthy donors. Taken together, these results may also explain the non-responsiveness of COPD ASM cells upon stimulation with recombinant Sema3E.

5.2. DIFFERENT SEMA3E ISOFORM PLAY DIFFERENT ROLE IN ASM CELLS

The results of the present study show that COPD cells express the Sema3E-P61 isoform in cell lysate and supernatants. This fragment of Sema3E is considered as a pro-metastatic molecule in cancer cell[204]. It also known that this isoform is the dominant endogenous type in cancer cells similar to our observation in HASMCs from COPD cells[201]. It is important to highlight that COPD cells used in this study were obtained from patients undergoing surgery to remove lung cancer nodules. The cells are isolated from non-involved healthy tissue. However, it is possible that these cells are primed in vivo by secreted factors released by cancerous neighboring cells. This priming cells may enhance their proteolytic capacity, has explaining their ability to cleave Sema3E-p87kDa to p61 isoform. In accordance with this suggestion, the full-length Sema3E is subject to furin proprotein-convertases (PPCs) cleavage in cancer [202].

5.3. EXOGENOUS SEMA3E COMPETE WITH THE RELEASED ISOFORM FROM ASM CELLS

Western blotting analysis showed that ASM cells release Sema3E protein in cell supernatants, that may be subjected to protease cleavage leading to truncated p61 isoform, which can bind cell surface receptor plexinD1 in autocrine manner. Casazza et. al. showed that, in cancer cells (melanoma) the release Sema3E-p61 was fully capable of binding plexinD1. They also found

that recombinant uncleaved-Sema3E, mutated at furin cleavage site, competes with endogenous p61-Sema3E produced by tumor cells. In their study uncleaved-Sema3E did not induce cancer cell migration whereas sema3E-p61 acts as pro-invasive and pro-metastatic. Furthermore, in endothelial cells full-length Sema3E strongly and rapidly induced the retraction of cellular processes and the rounding of human umbilical vein-derived endothelial cells (HUVEC) [204]. Moreover, it is shown that Sema3E inhibits integrin function and intracellular signaling in endothelial cell [239]. Unclveaved-Sema3E has been found to trigger a dramatic disassembly of focal adhesion complexes in cells treated for 5 min with uncleaved-Sema3E[204]. Unlike p61, uncleaved-Sema3E bound to PlexinD1 is able to elicit anti-angiogenic and repellent functions in endothelial cells [201]. We suggest, although not yet confirmed, that in COPD cells, Sema3E-p61 may drive the pro-proliferative activates of HASMCs, which may account for smooth muscle remodeling.

CHAPTER 6: FUTURE DIRECTIONS

1-Investigate the expression of neuropilin (Nrp-1) in HASMCs from COPD and determine whether Nrp-1, co receptor of Sema3E, play any role in Sema3E mediated function in HASMCs. This can be achieved by using flow cytomtery and RT-PCR.

2-Investigate whether the absence of Sema3E effect in COPD is due to the binding of the endogenous Sema3E p61kDa to surface receptor in autocrine manner.

3-Knock down Sema3E expression using ShRNA and then investigate the effect of recombinant3E on cell proliferation.

4-Investigate the regulatory mechanisms that govern Sema3E and plexinD1 expression in ASM

cells. In particular, determine the role of pro-inflammatory cytokines TNF and IL-1β on Sema3E expression by cell culture and ELISA as well as RT-PCR.

5-Confirm the expression of Sema3E *in vivo* within COPD muscle bundles using double immunostaining with ASM marker alpha smooth muscle actin and Sema3E antibody.

CHAPTER 7: REFRENCES

1. Boyd DR and Genuis SJ (2008) The environmental burden of disease in Canada: respiratory disease, cardiovascular disease, cancer, and congenital affliction. Environ Res 106:240-9. doi: 10.1016/j.envres.2007.08.009

2. Bang KM (2015) Chronic obstructive pulmonary disease in nonsmokers by occupation and exposure: a brief review. Curr Opin Pulm Med. doi: 10.1097/MCP.0000000000000135

3. Sanguinetti CM, Ambrosino N, Ando F, De Benedetto F, Donner CF, Nardini S, Polverino M, Torchio R, Vagheggini G, Visconti A and Consensus Conference G (2014) Standards of suitability for the management of chronic obstructive respiratory diseases. Multidiscip Respir Med 9:65. doi: 10.1186/2049-6958-9-65

4. Johns DP, Walters JA and Walters EH (2014) Diagnosis and early detection of COPD using spirometry. J Thorac Dis 6:1557-69. doi: 10.3978/j.issn.2072-1439.2014.08.18

5. Kankaanranta H, Harju T, Kilpelainen M, Mazur W, Lehto JT, Katajisto M, Peisa T, Meinander T and Lehtimaki L (2014) Diagnosis and Pharmacotherapy of Stable Chronic Obstructive Pulmonary Disease: The Finnish Guidelines Guidelines of the Finnish Medical Society Duodecim and the Finnish Respiratory Society. Basic Clin Pharmacol Toxicol. doi: 10.1111/bcpt.12366

6. Benetazzo MG, Gile LS, Bombieri C, Malerba G, Massobrio M, Pignatti PF and Luisetti M (1999) alpha 1-antitrypsin TAQ I polymorphism and alpha 1-antichymotrypsin mutations in patients with obstructive pulmonary disease. Respir Med 93:648-54.

7. Saferali A, Lee J, Sin DD, Rouhani FN, Brantly ML and Sandford AJ (2014) Longer telomere length in COPD patients with alpha1-antitrypsin deficiency independent of lung function. PLoS One 9:e95600. doi: 10.1371/journal.pone.0095600

8. Seifart C and Plagens A (2007) Genetics of chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 2:541-50.

9. Babalik A, Bakirci N, Taylan M, Bostan L, Kiziltas S, Basbug Y and Calisir HC (2013) Biomass smoke exposure as a serious health hazard for women. Tuberk Toraks 61:115-21.

10. Yoo S, Takikawa S, Geraghty P, Argmann C, Campbell J, Lin L, Huang T, Tu Z, Feronjy R, Spira A, Schadt EE, Powell CA and Zhu J (2015) Integrative Analysis of DNA Methylation and Gene Expression Data Identifies EPAS1 as a Key Regulator of COPD. PLoS Genet 11:e1004898. doi: 10.1371/journal.pgen.1004898

11. Ding Y, Yang D, Xun X, Wang Z, Sun P, Xu D, He P, Niu H and Jin T (2015) Association of genetic polymorphisms with chronic obstructive pulmonary disease in the Hainan

population: a case-control study. Int J Chron Obstruct Pulmon Dis 10:7-13. doi: 10.2147/COPD.S73042

12. Takiguchi Y, Sekine I, Iwasawa S, Kurimoto R and Tatsumi K (2014) Chronic obstructive pulmonary disease as a risk factor for lung cancer. World J Clin Oncol 5:660-6. doi: 10.5306/wjco.v5.i4.660

13. Campos MA and Lascano J (2014) alpha1 Antitrypsin deficiency: current best practice in testing and augmentation therapy. Ther Adv Respir Dis 8:150-61. doi: 10.1177/1753465814542243

14. Wallace AM and Sandford AJ (2002) Genetic polymorphisms of matrix metalloproteinases: functional importance in the development of chronic obstructive pulmonary disease? Am J Pharmacogenomics 2:167-75.

15. Fischer BM, Pavlisko E and Voynow JA (2011) Pathogenic triad in COPD: oxidative stress, protease-antiprotease imbalance, and inflammation. Int J Chron Obstruct Pulmon Dis 6:413-21. doi: 10.2147/COPD.S10770

16. Grainge CL and Davies DE (2013) Epithelial injury and repair in airways diseases. Chest 144:1906-12. doi: 10.1378/chest.12-1944

17. Borok Z, Whitsett JA, Bitterman PB, Thannickal VJ, Kotton DN, Reynolds SD, Krasnow MA, Bianchi DW, Morrisey EE, Hogan BL, Kurie JM, Walker DC, Radisky DC, Nishimura SL, Violette SM, Noble PW, Shapiro SD, Blaisdell CJ, Chapman HA, Kiley J, Gail D and Hoshizaki D (2011) Cell plasticity in lung injury and repair: report from an NHLBI workshop, April 19-20, 2010. Proc Am Thorac Soc 8:215-22. doi: 10.1513/pats.201012-067CB

18. Nakawah MO, Hawkins C and Barbandi F (2013) Asthma, chronic obstructive pulmonary disease (COPD), and the overlap syndrome. J Am Board Fam Med 26:470-7. doi: 10.3122/jabfm.2013.04.120256

19. Barnes PJ (2004) Mediators of chronic obstructive pulmonary disease. Pharmacol Rev 56:515-48. doi: 10.1124/pr.56.4.2

20. Pini L, Pinelli V, Modina D, Bezzi M, Tiberio L and Tantucci C (2014) Central airways remodeling in COPD patients. Int J Chron Obstruct Pulmon Dis 9:927-32. doi: 10.2147/COPD.S52478

21. Najafi A, Masoudi-Nejad A, Ghanei M, Nourani MR and Moeini A (2014) Pathway reconstruction of airway remodeling in chronic lung diseases: a systems biology approach. PLoS One 9:e100094. doi: 10.1371/journal.pone.0100094

22. (2010). How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General, Atlanta (GA)

23. Hogg JC, Macklem PT and Thurlbeck WM (1968) Site and nature of airway obstruction in chronic obstructive lung disease. N Engl J Med 278:1355-60. doi: 10.1056/NEJM196806202782501

24. Burgel PR and Martin C (2010) Mucus hypersecretion in COPD: should we only rely on symptoms? Eur Respir Rev 19:94-6. doi: 10.1183/09059180.00004410

25. O'Donnell R, Breen D, Wilson S and Djukanovic R (2006) Inflammatory cells in the airways in COPD. Thorax 61:448-54. doi: 10.1136/thx.2004.024463

26. Rahman I (2005) The role of oxidative stress in the pathogenesis of COPD: implications for therapy. Treat Respir Med 4:175-200.

27. Henson PM, Vandivier RW and Douglas IS (2006) Cell death, remodeling, and repair in chronic obstructive pulmonary disease? Proc Am Thorac Soc 3:713-7. doi: 10.1513/pats.200605-104SF

28. Greene CM and McElvaney NG (2009) Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery. Br J Pharmacol 158:1048-58. doi: 10.1111/j.1476-5381.2009.00448.x

29. Chung KF (2005) The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease. Proc Am Thorac Soc 2:347-54; discussion 371-2. doi: 10.1513/pats.200504-028SR

30. Mortaz E, Masjedi MR and Barnes P (2011) Identification of Novel Therapeutic Targets in COPD. Tanaffos 10:9-14.

31. Agusti A (2009) [COPD and systemic inflammation. A link for comorbidity]. Arch Bronconeumol 45 Suppl 4:14-7. doi: 10.1016/S0300-2896(09)72858-3

32. Yao H and Rahman I (2011) Current concepts on oxidative/carbonyl stress, inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. Toxicol Appl Pharmacol 254:72-85. doi: 10.1016/j.taap.2009.10.022

33. Haczku A (2012) The dendritic cell niche in chronic obstructive pulmonary disease. Respir Res 13:80. doi: 10.1186/1465-9921-13-80

34. Majo J, Ghezzo H and Cosio MG (2001) Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. Eur Respir J 17:946-53.

35. Di Stefano A, Caramori G, Ricciardolo FL, Capelli A, Adcock IM and Donner CF (2004) Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. Clin Exp Allergy 34:1156-67. doi: 10.1111/j.1365-2222.2004.02030.x

36. Grumelli S, Corry DB, Song LZ, Song L, Green L, Huh J, Hacken J, Espada R, Bag R, Lewis DE and Kheradmand F (2004) An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. PLoS Med 1:e8. doi: 10.1371/journal.pmed.0010008

37. Miossec P, Korn T and Kuchroo VK (2009) Interleukin-17 and type 17 helper T cells. N Engl J Med 361:888-98. doi: 10.1056/NEJMra0707449

38. Isajevs S, Taivans I, Strazda G, Kopeika U, Bukovskis M, Gordjusina V and Kratovska A (2009) Decreased FOXP3 expression in small airways of smokers with COPD. Eur Respir J 33:61-7. doi: 10.1183/09031936.00145307

39. Plumb J, Smyth LJ, Adams HR, Vestbo J, Bentley A and Singh SD (2009) Increased T-regulatory cells within lymphocyte follicles in moderate COPD. Eur Respir J 34:89-94. doi: 10.1183/09031936.00100708

40. Rovina N, Koutsoukou A and Koulouris NG (2013) Inflammation and immune response in COPD: where do we stand? Mediators Inflamm 2013:413735. doi: 10.1155/2013/413735

41. Litsiou E, Semitekolou M, Galani IE, Morianos I, Tsoutsa A, Kara P, Rontogianni D, Bellenis I, Konstantinou M, Potaris K, Andreakos E, Sideras P, Zakynthinos S and Tsoumakidou M (2013) CXCL13 production in B cells via Toll-like receptor/lymphotoxin receptor signaling is involved in lymphoid neogenesis in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 187:1194-202. doi: 10.1164/rccm.201208-1543OC

42. Daffa NI, Tighe PJ, Corne JM, Fairclough LC and Todd I (2014) Natural and disease-specific autoantibodies in chronic obstructive pulmonary disease. Clin Exp Immunol. doi: 10.1111/cei.12565

43. Feghali-Bostwick CA, Gadgil AS, Otterbein LE, Pilewski JM, Stoner MW, Csizmadia E, Zhang Y, Sciurba FC and Duncan SR (2008) Autoantibodies in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 177:156-63. doi: 10.1164/rccm.200701-014OC

44. Demedts IK, Bracke KR, Van Pottelberge G, Testelmans D, Verleden GM, Vermassen FE, Joos GF and Brusselle GG (2007) Accumulation of dendritic cells and increased CCL20 levels in the airways of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 175:998-1005. doi: 10.1164/rccm.200608-1113OC

45. Stoll P, Heinz AS, Bratke K, Bier A, Garbe K, Kuepper M, Virchow JC and Lommatzsch M (2014) Impact of smoking on dendritic cell phenotypes in the airway lumen of patients with COPD. Respir Res 15:48. doi: 10.1186/1465-9921-15-48

46. Bratke K, Klug M, Bier A, Julius P, Kuepper M, Virchow JC and Lommatzsch M (2008) Function-associated surface molecules on airway dendritic cells in cigarette smokers. Am J Respir Cell Mol Biol 38:655-60. doi: 10.1165/rcmb.2007-0400OC

47. D'Hulst A I, Vermaelen KY, Brusselle GG, Joos GF and Pauwels RA (2005) Time course of cigarette smoke-induced pulmonary inflammation in mice. Eur Respir J 26:204-13. doi: 10.1183/09031936.05.00095204

48. Liao SX, Ding T, Rao XM, Sun DS, Sun PP, Wang YJ, Fu DD, Liu XL and Ou-Yang Y (2015) Cigarette smoke affects dendritic cell maturation in the small airways of patients with chronic obstructive pulmonary disease. Mol Med Rep 11:219-25. doi: 10.3892/mmr.2014.2759

49. Bracke K, Cataldo D, Maes T, Gueders M, Noel A, Foidart JM, Brusselle G and Pauwels RA (2005) Matrix metalloproteinase-12 and cathepsin D expression in pulmonary macrophages and dendritic cells of cigarette smoke-exposed mice. Int Arch Allergy Immunol 138:169-79. doi: 10.1159/000088439

50. Reynolds HY (1987) Bronchoalveolar lavage. Am Rev Respir Dis 135:250-63.

51. Tetley TD (2002) Macrophages and the pathogenesis of COPD. Chest 121:156S-159S.

52. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO and Pare PD (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med 350:2645-53. doi: 10.1056/NEJMoa032158

53. Damiano VV, Tsang A, Kucich U, Abrams WR, Rosenbloom J, Kimbel P, Fallahnejad M and Weinbaum G (1986) Immunolocalization of elastase in human emphysematous lungs. J Clin Invest 78:482-93. doi: 10.1172/JCI112600

54. Wang Z, Zheng T, Zhu Z, Homer RJ, Riese RJ, Chapman HA, Jr., Shapiro SD and Elias JA (2000) Interferon gamma induction of pulmonary emphysema in the adult murine lung. J Exp Med 192:1587-600.

55. Finlay GA, O'Driscoll LR, Russell KJ, D'Arcy EM, Masterson JB, FitzGerald MX and O'Connor CM (1997) Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. Am J Respir Crit Care Med 156:240-7. doi: 10.1164/ajrccm.156.1.9612018

56. D'Armiento J, Dalal SS, Okada Y, Berg RA and Chada K (1992) Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema. Cell 71:955-61.

57. Hu B, Sonstein J, Christensen PJ, Punturieri A and Curtis JL (2000) Deficient in vitro and in vivo phagocytosis of apoptotic T cells by resident murine alveolar macrophages. J Immunol 165:2124-33.

58. Curtis JL, Todt JC, Hu B, Osterholzer JJ and Freeman CM (2009) Tyro3 receptor tyrosine kinases in the heterogeneity of apoptotic cell uptake. Front Biosci (Landmark Ed) 14:2631-46.

59. Stockley RA (2002) Neutrophils and the pathogenesis of COPD. Chest 121:151S-155S.

60. Selby C, Drost E, Lannan S, Wraith PK and MacNee W (1991) Neutrophil retention in the lungs of patients with chronic obstructive pulmonary disease. Am Rev Respir Dis 143:1359-64. doi: 10.1164/ajrccm/143.6.1359

61. Stanescu D, Sanna A, Veriter C, Kostianev S, Calcagni PG, Fabbri LM and Maestrelli P (1996) Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. Thorax 51:267-71.

62. Martin TR, Raghu G, Maunder RJ and Springmeyer SC (1985) The effects of chronic bronchitis and chronic air-flow obstruction on lung cell populations recovered by bronchoalveolar lavage. Am Rev Respir Dis 132:254-60.

63. Chalmers GW, MacLeod KJ, Thomson L, Little SA, McSharry C and Thomson NC (2001) Smoking and airway inflammation in patients with mild asthma. Chest 120:1917-22.

64. Owen CA (2008) Roles for proteinases in the pathogenesis of chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 3:253-68.

65. Gompertz S, O'Brien C, Bayley DL, Hill SL and Stockley RA (2001) Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. Eur Respir J 17:1112-9.

66. Takeyama K, Agusti C, Ueki I, Lausier J, Cardell LO and Nadel JA (1998) Neutrophil-dependent goblet cell degranulation: role of membrane-bound elastase and adhesion molecules. Am J Physiol 275:L294-302.

67. Kafienah W, Buttle DJ, Burnett D and Hollander AP (1998) Cleavage of native type I collagen by human neutrophil elastase. Biochem J 330 (Pt 2):897-902.

68. Vogt W (2000) Cleavage of the fifth component of complement and generation of a functionally active C5b6-like complex by human leukocyte elastase. Immunobiology 201:470-7. doi: 10.1016/S0171-2985(00)80099-6

69. Yamamoto C, Yoneda T, Yoshikawa M, Fu A, Tokuyama T, Tsukaguchi K and Narita N (1997) Airway inflammation in COPD assessed by sputum levels of interleukin-8. Chest 112:505-10.

70. Balzano G, Stefanelli F, Iorio C, De Felice A, Melillo EM, Martucci M and Melillo G (1999) Eosinophilic inflammation in stable chronic obstructive pulmonary disease. Relationship with neutrophils and airway function. Am J Respir Crit Care Med 160:1486-92. doi: 10.1164/ajrccm.160.5.9810105

71. Bhowmik A, Seemungal TA, Sapsford RJ and Wedzicha JA (2000) Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. Thorax 55:114-20.

72. Rutgers SR, Timens W, Kaufmann HF, van der Mark TW, Koeter GH and Postma DS (2000) Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. Eur Respir J 15:109-15.

73. Saetta M, Di Stefano A, Maestrelli P, Turato G, Mapp CE, Pieno M, Zanguochi G, Del Prete G and Fabbri LM (1996) Airway eosinophilia and expression of interleukin-5 protein in asthma and in exacerbations of chronic bronchitis. Clin Exp Allergy 26:766-74.

74. Zhu J, Qiu YS, Majumdar S, Gamble E, Matin D, Turato G, Fabbri LM, Barnes N, Saetta M and Jeffery PK (2001) Exacerbations of Bronchitis: bronchial eosinophilia and gene expression for interleukin-4, interleukin-5, and eosinophil chemoattractants. Am J Respir Crit Care Med 164:109-16. doi: 10.1164/ajrccm.164.1.2007050

75. Bocchino V, Bertorelli G, Bertrand CP, Ponath PD, Newman W, Franco C, Marruchella A, Merlini S, Del Donno M, Zhuo X and Olivieri D (2002) Eotaxin and CCR3 are up-regulated in exacerbations of chronic bronchitis. Allergy 57:17-22.

76. Papi A, Luppi F, Franco F and Fabbri LM (2006) Pathophysiology of exacerbations of chronic obstructive pulmonary disease. Proc Am Thorac Soc 3:245-51. doi: 10.1513/pats.200512-125SF

77. Keatings VM and Barnes PJ (1997) Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. Am J Respir Crit Care Med 155:449-53. doi: 10.1164/ajrccm.155.2.9032177

78. Tetley TD (2005) Inflammatory cells and chronic obstructive pulmonary disease. Curr Drug Targets Inflamm Allergy 4:607-18.

79. Pizzichini E, Pizzichini MM, Gibson P, Parameswaran K, Gleich GJ, Berman L, Dolovich J and Hargreave FE (1998) Sputum eosinophilia predicts benefit from prednisone in smokers with chronic obstructive bronchitis. Am J Respir Crit Care Med 158:1511-7. doi: 10.1164/ajrccm.158.5.9804028

80. Barnes PJ (2008) The cytokine network in asthma and chronic obstructive pulmonary disease. J Clin Invest 118:3546-56. doi: 10.1172/JCI36130

81. Kent L, Smyth L, Clayton C, Scott L, Cook T, Stephens R, Fox S, Hext P, Farrow S and Singh D (2008) Cigarette smoke extract induced cytokine and chemokine gene expression changes in COPD macrophages. Cytokine 42:205-16. doi: 10.1016/j.cyto.2008.02.001

82. Chung KF (2001) Cytokines in chronic obstructive pulmonary disease. Eur Respir J Suppl 34:50s-59s.

83. Koelink PJ, Overbeek SA, Braber S, de Kruijf P, Folkerts G, Smit MJ and Kraneveld AD (2012) Targeting chemokine receptors in chronic inflammatory diseases: an extensive review. Pharmacol Ther 133:1-18. doi: 10.1016/j.pharmthera.2011.06.008

84. Larsson K (2007) Aspects on pathophysiological mechanisms in COPD. J Intern Med 262:311-40. doi: 10.1111/j.1365-2796.2007.01837.x

85. Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A and Rennard SI (1997) Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. Am J Respir Crit Care Med 155:1770-6. doi: 10.1164/ajrccm.155.5.9154890

86. Khair OA, Devalia JL, Abdelaziz MM, Sapsford RJ, Tarraf H and Davies RJ (1994) Effect of Haemophilus influenzae endotoxin on the synthesis of IL-6, IL-8, TNF-alpha and expression of ICAM-1 in cultured human bronchial epithelial cells. Eur Respir J 7:2109-16.

87. Wang B, Cleary PP, Xu H and Li JD (2003) Up-regulation of interleukin-8 by novel small cytoplasmic molecules of nontypeable Haemophilus influenzae via p38 and extracellular signal-regulated kinase pathways. Infect Immun 71:5523-30.

88. Koto H, Mak JC, Haddad EB, Xu WB, Salmon M, Barnes PJ and Chung KF (1996) Mechanisms of impaired beta-adrenoceptor-induced airway relaxation by interleukin-1beta in vivo in the rat. J Clin Invest 98:1780-7. doi: 10.1172/JCI118977

89. Caramori G, Adcock IM, Di Stefano A and Chung KF (2014) Cytokine inhibition in the treatment of COPD. Int J Chron Obstruct Pulmon Dis 9:397-412. doi: 10.2147/COPD.S42544

90. Mukhopadhyay S, Hoidal JR and Mukherjee TK (2006) Role of TNFalpha in pulmonary pathophysiology. Respir Res 7:125. doi: 10.1186/1465-9921-7-125

91. Yoshida T and Tuder RM (2007) Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. Physiol Rev 87:1047-82. doi: 10.1152/physrev.00048.2006

92. Boorsma CE, Draijer C and Melgert BN (2013) Macrophage heterogeneity in respiratory diseases. Mediators Inflamm 2013:769214. doi: 10.1155/2013/769214

93. Churg A, Wang RD, Tai H, Wang X, Xie C and Wright JL (2004) Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. Am J Respir Crit Care Med 170:492-8. doi: 10.1164/rccm.200404-511OC

94. Rusznak C, Mills PR, Devalia JL, Sapsford RJ, Davies RJ and Lozewicz S (2000) Effect of cigarette smoke on the permeability and IL-1beta and sICAM-1 release from cultured human bronchial epithelial cells of never-smokers, smokers, and patients with chronic obstructive pulmonary disease. Am J Respir Cell Mol Biol 23:530-6. doi: 10.1165/ajrcmb.23.4.3959

95. Hackett TL, Stefanowicz D, Aminuddin F, Sin DD, Connett JE, Anthonisen NR, Pare PD and Sandford AJ (2011) Effect of gene environment interactions on lung function and

cardiovascular disease in COPD. Int J Chron Obstruct Pulmon Dis 6:277-87. doi: 10.2147/COPD.S18279

96. Lee IT and Yang CM (2013) Inflammatory signalings involved in airway and pulmonary diseases. Mediators Inflamm 2013:791231. doi: 10.1155/2013/791231

97. Alam S, Li Z, Atkinson C, Jonigk D, Janciauskiene S and Mahadeva R (2014) Z alpha1-antitrypsin confers a proinflammatory phenotype that contributes to chronic obstructive pulmonary disease. Am J Respir Crit Care Med 189:909-31. doi: 10.1164/rccm.201308-1458OC

98. Al-Omari M, Korenbaum E, Ballmaier M, Lehmann U, Jonigk D, Manstein DJ, Welte T, Mahadeva R and Janciauskiene S (2011) Acute-phase protein alpha1-antitrypsin inhibits neutrophil calpain I and induces random migration. Mol Med 17:865-74. doi: 10.2119/molmed.2011.00089

99. Stoller JK, Lacbawan FL and Aboussouan LS (1993) Alpha-1 Antitrypsin Deficiency. In: Pagon RA, Adam MP, Ardinger HH, Bird TD, Dolan CR, Fong CT, Smith RJH and Stephens K (eds) GeneReviews(R), Seattle (WA)

100. Thun GA, Ferrarotti I, Imboden M, Rochat T, Gerbase M, Kronenberg F, Bridevaux PO, Zemp E, Zorzetto M, Ottaviani S, Russi EW, Luisetti M and Probst-Hensch NM (2012) SERPINA1 PiZ and PiS heterozygotes and lung function decline in the SAPALDIA cohort. PLoS One 7:e42728. doi: 10.1371/journal.pone.0042728

101. Joos L, Pare PD and Sandford AJ (2002) Genetic risk factors of chronic obstructive pulmonary disease. Swiss Med Wkly 132:27-37. doi: 2002/03/smw-09752

102. Stoller JK and Aboussouan LS (2012) A review of alpha1-antitrypsin deficiency. Am J Respir Crit Care Med 185:246-59. doi: 10.1164/rccm.201108-1428CI

103. Woodruff PG, Koth LL, Yang YH, Rodriguez MW, Favoreto S, Dolganov GM, Paquet AC and Erle DJ (2005) A distinctive alveolar macrophage activation state induced by cigarette smoking. Am J Respir Crit Care Med 172:1383-92. doi: 10.1164/rccm.200505-686OC

104. Gill SE and Parks WC (2008) Metalloproteinases and their inhibitors: regulators of wound healing. Int J Biochem Cell Biol 40:1334-47. doi: 10.1016/j.biocel.2007.10.024

105. Shapiro SD (1998) Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 10:602-8.

106. Dvir-Ginzberg M, Gagarina V, Lee EJ, Booth R, Gabay O and Hall DJ (2011) Tumor necrosis factor alpha-mediated cleavage and inactivation of SirT1 in human osteoarthritic chondrocytes. Arthritis Rheum 63:2363-73. doi: 10.1002/art.30279

107. Minematsu N, Nakamura H, Tateno H, Nakajima T and Yamaguchi K (2001) Genetic polymorphism in matrix metalloproteinase-9 and pulmonary emphysema. Biochem Biophys Res Commun 289:116-9. doi: 10.1006/bbrc.2001.5936

108. Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ and Chung KF (2000) Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. Am J Respir Crit Care Med 162:1355-60. doi: 10.1164/ajrccm.162.4.9910097

109. Pryor WA and Stone K (1993) Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Ann N Y Acad Sci 686:12-27; discussion 27-8.

110. Rahman I and MacNee W (1999) Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. Am J Physiol 277:L1067-88.

111. Rahman I and MacNee W (1996) Role of oxidants/antioxidants in smoking-induced lung diseases. Free Radic Biol Med 21:669-81.

112. Hallstrand TS, Hackett TL, Altemeier WA, Matute-Bello G, Hansbro PM and Knight DA (2014) Airway epithelial regulation of pulmonary immune homeostasis and inflammation. Clin Immunol 151:1-15. doi: 10.1016/j.clim.2013.12.003

113. Keane MP and Strieter RM (2002) The importance of balanced pro-inflammatory and anti-inflammatory mechanisms in diffuse lung disease. Respir Res 3:5.

114. Kato A and Schleimer RP (2007) Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. Curr Opin Immunol 19:711-20. doi: 10.1016/j.coi.2007.08.004

115. Barnes PJ (2009) The cytokine network in chronic obstructive pulmonary disease. Am J Respir Cell Mol Biol 41:631-8. doi: 10.1165/rcmb.2009-0220TR

116. Spagnolo P, Grunewald J and du Bois RM (2014) Genetic determinants of pulmonary fibrosis: evolving concepts. Lancet Respir Med 2:416-28. doi: 10.1016/S2213-2600(14)70047-5

117. Randell SH, Boucher RC and University of North Carolina Virtual Lung G (2006) Effective mucus clearance is essential for respiratory health. Am J Respir Cell Mol Biol 35:20-8. doi: 10.1165/rcmb.2006-0082SF

118. Araya J, Cambier S, Markovics JA, Wolters P, Jablons D, Hill A, Finkbeiner W, Jones K, Broaddus VC, Sheppard D, Barzcak A, Xiao Y, Erle DJ and Nishimura SL (2007) Squamous metaplasia amplifies pathologic epithelial-mesenchymal interactions in COPD patients. J Clin Invest 117:3551-62. doi: 10.1172/JCI32526

119. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D and Chapman HA (2006) Alveolar epithelial cell mesenchymal transition develops in vivo

during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci U S A 103:13180-5. doi: 10.1073/pnas.0605669103

120. Hallgren O, Nihlberg K, Dahlback M, Bjermer L, Eriksson LT, Erjefalt JS, Lofdahl CG and Westergren-Thorsson G (2010) Altered fibroblast proteoglycan production in COPD. Respir Res 11:55. doi: 10.1186/1465-9921-11-55

121. Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M and Pardo A (2001) Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. Am J Respir Cell Mol Biol 24:591-8. doi: 10.1165/ajrcmb.24.5.4333

122. Desmouliere A, Geinoz A, Gabbiani F and Gabbiani G (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 122:103-11.

123. Chambers RC, Leoni P, Kaminski N, Laurent GJ and Heller RA (2003) Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. Am J Pathol 162:533-46.

124. Li Q, Park PW, Wilson CL and Parks WC (2002) Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell 111:635-46.

125. Sheppard D (2004) Roles of alphav integrins in vascular biology and pulmonary pathology. Curr Opin Cell Biol 16:552-7. doi: 10.1016/j.ceb.2004.06.017

126. Puente-Maestu L and Stringer WW (2006) Hyperinflation and its management in COPD. Int J Chron Obstruct Pulmon Dis 1:381-400.

127. Khan MA (2013) Inflammation signals airway smooth muscle cell proliferation in asthma pathogenesis. Multidiscip Respir Med 8:11. doi: 10.1186/2049-6958-8-11

128. Chung KF (2000) Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? Eur Respir J 15:961-8.

129. Hirst SJ (1996) Airway smooth muscle cell culture: application to studies of airway wall remodelling and phenotype plasticity in asthma. Eur Respir J 9:808-20.

130. James A and Carroll N (2000) Airway smooth muscle in health and disease; methods of measurement and relation to function. Eur Respir J 15:782-9.

131. Damera G and Panettieri RA, Jr. (2011) Does airway smooth muscle express an inflammatory phenotype in asthma? Br J Pharmacol 163:68-80. doi: 10.1111/j.1476-5381.2010.01165.x

132. Roscioni SS, Prins AG, Elzinga CR, Menzen MH, Dekkers BG, Halayko AJ, Meurs H, Maarsingh H and Schmidt M (2011) Protein kinase A and the exchange protein directly activated by cAMP (Epac) modulate phenotype plasticity in human airway smooth muscle. Br J Pharmacol 164:958-69. doi: 10.1111/j.1476-5381.2011.01354.x

133. Berair R, Saunders R and Brightling CE (2013) Origins of increased airway smooth muscle mass in asthma. BMC Med 11:145. doi: 10.1186/1741-7015-11-145

134. Gerthoffer WT, Schaafsma D, Sharma P, Ghavami S and Halayko AJ (2012) Motility, survival, and proliferation. Compr Physiol 2:255-81. doi: 10.1002/cphy.c110018

135. Redhu NS, Shan L, Al-Subait D, Ashdown HL, Movassagh H, Lamkhioued B and Gounni AS (2013) IgE induces proliferation in human airway smooth muscle cells: role of MAPK and STAT3 pathways. Allergy Asthma Clin Immunol 9:41. doi: 10.1186/1710-1492-9-41

136. Gerthoffer WT (2008) Migration of airway smooth muscle cells. Proc Am Thorac Soc 5:97-105. doi: 10.1513/pats.200704-051VS

137. Johnson PR, Black JL, Carlin S, Ge Q and Underwood PA (2000) The production of extracellular matrix proteins by human passively sensitized airway smooth-muscle cells in culture: the effect of beclomethasone. Am J Respir Crit Care Med 162:2145-51. doi: 10.1164/ajrccm.162.6.9909111

138. Panettieri RA, Jr. (1998) Cellular and molecular mechanisms regulating airway smooth muscle proliferation and cell adhesion molecule expression. Am J Respir Crit Care Med 158:S133-40. doi: 10.1164/ajrccm.158.supplement_2.13tac900

139. Billington CK and Penn RB (2003) Signaling and regulation of G protein-coupled receptors in airway smooth muscle. Respir Res 4:2.

140. Black JL and Johnson PR (2002) Factors controlling smooth muscle proliferation and airway remodelling. Curr Opin Allergy Clin Immunol 2:47-51.

141. Hirst SJ, Barnes PJ and Twort CH (1996) PDGF isoform-induced proliferation and receptor expression in human cultured airway smooth muscle cells. Am J Physiol 270:L415-28.

142. Cohen MD, Ciocca V and Panettieri RA, Jr. (1997) TGF-beta 1 modulates human airway smooth-muscle cell proliferation induced by mitogens. Am J Respir Cell Mol Biol 16:85-90. doi: 10.1165/ajrcmb.16.1.8998083

143. Stamatiou R, Paraskeva E, Gourgoulianis K, Molyvdas PA and Hatziefthimiou A (2012) Cytokines and growth factors promote airway smooth muscle cell proliferation. ISRN Inflamm 2012:731472. doi: 10.5402/2012/731472

144. Wang Q, Li H, Yao Y, Xia D and Zhou J (2010) The overexpression of heparin-binding epidermal growth factor is responsible for Th17-induced airway remodeling in an experimental asthma model. J Immunol 185:834-41. doi: 10.4049/jimmunol.0901490

145. Cargnello M and Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 75:50-83. doi: 10.1128/MMBR.00031-10

146. Clarke D, Damera G, Sukkar MB and Tliba O (2009) Transcriptional regulation of cytokine function in airway smooth muscle cells. Pulm Pharmacol Ther 22:436-45. doi: 10.1016/j.pupt.2009.04.003

147. Gosens R, Zaagsma J, Meurs H and Halayko AJ (2006) Muscarinic receptor signaling in the pathophysiology of asthma and COPD. Respir Res 7:73. doi: 10.1186/1465-9921-7-73

148. Black JL, Panettieri RA, Jr., Banerjee A and Berger P (2012) Airway smooth muscle in asthma: just a target for bronchodilation? Clin Chest Med 33:543-58. doi: 10.1016/j.ccm.2012.05.002

149. Dickson EJ, Falkenburger BH and Hille B (2013) Quantitative properties and receptor reserve of the IP(3) and calcium branch of G(q)-coupled receptor signaling. J Gen Physiol 141:521-35. doi: 10.1085/jgp.201210886

150. Rebecchi MJ and Pentyala SN (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. Physiol Rev 80:1291-335.

151. Fogli S, Stefanelli F, Picchianti L, Del Re M, Mey V, Bardelli C, Danesi R and Breschi MC (2013) Synergistic interaction between PPAR ligands and salbutamol on human bronchial smooth muscle cell proliferation. Br J Pharmacol 168:266-75. doi: 10.1111/j.1476-5381.2012.02180.x

152. Goncharova EA, Ammit AJ, Irani C, Carroll RG, Eszterhas AJ, Panettieri RA and Krymskaya VP (2002) PI3K is required for proliferation and migration of human pulmonary vascular smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 283:L354-63. doi: 10.1152/ajplung.00010.2002

153. Fingar DC, Salama S, Tsou C, Harlow E and Blenis J (2002) Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev 16:1472-87. doi: 10.1101/gad.995802

154. Altomare DA and Khaled AR (2012) Homeostasis and the importance for a balance between AKT/mTOR activity and intracellular signaling. Curr Med Chem 19:3748-62.

155. Krymskaya VP, Orsini MJ, Eszterhas AJ, Brodbeck KC, Benovic JL, Panettieri RA, Jr. and Penn RB (2000) Mechanisms of proliferation synergy by receptor tyrosine kinase and G

protein-coupled receptor activation in human airway smooth muscle. Am J Respir Cell Mol Biol 23:546-54. doi: 10.1165/ajrcmb.23.4.4115

156. Nie M, Pang L, Inoue H and Knox AJ (2003) Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: involvement of different promoter elements, transcription factors, and histone h4 acetylation. Mol Cell Biol 23:9233-44.

157. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS, Jr. and Mayo MW (2000) Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. Mol Cell Biol 20:1626-38.

158. Aravamudan B, Thompson M, Pabelick C and Prakash YS (2012) Brain-derived neurotrophic factor induces proliferation of human airway smooth muscle cells. J Cell Mol Med 16:812-23. doi: 10.1111/j.1582-4934.2011.01356.x

159. Jude JA, Tirumurugaan KG, Kang BN, Panettieri RA, Walseth TF and Kannan MS (2012) Regulation of CD38 expression in human airway smooth muscle cells: role of class I phosphatidylinositol 3 kinases. Am J Respir Cell Mol Biol 47:427-35. doi: 10.1165/rcmb.2012-0025OC

160. Koncina E, Roth L, Gonthier B and Bagnard D (2007) Role of semaphorins during axon growth and guidance. Adv Exp Med Biol 621:50-64. doi: 10.1007/978-0-387-76715-4_4

161. Pasterkamp RJ and Verhaagen J (2006) Semaphorins in axon regeneration: developmental guidance molecules gone wrong? Philos Trans R Soc Lond B Biol Sci 361:1499-511. doi: 10.1098/rstb.2006.1892

162. Dent EW, Gupton SL and Gertler FB (2011) The growth cone cytoskeleton in axon outgrowth and guidance. Cold Spring Harb Perspect Biol 3. doi: 10.1101/cshperspect.a001800

163. Yazdani U and Terman JR (2006) The semaphorins. Genome Biol 7:211. doi: 10.1186/gb-2006-7-3-211

164. O'Malley AM, Shanley DK, Kelly AT and Barry DS (2014) Towards an understanding of semaphorin signalling in the spinal cord. Gene 553:69-74. doi: 10.1016/j.gene.2014.10.005

165. Kumanogoh A and Kikutani H (2003) Immune semaphorins: a new area of semaphorin research. J Cell Sci 116:3463-70. doi: 10.1242/jcs.00674

166. Takamatsu H and Kumanogoh A (2012) Diverse roles for semaphorin-plexin signaling in the immune system. Trends Immunol 33:127-35. doi: 10.1016/j.it.2012.01.008

167. Roth L, Koncina E, Satkauskas S, Cremel G, Aunis D and Bagnard D (2009) The many faces of semaphorins: from development to pathology. Cell Mol Life Sci 66:649-66. doi: 10.1007/s00018-008-8518-z

168. Gitler AD, Lu MM and Epstein JA (2004) PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. Dev Cell 7:107-16. doi: 10.1016/j.devcel.2004.06.002

169. Capparuccia L and Tamagnone L (2009) Semaphorin signaling in cancer cells and in cells of the tumor microenvironment--two sides of a coin. J Cell Sci 122:1723-36. doi: 10.1242/jcs.030197

170. Messina A and Giacobini P (2013) Semaphorin signaling in the development and function of the gonadotropin hormone-releasing hormone system. Front Endocrinol (Lausanne) 4:133. doi: 10.3389/fendo.2013.00133

171. Adams RH and Eichmann A (2010) Axon guidance molecules in vascular patterning. Cold Spring Harb Perspect Biol 2:a001875. doi: 10.1101/cshperspect.a001875

172. Tran TS, Kolodkin AL and Bharadwaj R (2007) Semaphorin regulation of cellular morphology. Annu Rev Cell Dev Biol 23:263-92. doi: 10.1146/annurev.cellbio.22.010605.093554

173. Vanderhaeghen P and Cheng HJ (2010) Guidance molecules in axon pruning and cell death. Cold Spring Harb Perspect Biol 2:a001859. doi: 10.1101/cshperspect.a001859

174. Neufeld G, Sabag AD, Rabinovicz N and Kessler O (2012) Semaphorins in angiogenesis and tumor progression. Cold Spring Harb Perspect Med 2:a006718. doi: 10.1101/cshperspect.a006718

175. Maione F, Molla F, Meda C, Latini R, Zentilin L, Giacca M, Seano G, Serini G, Bussolino F and Giraudo E (2009) Semaphorin 3A is an endogenous angiogenesis inhibitor that blocks tumor growth and normalizes tumor vasculature in transgenic mouse models. J Clin Invest 119:3356-72. doi: 10.1172/JCI36308

176. Worzfeld T and Offermanns S (2014) Semaphorins and plexins as therapeutic targets. Nat Rev Drug Discov 13:603-21. doi: 10.1038/nrd4337

177. Kumanogoh A and Kikutani H (2013) Immunological functions of the neuropilins and plexins as receptors for semaphorins. Nat Rev Immunol 13:802-14.

178. Mann F and Rougon G (2007) Mechanisms of axon guidance: membrane dynamics and axonal transport in semaphorin signalling. J Neurochem 102:316-23. doi: 10.1111/j.1471-4159.2007.04578.x

179. Nasarre P, Gemmill RM and Drabkin HA (2014) The emerging role of class-3 semaphorins and their neuropilin receptors in oncology. Onco Targets Ther 7:1663-87. doi: 10.2147/OTT.S37744

180. Treps L, Le Guelte A and Gavard J (2013) Emerging roles of Semaphorins in the regulation of epithelial and endothelial junctions. Tissue Barriers 1:e23272. doi: 10.4161/tisb.23272

181. Nakamura F and Goshima Y (2002) Structural and functional relation of neuropilins. Adv Exp Med Biol 515:55-69.

182. Roney K, Holl E and Ting J (2013) Immune plexins and semaphorins: old proteins, new immune functions. Protein Cell 4:17-26. doi: 10.1007/s13238-012-2108-4

183. Gelfand MV, Hong S and Gu C (2009) Guidance from above: common cues direct distinct signaling outcomes in vascular and neural patterning. Trends Cell Biol 19:99-110. doi: 10.1016/j.tcb.2009.01.001

184. Banno A and Ginsberg MH (2008) Integrin activation. Biochem Soc Trans 36:229-34. doi: 10.1042/BST0360229

185. Serini G, Maione F, Giraudo E and Bussolino F (2009) Semaphorins and tumor angiogenesis. Angiogenesis 12:187-93. doi: 10.1007/s10456-009-9138-4

186. Artigiani S, Comoglio PM and Tamagnone L (1999) Plexins, semaphorins, and scatter factor receptors: a common root for cell guidance signals? IUBMB Life 48:477-82. doi: 10.1080/713803563

187. Benvenuti S and Comoglio PM (2007) The MET receptor tyrosine kinase in invasion and metastasis. J Cell Physiol 213:316-25. doi: 10.1002/jcp.21183

188. Cagnoni G and Tamagnone L (2014) Semaphorin receptors meet receptor tyrosine kinases on the way of tumor progression. Oncogene 33:4795-802. doi: 10.1038/onc.2013.474

189. Masuda T and Taniguchi M (2015) Congenital diseases and semaphorin signaling: Overview to date of the evidence linking them. Congenit Anom (Kyoto) 55:26-30. doi: 10.1111/cga.12095

190. Gay CM, Zygmunt T and Torres-Vazquez J (2011) Diverse functions for the semaphorin receptor PlexinD1 in development and disease. Dev Biol 349:1-19. doi: 10.1016/j.ydbio.2010.09.008

191. Togashi H, Schmidt EF and Strittmatter SM (2006) RanBPM contributes to Semaphorin3A signaling through plexin-A receptors. J Neurosci 26:4961-9. doi: 10.1523/JNEUROSCI.0704-06.2006

192. Neufeld G and Kessler O (2008) The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nat Rev Cancer 8:632-45. doi: 10.1038/nrc2404

193. Feiner L, Webber AL, Brown CB, Lu MM, Jia L, Feinstein P, Mombaerts P, Epstein JA and Raper JA (2001) Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. Development 128:3061-70.

194. Hayashi M, Nakashima T, Taniguchi M, Kodama T, Kumanogoh A and Takayanagi H (2012) Osteoprotection by semaphorin 3A. Nature 485:69-74. doi: 10.1038/nature11000

195. Dontchev VD and Letourneau PC (2003) Growth cones integrate signaling from multiple guidance cues. J Histochem Cytochem 51:435-44.

196. Fukuda T, Takeda S, Xu R, Ochi H, Sunamura S, Sato T, Shibata S, Yoshida Y, Gu Z, Kimura A, Ma C, Xu C, Bando W, Fujita K, Shinomiya K, Hirai T, Asou Y, Enomoto M, Okano H, Okawa A and Itoh H (2013) Sema3A regulates bone-mass accrual through sensory innervations. Nature 497:490-3. doi: 10.1038/nature12115

197. Catalano A (2010) The neuroimmune semaphorin-3A reduces inflammation and progression of experimental autoimmune arthritis. J Immunol 185:6373-83. doi: 10.4049/jimmunol.0903527

198. Schmidt AM and Moore KJ (2013) The Semaphorin 3E/PlexinD1 axis regulates macrophage inflammation in obesity. Cell Metab 18:461-2. doi: 10.1016/j.cmet.2013.09.011

199. Sabag AD, Bode J, Fink D, Kigel B, Kugler W and Neufeld G (2012) Semaphorin-3D and semaphorin-3E inhibit the development of tumors from glioblastoma cells implanted in the cortex of the brain. PLoS One 7:e42912. doi: 10.1371/journal.pone.0042912

200. Klagsbrun M and Shimizu A (2010) Semaphorin 3E, an exception to the rule. J Clin Invest 120:2658-60. doi: 10.1172/JCI44110

201. Oh WJ and Gu C (2013) The role and mechanism-of-action of Sema3E and Plexin-D1 in vascular and neural development. Semin Cell Dev Biol 24:156-62. doi: 10.1016/j.semcdb.2012.12.001

202. Casazza A, Finisguerra V, Capparuccia L, Camperi A, Swiercz JM, Rizzolio S, Rolny C, Christensen C, Bertotti A, Sarotto I, Risio M, Trusolino L, Weitz J, Schneider M, Mazzone M, Comoglio PM and Tamagnone L (2010) Sema3E-Plexin D1 signaling drives human cancer cell invasiveness and metastatic spreading in mice. J Clin Invest 120:2684-98. doi: 10.1172/JCI42118

203. Luchino J, Hocine M, Amoureux MC, Gibert B, Bernet A, Royet A, Treilleux I, Lecine P, Borg JP, Mehlen P, Chauvet S and Mann F (2013) Semaphorin 3E suppresses tumor cell death triggered by the plexin D1 dependence receptor in metastatic breast cancers. Cancer Cell 24:673-85. doi: 10.1016/j.ccr.2013.09.010

204. Casazza A, Kigel B, Maione F, Capparuccia L, Kessler O, Giraudo E, Mazzone M, Neufeld G and Tamagnone L (2012) Tumour growth inhibition and anti-metastatic activity of a

mutated furin-resistant Semaphorin 3E isoform. EMBO Mol Med 4:234-50. doi: 10.1002/emmm.201100205

205. Tseng CH, Murray KD, Jou MF, Hsu SM, Cheng HJ and Huang PH (2011) Sema3E/plexin-D1 mediated epithelial-to-mesenchymal transition in ovarian endometrioid cancer. PLoS One 6:e19396. doi: 10.1371/journal.pone.0019396

206. Chauvet S, Cohen S, Yoshida Y, Fekrane L, Livet J, Gayet O, Segu L, Buhot MC, Jessell TM, Henderson CE and Mann F (2007) Gating of Sema3E/PlexinD1 signaling by neuropilin-1 switches axonal repulsion to attraction during brain development. Neuron 56:807-22. doi: 10.1016/j.neuron.2007.10.019

207. Thomas G (2002) Furin at the cutting edge: from protein traffic to embryogenesis and disease. Nat Rev Mol Cell Biol 3:753-66. doi: 10.1038/nrm934

208. Varshavsky A, Kessler O, Abramovitch S, Kigel B, Zaffryar S, Akiri G and Neufeld G (2008) Semaphorin-3B is an angiogenesis inhibitor that is inactivated by furin-like pro-protein convertases. Cancer Res 68:6922-31. doi: 10.1158/0008-5472.CAN-07-5408

209. van der Zwaag B, Hellemons AJ, Leenders WP, Burbach JP, Brunner HG, Padberg GW and Van Bokhoven H (2002) PLEXIN-D1, a novel plexin family member, is expressed in vascular endothelium and the central nervous system during mouse embryogenesis. Dev Dyn 225:336-43. doi: 10.1002/dvdy.10159

210. Kim J, Oh WJ, Gaiano N, Yoshida Y and Gu C (2011) Semaphorin 3E-Plexin-D1 signaling regulates VEGF function in developmental angiogenesis via a feedback mechanism. Genes Dev 25:1399-411. doi: 10.1101/gad.2042011

211. Eichmann A, Makinen T and Alitalo K (2005) Neural guidance molecules regulate vascular remodeling and vessel navigation. Genes Dev 19:1013-21. doi: 10.1101/gad.1305405

212. Zygmunt T, Gay CM, Blondelle J, Singh MK, Flaherty KM, Means PC, Herwig L, Krudewig A, Belting HG, Affolter M, Epstein JA and Torres-Vazquez J (2011) Semaphorin-PlexinD1 signaling limits angiogenic potential via the VEGF decoy receptor sFlt1. Dev Cell 21:301-14. doi: 10.1016/j.devcel.2011.06.033

213. Roodink I, Kats G, van Kempen L, Grunberg M, Maass C, Verrijp K, Raats J and Leenders W (2008) Semaphorin 3E expression correlates inversely with Plexin D1 during tumor progression. Am J Pathol 173:1873-81. doi: 10.2353/ajpath.2008.080136

214. Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M and Comoglio PM (1999) Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99:71-80.

215. Gosens R, Stelmack GL, Dueck G, McNeill KD, Yamasaki A, Gerthoffer WT, Unruh H, Gounni AS, Zaagsma J and Halayko AJ (2006) Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 291:L523-34. doi: 10.1152/ajplung.00013.2006

216. Yang J, Chang E, Cherry AM, Bangs CD, Oei Y, Bodnar A, Bronstein A, Chiu CP and Herron GS (1999) Human endothelial cell life extension by telomerase expression. J Biol Chem 274:26141-8.

217. Gosens R, Baarsma HA, Heijink IH, Oenema TA, Halayko AJ, Meurs H and Schmidt M (2010) De novo synthesis of {beta}-catenin via H-Ras and MEK regulates airway smooth muscle growth. FASEB J 24:757-68. doi: 10.1096/fj.09-136325

218. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S and Wright WE (1998) Extension of life-span by introduction of telomerase into normal human cells. Science 279:349-52.

219. Smith EP, Shanks K, Lipsky MM, DeTolla LJ, Keegan AD and Chapoval SP (2011) Expression of neuroimmune semaphorins 4A and 4D and their receptors in the lung is enhanced by allergen and vascular endothelial growth factor. BMC Immunol 12:30. doi: 10.1186/1471-2172-12-30

220. Kigel B, Varshavsky A, Kessler O and Neufeld G (2008) Successful inhibition of tumor development by specific class-3 semaphorins is associated with expression of appropriate semaphorin receptors by tumor cells. PLoS One 3:e3287. doi: 10.1371/journal.pone.0003287

221. Sakurai A, Gavard J, Annas-Linhares Y, Basile JR, Amornphimoltham P, Palmby TR, Yagi H, Zhang F, Randazzo PA, Li X, Weigert R and Gutkind JS (2010) Semaphorin 3E initiates antiangiogenic signaling through plexin D1 by regulating Arf6 and R-Ras. Mol Cell Biol 30:3086-98. doi: 10.1128/MCB.01652-09

222. Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL and Ginty DD (2005) Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. Science 307:265-8. doi: 10.1126/science.1105416

223. Movassagh H, Shan L, Halayko AJ, Roth M, Tamm M, Chakir J and Gounni AS (2014) Neuronal chemorepellent Semaphorin 3E inhibits human airway smooth muscle cell proliferation and migration. J Allergy Clin Immunol 133:560-7. doi: 10.1016/j.jaci.2013.06.011

224. Yawn BP (2009) Differential assessment and management of asthma vs chronic obstructive pulmonary disease. Medscape J Med 11:20.

225. Christensen C, Ambartsumian N, Gilestro G, Thomsen B, Comoglio P, Tamagnone L, Guldberg P and Lukanidin E (2005) Proteolytic processing converts the repelling signal Sema3E into an inducer of invasive growth and lung metastasis. Cancer Res 65:6167-77. doi: 10.1158/0008-5472.CAN-04-4309

226. Korkmaz B, Horwitz MS, Jenne DE and Gauthier F (2010) Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev 62:726-59. doi: 10.1124/pr.110.002733

227. Lalou C, Basak A, Mishra P, Mohanta BC, Banik R, Dinda B and Khatib AM (2013) Inhibition of tumor cells proliferation and migration by the flavonoid furin inhibitor isolated from Oroxylum indicum. Curr Med Chem 20:583-91.

228. Ma YC, Fan WJ, Rao SM, Gao L, Bei ZY and Xu ST (2014) Effect of Furin inhibitor on lung adenocarcinoma cell growth and metastasis. Cancer Cell Int 14:43. doi: 10.1186/1475-2867-14-43

229. Mercapide J, Lopez De Cicco R, Bassi DE, Castresana JS, Thomas G and Klein-Szanto AJ (2002) Inhibition of furin-mediated processing results in suppression of astrocytoma cell growth and invasiveness. Clin Cancer Res 8:1740-6.

230. Jeffery PK (2004) Remodeling and inflammation of bronchi in asthma and chronic obstructive pulmonary disease. Proc Am Thorac Soc 1:176-83. doi: 10.1513/pats.200402-009MS

231. Erle DJ and Sheppard D (2014) The cell biology of asthma. J Cell Biol 205:621-31. doi: 10.1083/jcb.201401050

232. Liesker JJ, Ten Hacken NH, Zeinstra-Smith M, Rutgers SR, Postma DS and Timens W (2009) Reticular basement membrane in asthma and COPD: similar thickness, yet different composition. Int J Chron Obstruct Pulmon Dis 4:127-35.

233. Moldoveanu B, Otmishi P, Jani P, Walker J, Sarmiento X, Guardiola J, Saad M and Yu J (2009) Inflammatory mechanisms in the lung. J Inflamm Res 2:1-11.

234. Allen JC, Seidel P, Schlosser T, Ramsay EE, Ge Q and Ammit AJ (2012) Cyclin D1 in ASM Cells from Asthmatics Is Insensitive to Corticosteroid Inhibition. J Allergy (Cairo) 2012:307838. doi: 10.1155/2012/307838

235. Panettieri RA, Jr. (2004) Effects of corticosteroids on structural cells in asthma and chronic obstructive pulmonary disease. Proc Am Thorac Soc 1:231-4. doi: 10.1513/pats.200402-021MS

236. Bergeron C, Tulic MK and Hamid Q (2007) Tools used to measure airway remodelling in research. Eur Respir J 29:596-604. doi: 10.1183/09031936.00019906

237. Tliba O and Amrani Y (2008) Airway smooth muscle cell as an inflammatory cell: lessons learned from interferon signaling pathways. Proc Am Thorac Soc 5:106-12. doi: 10.1513/pats.200705-060VS

238. Bribian A, Nocentini S, Llorens F, Gil V, Mire E, Reginensi D, Yoshida Y, Mann F and del Rio JA (2014) Sema3E/PlexinD1 regulates the migration of hem-derived Cajal-Retzius cells in developing cerebral cortex. Nat Commun 5:4265. doi: 10.1038/ncomms5265

239. Sakurai A, Doci CL and Gutkind JS (2012) Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer. Cell Res 22:23-32. doi: 10.1038/cr.2011.198