Université de Montréal

Epithelial-Mesenchymal-Transition: a proposed mechanism in the development of Bronchiolitis Obliterans

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Epithelial-Mesenchymal-Transition: a proposed mechanism in the development of Bronchiolitis Obliterans

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Résumé

La transplantation pulmonaire pour les patients avec une maladie pulmonaire en phase terminale est leur seul espoir de survie. Malheureusement, certains greffés du poumon rencontrent des difficultés après la transplantation du poumon, dont l'un est le rejet chronique du greffon pulmonaire également connu histologiquement comme la bronchiolite oblitérante et cliniquement comme syndrome de bronchiolite oblitérante. L'étiologie exacte de la BO reste mal comprise. Certaines hypothèses suggèrent l'implication des cellules épithéliales dans le processus de remodelage des voies respiratoires, conduisant à l'obstruction des voies aériennes. Un des mécanismes proposés est un processus de transition, connue sous le nom de transition épithéliale-mésenchymateuse (TEM). Lors de ce processus, les cellules perdent leurs propriétés épithéliales, acquièrent un phénotype mésenchymateux et deviennent plus mobiles et envahissantes. Cette transformation leur permet de participer activement au processus de remodelage bronchique dans la bronchiolite oblitérante. L'induction de la TEM peut être due à certains facteurs tels que l'inflammation et l'apoptose. Le principal objectif de ce travail de maîtrise est de détecter in vivo la présence de la TEM dans des biopsies transbronchiques obtenues chez des greffés et de l'associer à leurs conditions cliniques. Le deuxième objectif est d'induire la TEM in vitro dans les cellules épithéliales des petites voies aériennes à l'aide de milieux conditionnés apoptotiques et non apoptotiques produits par les cellules endothéliales microvasculaires humaines du poumon. D'autre part, nous avons évalué si des médiateurs connus pour participer au processus de TEM tels que le facteur de croissance du tissu conjonctif (CTGF)et le facteur de croissance transformant bêta

(TGF-beta) ainsi que le perlecan sont présents dans les milieux conditionnés utilisés.

Mots-clés: transplantation pulmonaire, rejet chronique, transition épithéliale-mésenchymateuse, biopsies transbronchiques, apoptose.

Summary

For patients with end-stage lung disease, lung transplantation is their only hope for survival. Unfortunately, some of the lung transplant recipients (LTRs) might face obstacles following lung transplantation, one of which is chronic lung transplant rejection also known as bronchiolitis obliterans (BO) histologically and bronchiolitis obliterans syndrome (BOS) clinically. The exact etiology behind BO development remains poorly understood. Speculations have suggested the involvement of epithelial cells in the airway remodeling process leading to airway obstruction. One of the proposed mechanisms is a transitional process, known as epithelial-mesenchymal-transition (EMT). In this process epithelial cells lose their properties and acquire mesenchymal ones causing them to be more mobile and invasive which allow them to take part of the airway remodeling process in BO. Induction of EMT can be due to several factors such as inflammation, apoptosis. In our study we try to detect in vivo the presence of EMT in transbronchial biopsies (TBB) obtained from LTRs and associates it with their clinical conditions. We also try to manipulate and induce EMT in vitro in small airway epithelial cells (SAEC) using conditioned apoptotic (SSC4h) and non apoptotic (ZVAD) media produced from human microvascular endothelial cells (HMVEC) from lung. In addition, we worked on detecting possible mediators such as connective-tissue growth factor (CTGF), transforming growth factor-beta (TGF-β), and perlecan in produced media.

Keywords: lung transplantation, chronic rejection, epithelial-mesenchymal-transition, transbronchial biopsies, and apoptosis.

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AEC	Airway Epithelial Cells
AIDS	Acquired Immune Deficiency Syndrome
AP-1	Activator Protein-1
Apo2L	Apo2 Ligand
ARDS	Acute Respiratory Distress Syndrome
α-SMA	Alpha-Smooth Muscle Actin
AT1	Alveolar type I Epithelium
AT2	Alveolar type II Epithelium
BALF	Bronchoalveolar Lavage Fluid
BALT	Bronchus-Associated Lymphoid Tissue
bFGF	basic Fibroblast Growth Factor
bHLH	basic Helix-Loop-Helix
BLT	Bilateral Lung Transplantation
BO	Bronchiolitis Obliterans
BOS	Bronchiolitis Obliterans Syndrome
Caspase	*C*ysteine-*Asp*artic Prote*ase*
CD	Cluster of Differentiation
cDNA	complementary Deoxyribonucleic Acid
CF	Cystic Fibrosis
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disorder
CS	Chondroitin 4-Sulfate
CTGF	Connective Tissue Growth Factor
DDR2	Discodin Domain Receptor 2

DISC	Death Inducing Signaling Complex
DR	Death Receptor
EC	Endothelial Cells
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal-Transition
FADD	Fas-Associated Death Domain
FasL	Fas Ligand
FEFL25-75	Mid-Expiratory Flow rate
FEV1	Forced Expiratory Volume in 1 second
FGF	Fibroblast Growth Factor
FSP-1	Fibroblast-Specific Protein-1
GERD	Gastroesophageal Reflux Disease
GM-CSF	Granulocyte-Monocyte Colony-Stimulating Factor
GSK-3β	Glycogen Synthase Kinase-3 beta
HB-EGF	Heparin Binding Epidermal Growth Factor
HLA	Human Leukocyte Atigen
HLT	Heart-Lung Transplant
HMGA2	High-Mobility Group A Protein 2
HMVEC-L	Human Microvascular Endothelial Cells-Lung
IGF-1	Insulin-Like Growth Factor-1
IL	Interleukin
ILEI	Interleukin-Like EMT Inducer
IPF	Idiopathic Pulmonary Fibrosis

LDLL	Living Donor Lobar Lung Transplant
MHC	Major Histocompatibility Complex
MMPs	Matrix Metalloproteinases
mRNA	messenger Ribonucleic Acid
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PAH	Pulmonary Arterial Hypertension
PDGF	Platelet Derived Growth Factor
PGD	Primary Graft Disfunction
PLT	Primed Lymphocyte Testing
Rbm	Reticular Basement Membrane
Rbm	Reticular basement membrane
RTK	Receptor Tyrosine Kinase
SAEC	Small Airway Epithelial Cells
SLBL	Split Lung Bilateral Lobar Transplant
SLT	Single Lung Transplantation
SSC4h	Conditioned Apoptotic Medium
SSC4h+Ab-CTGF	Conditioned Apoptotic Medium + neutralizing antibody against CTGF
SSC4h-ZVAD	Conditioned Non-Apoptotic Medium
TBB	Transbronchial Biopsies
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TRAIL	(TNF)-Related Apoptosis-Inducing Ligand
VSMC	Vascular Smooth Muscle Cells
ZVAD-FMK	Caspase Inhibitor

Dedication

I would like to dedicate my thesis to my supporting parents, whom without; I would not have reached this dream today. Dad, you are the greatest father in the entire world! Saying “thank you” would not be enough for what you have given me. You should know that your constant prayers, precious sacrifices, and endless support did not go to waste, and they were the hidden force that kept me going.

Mom, thank you for being there for me not only as a mother, but also as a friend. Your love, support and most importantly believing in me were my sources of strength when I was weak. Mom, people used to say “it is just a dream” that I will never be able to reach, but guess what! The dream did come true and you are the one who helped in that.

I love you both so much, and I want you both to be proud of me just as I am very proud of you both. This thesis is dedicated to you both, because only you would deserve it and no one else.

Love you,

Areej

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Chapter 1:

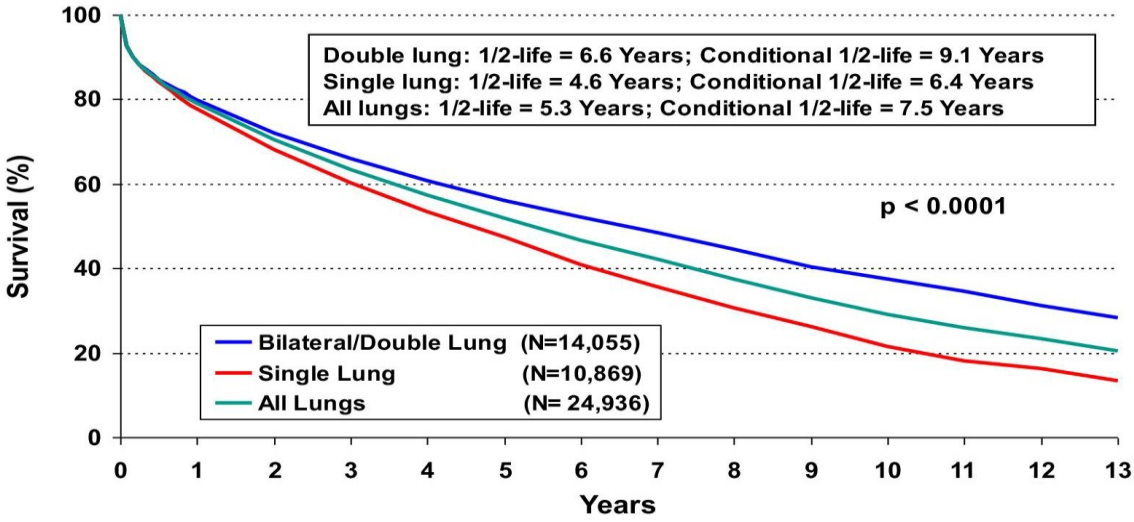
Introduction

1. Introduction:

1.1. Lung transplant as a therapeutical approach

Lung transplantation has been widely accepted as a therapeutic option for patients with end-stage lung disease in order to improve their survival. Up until the year of 1989 the most common form of lung transplantation was the heart-lung transplant (HLT) for patients who suffered pulmonary or cardiac combined with pulmonary end stage disease. Afterwards, single lung transplantation (SLT) and bilateral lung transplantation (BLT) started to pick up to be the most common procedures; however, nowadays the proportion of BLT is over passing the rate of SLT[1, 2] (see figure 1). In addition to previously mentioned procedures, other procedures are being considered as well such as split lung bilateral lobar transplants (SLBL) and living donor lobar lung (LDLL) transplants. According to the annual report 2010 of the International Society for Heart and Lung Transplant registry, the median survival for all adult recipients is 5.3 years, with the double lung procedure having a better survival rate compared to single lung procedure 6.6 years vs. 4.6 years, respectively (Figure 1)[2].

Figure 1: Survival by procedure type for adult lung transplants performed between January 1994 and June 2008:



Kaplan-Meier survival by procedure type for adult lung transplants performed between January 1994 and June 2008. Conditional half-life is the time to 50% survival for the sub-set of recipients who were alive 1 year after transplantation. J.D Christie 2010. J Heart Lung Transplant. 29:1104-1118 © International Society for Heart and Lung Transplantation

End-stage lung disease can be treated with lung transplant; however, types of lung transplant procedures vary and based depending on the type of disease. End-stage lung diseases include: chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), idiopathic interstitial pneumonitis, pulmonary arterial hypertension (PAH), and other rare diseases. COPD is considered to be the most common indication for lung transplantation [2]. For this type of disorder, both SLT and BLT have been suggested as procedures of lung transplant [3-9]. In the United States, the foremost cause of end-stage lung disease is cystic fibrosis (CF); it is the third most common indication for lung transplantation [10, 11]. For most adults with CF, bilateral lung transplant (BLT) would be their best option of lung transplant

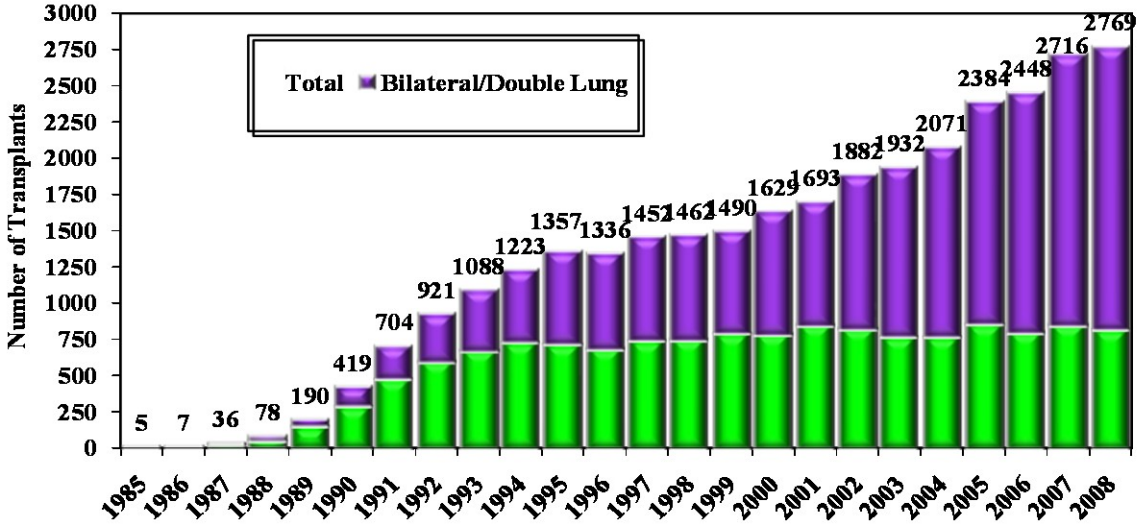
procedure. The heart-lung transplant is rarely considered, depending on the patient's needs, and association with left heart failure [12-15]. Another alternative of lung transplant procedure for CF patients would be living donor lobar lung transplant (LDLL) [16, 17]. This type of procedure is appropriate for children and young patients with CF who are small in size and would attain sufficient lung function from having two adult donor lobes [18]. Furthermore, another end-stage lung disease is idiopathic interstitial pneumonitis (fibrosis) which accounts for 20 percent of lung transplant [2, 19]. One common form of idiopathic interstitial pneumonitis is idiopathic pulmonary fibrosis (IPF) which commonly requires lung transplantation followed by nonspecific interstitial pneumonitis (NSIP) [20-22]. For patients with IPF, single lung transplant was the standard procedure; however, there is an increase in the number of patients undergoing BLT [2, 23-27]. The pulmonary arterial hypertension (PAH) accounts for less than 5 percent of lung transplant[2, 28]. It has been noted that patients with PAH have the highest 30-day mortality rate following lung transplantation In PAH patients, SLT and BLT have been considered as lung transplant procedures, where both were successful but survival comparisons revealed BLT to be more favorable over SLT. The cause and type of end-stage lung disease vary, which influences the approach and procedure of lung transplantation in leading for best results of survival for patients with end-stage lung diseases.

1.1.1. Factors influencing the rate of survival

Despite the fact that lung transplantation serves as the only therapeutic way for patients with end-stage lung diseases, however; it has limitations that might influence its successful outcome. One of the influencing factors on the survival rate of lung

transplant is the type of lung transplant that was chosen to be performed. Most common types of lung transplant include: single lung transplant (SLT), and bilateral lung transplant (BLT). The number of SLT performed annually has remained stable, though; the number of BLT has increased to surpass that of the SLT (Figure 2). As noted before, the original lung disease is a main factor in the selection of preferred lung transplant type which in turn influences the survival of LTR.

Figure 2: Number of lung transplants reported by year and procedure type:

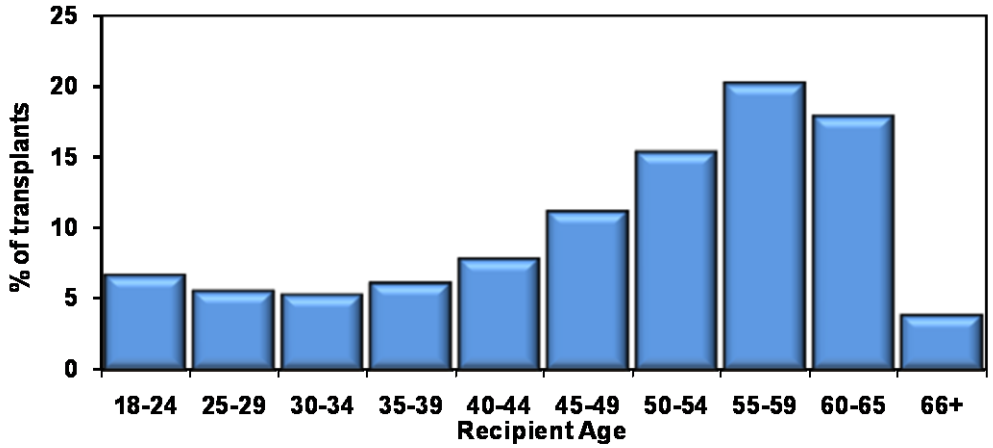


Number of lung transplants reported by year and procedure type. J Heart Lung Transplant. 2010 Oct; 29 (10): 1083-1141[29]

Age of the lung transplant recipient has an influence on the survival rate as well. Since younger recipients have a longer survival than older ones [2, 30], the American Thoracic Society (ATS) has proposed international guidelines to assist in selecting lung transplant candidates [31]. According to the ATS guidelines the upper age limits are as follow: approximately 55 years for HLT, approximately 65 years for

SLT, and approximately 60 years for BLT. This would help in maximizing the chances of survival (Figure 3).

Figure 3: Age distribution of adult lung transplant recipients (1/2985-6/2009):

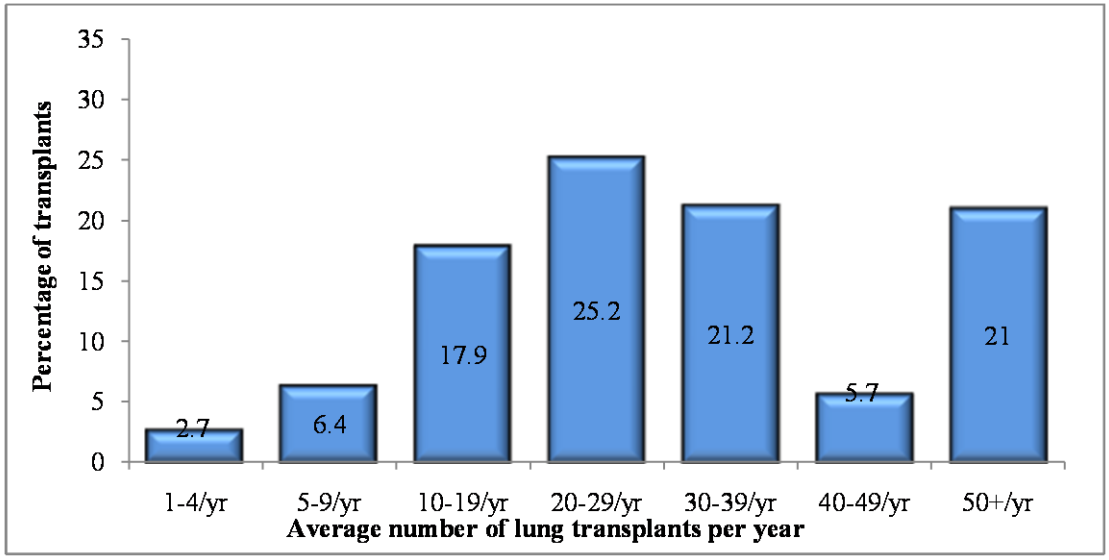


Age distribution of adult lung transplant recipients (1/2985-6/2009). J Heart Lung Transplant. 2010 Oct; 29 (10): 1083-1141[29]

Another key factor that influences the success of lung transplantation is the involvement of transplant organizations and centers in recruiting, placing, and helping patients on the waiting list in getting lung transplant. The process of placement is based on Lung Allocation Score (LAS) in which it would help in addressing high waitlist mortality and earlier placement of patients on the waiting list [32-34]. The LAS system takes into account the urgency measure, which is defined as expected days lived without a transplant with an additional year on the waitlist. In addition, post-transplant survival measure is taken into consideration, which is defined as expected number of days lived during the first year post-transplant. These types of

measures are based and calculated using individual candidate's clinical and physiological characteristics and statistical models [35]. Although the LAS system has been established in the year of 2005, two years following its establishment it has been noted that there was a decrease in the wait-list times and the mean LAS score of transplant recipients has increased. There was an increase in urgency for transplantation, and in the total number of transplanted patients as well [36]. The international society for heart-lung transplant registry (ISHLT) has indicated the distribution of lung transplant by center volume (Figure 4).

Figure 4: Distribution of transplant by center volume. Lung transplants: January 1, 2000- June 30, 2009:



Distribution of transplant by center volume. Lung transplants: January 1, 2000- June 30, 2009. J Heart and Lung Transplant. 2010 Oct; 29 (10): 1083-1141[29]

1.1.2 Complications of lung transplant (LT)

Despite the improvement in the lung transplantation procedure and survival, there have been reports of the occurrence of different types of complications following lung transplant. One major complication following lung transplant (LT) is primary graft dysfunction (PGD), which represents a mulitfactorial injury developing in the first 72 hours following lung transplantation. It has been referred to PGD as "ischemia-reperfusion injury", "early graft dysfunction", and "reimplantation edema" [37]. Characteristics of PGD include: sever hypoxemia, lung edema, and appearance of diffuse pulmonary opacities without other identifiable cause. Furthermore, diffuse alveolar damage serves as the typical pathologic pattern of PGD. It is worth mentioning that PGD is responsible for significant morbidity and mortality after lung transplantation, in spite of advances in organ preservation, surgical technique, and perioperative care [38-41].

Acute rejection is another possible complication that can occur following a lung transplant. Based on previous reports, acute cellular rejection appears less in both organs of the heart-lung transplantation compared to the rate of rejection in lungs or heart that are transplanted alone [2, 42, 43]. Furthermore, it has been noted that the rate of acute cellular rejection in the lung is higher compared to that in the heart following HLT [2, 42, 44, 45]. A proposed explanation of this increase is due to the presence of donor bronchus-associated lymphoid tissue (BALT), increase in the immunogenicity of the lungs, and frequent infectious insults with suppressed defense mechanisms [46].

Chronic rejection is also considered as one of the complications that might be faced following lung transplant. Chronic rejection of the lung is manifested histologically as bronchiolitis obliterans (BO), whereas grading via spirometry testing is known as bronchiolitis obliterans syndrome (BOS)[47] (will be discussed further in 1.2. Chronic Lung Transplant Rejection).

Infection, whether it is viral or bacterial, is considered as a possible complication following lung transplant as well. For instance, infection with cytomegalovirus (CMV) has been suggested as a risk factor for BO in patients undergoing lung transplantation [48-50].

In addition to previously mentioned complications, there are complications that might rise and are not necessarily unique to lung transplantation but rather are side effects of the immunosuppressive medications or general medical problems that are aggravated by the posttransplantation regimen [51]. One of these complications is chronic renal failure that is caused by immunosuppressant such as tacrolimus or cyclosporine [52]. Other complications include: osteoporosis [53-55], systemic hypertension [56], diabetes mellitus [57], obesity, anemia, gastroesophageal reflux disease (GERD) [58, 59], gastroparesis [60, 61], hypercholesterolemia and hypertriglyceridemia, cholecystitis, diverticulitis, weakness of respiratory and limb muscles [62-64], and pulmonary capillaritis [65].

1.2. Chronic Lung Transplant Rejection

Chronic rejection of the lung remains the major source of morbidity and mortality following lung transplant. The clinical syndrome of chronic rejection and

the infectious complications related to its treatment have been defined as major sources of late morbidity and mortality following lung transplant [66]. Chronic rejection has been classified pathologically into two types: chronic vascular rejection and chronic airway rejection [67]. The first type, less common to manifest, refers to a form of atherosclerosis developing in the pulmonary vasculature. The second type, which is more common and morbid, refers to presence of bronchiolitis obliterans (BO) histologically [68].

1.2.1 Bronchiolitis Obliterans (BO) vs. Bronchiolitis Obliterans Syndrome (BOS)

Understanding etiology and mechanism behind chronic lung transplant rejection have been puzzling researchers for several years due to its complexity in its mechanisms, diagnosis and causes of its development. As results of this confusion, a group of investigators from the International Society for Heart and Lung Transplantation has set a standardized nomenclature to help in classifying and diagnosing chronic rejection of lung transplant (see Table I)[47, 69]. They have also made a distinction between bronchiolitis obliterans (BO) and bronchiolitis obliterans syndrome (BOS). The earlier term refers to histological proofs of chronic rejection with scarring and fibrosis of the airways [70, 71]. The latter term refers to deterioration of graft function secondary to progressive airway disease with absence of histologic evidence of BO and no indication of other causes: infection, acute rejection, or anastomotic complications [69, 72].

Table I: Original and proposed classification of BOS:

Original classification		Current proposition	
BOS 0	FEV_1 80 percent or more baseline	**BOS 0**	FEV_1> 90 percent of baseline and FEF_{25-75}> 75 percent of baseline
		BOS 0-p	FEV_1 81 to 90 percent of baseline and/or $FEF_{25-75} \leq$ 75 percent of baseline
BOS 1	FEV_1 66 to 80 percent of baseline	**BOS 1**	FEV_1 66 to 80 percent of baseline
BOS 2	FEV_1 51 to 65 percent of baseline	**BOS 2**	FEV_1 51 to 65 percent of baseline
BOS 3	FEV_1 50 percent or less of baseline	**BOS 3**	FEV_1 50 percent or less of baseline

BOS, bronchiolitis obliterans syndrome; FEF25–75, mid-expiratory flow rate; FEV1, forced expiratory volume in 1 second. Reproduced from: Estenne, M, Maurer, JR, Boehler, A et al. Bronchiolitis Obliterans Syndrome 2001: an update of the diagnostic criteria. J Heart Lung Transplant 2002; 21:297. Copyright©2006 the international Society for Heart and Lung Transplantation.

1.2.2 Risk Factors contributing in the Development of Bronchiolitis Obliterans

Although BO is a manifestation of chronic allograft rejection, other events can contribute in the development of BO. Severe acute rejection has been considered as a risk factor for BO development. Retrospective epidemiologic analyses have demonstrated that occurrence of three or more episodes of acute rejection is a major risk factor for BO development [66, 73, 74]. Moreover, cytomegalovirus (CMV) infection has been described as well as a cause of BO, where retrospective analyses have showed that it might be a risk factor for BO in patients undergoing lung transplantation; however, it was not confirmed in all studies [48-50, 75]. Primary graft dysfunction (PGD), also known as ischemia reperfusion injury, has been

associated with the development of BO, where there is a correlation between the severity of initial PGD with the risk of BO development [76-78]. This correlation can be explained due to presence of oxidative damage, impairment of nitric oxide synthesis by pulmonary endothelial cells, or by upregulation of HLA class II antigens on the allograft leading to production of anti-donor antibodies [79-82]. Another factor that may contribute to chronic allograft rejection is gastroesophageal reflux disease (GERD) which appears to be common in patients following lung transplant. Gastroesophageal dysfunction disease is common in patients with end-stage lung disease prior to lung transplantation and appears to increase following transplant [59, 83-88]. Possible mechanism underlying the risk of GERD may include injury to the vagus nerve and esophagus duringtransplant surgery [89]. Furthermore, type of lung transplant can be a key factor in the development of BO. For example, COPD patients whom underwent double lung transplant were more likely to be free of BO compared to those whom underwent single lung transplant three-years and five-years after transplantation [90]. Finally, autoimmunity has been a possible theory concerning the pathology of BO, which suggests that collagen type V epitopes resulted from ischemia/reperfusion injury or other type of an injury, cause damage of the epithelium of the allograft airway. Another key factor in the autoimmunity development of BO is human leukocyte antigen (HLA) mismatch. This process is characterized by recipient's lymphocytes reactivity towards the donor antigen-specific class I antigens, which was reported by primed lymphocyte testing (PLT) in patients with BO [91, 92]. Production of anti-HLA class I antibodies precedes BO development, and it has been indicated that there is a correlation between an increase in anti-HLA antibodies with loss of pulmonary function [93]. Increase in HLA mismatches between graft and host,

more specifically mismatches at the HLA-A locus, are associated with an enhanced risk of BO [48, 49, 94-96]. It has been mentioned as well that BOS is, for many patients, a recapitulation of the original lung disease for which the transplant was done. Risk factors contributing in the development of BO have been summarized in Table II.

Table II: Risk factors associated with the development of Bronchiolitis Obliterans:

Probable	Potential	Hypothetical
Acute rejection	CMV infection (without pneumonitis)	Underlying cause of lung disease
Lymphocytic bronchitis/bronchiolitis	Organizing pneumonia	HLA-mismatching
CMV pneumonitis	Recurrent infection other than CMV	Gastroesophageal reflux with aspiration
Medication noncompliance	Older donor age	
Primary graft dysfunction	Prolonged allograft ischemia	
	Donor antigen-specific reactivity	

Reproduced from: Estenne, M, Maurer, JR, Bohler, A, et al. Bronchiolitis Obliterans syndrome 2001: An update of thediagnostic criteria J Heart Lung Transplant 2002; 21: 297[47]

1.2.3. Clinical presentation and prognosis of BO

Symptoms that were associated during the development of BO still remain nonspecific, and indolent compared to those of acute rejection [97]. In Table III, the symptoms and signs at both early and late phase of BO are summarized. In usual cases, patients present a syndrome that resembles an upper respiratory tract infection. It remains unclear whether this presentation was based on a misinterpretation of the symptoms or it is further evidence of viral infection role in the etiology of BO. An increase in exertional dyspnea and decline in spirometry are usually noticed in patients. Pulmonary functioning test assists in detecting airflow obstruction. One possible early and sensitive indicator of airflow obstruction is forced expiratory flow between 25 and 75 percent of the vital capacity (FEF 25-75). This indicator appears to be more sensitive than decline in forced expiratory volume in 1 second (FEV1) [47, 98-100].

Table III: Clinical presentation of bronchiolitis obliterans after lung transplantation:

	Early	Late
Symptoms	Non-productive cough; dyspnea on exertion	Productive cough; dyspnea at rest
Physical examination	Clear chest	"Pops and squeaks"
Chest radiography	Clear	Bronchiectasis, hyperinflation
Pulmonary function tests	Obstruction; most marked in mild flows (FEF (25-75))	Severe obstructive
Sputum culture	Negative	Pseudomonas

Reproduced from Reilly, JJ. Chronic lung transplant rejection: Bronchiolitis obliterans. In: UpToDate, Trulock, EP (Ed), UpToDate, Waltham, MA, 2011.

During early stages of BO, physical examination and radiography help in excluding other potential explanation for symptoms. Some of the early BO characteristics include: normal physical examination, clear chest radiograph, sterile sputum cultures or "oral flora" growth. Once BO reaches advanced stages the following characteristics are demonstrated: abnormal chest examination, and end inspiratory pops and squeaks. In addition, brnochiectasis and hyperinflation might be revealed by chest radiograph and computed tomography (CT) [101, 102]. Bronchiectasis presents the symptom at advanced stages with chronic productive

cough, breathlessness, and severe airflow obstruction on pulmonary function testing (Table III).

In the diagnosis of BO two approaches have been set: diagnosis by exclusion and definitive proof. It has been demonstrated in part 1.2.1 that histological proof refers to BO, whereas BOS refers to deterioration of the lung function secondary to an airway disease. In the histological part transbronchial biopsies (TBB) are used in confirming the diagnosis of BO. In one study, it has been reported a sensitivity of 17 percent and specificity of 94.5 percent of a single set of TBB [103]. In a second study, it has been reported a 15 percent histologic confirmation in patients clinically diagnosed with BOS[98]. Furthermore, a third study where TBB were used demonstrated a diagnosis confirmation in 82 percent of patients who developed clinical BOS [74]. Finally, in a fourth study it was noted that among 77 patients who were diagnosed with chronic rejection 52 percent of them had decline in FEV1 [70]. In this study, 9 percent of patients (7 out of 77) had diagnostic biopsies without accompanying physiological abnormalities, whereas 39 percent of patients (30 out of 77) revealed both positive histology and decline in spirometry. Other than the use of TBB and clinical data, several markers have been suggested as potential markers of early BOS. These markers include: neutrophil-predominant alveolitis with an increased levels of interleukin-12 (IL-12) in bronchoalveolar lavage fluid (BALF) [104-107], elevated levels of exhaled nitric oxide [108-110], evidence of air trapping on chest CT scan [111-114], bronchial hyperresponsiveness [115], and soluble CD 30 levels [116].

It is worth mentioning as well that bronchiolitis obliterans (BO) might also be idiopathic. It is characterized by a progressive airflow obstruction that leads to dyspnea, hypercapnia, and death. The importance and severity of BO rises due to its irreversible condition which limits the chances of survival in lung transplant recipients. According to the ISHLT registry[29], BO is considered as a leading cause of mortality with 25.4% in period between 1-3 years posttransplant, and 29.2% in period between 3-5 years posttranstplant.

1.2.4. Treatments of BO

Various approaches have been proposed in the treatment of BO, however; there is no well-established protocol in treating BO. For instance, in one study it was demonstrated that 32 patients with BO presented spirometric stabilization after switching from cyclosporine to tacrolimus over 12 months of follow-up [117]. In another study, similar results were observed when mycophenolate mofetil was introduced[118]. Other data revealed photopheresis to help in stabilizing some BO patients [119]. A report provided by a single center demonstrated possible benefit from aerosolized cyclosporine usage [120]. In an open study, it was noted that substitution of sirolimus by azathioprine was likely to lead of BOS progression in 37 subjects receiving either cyclosporine or tacrolimus [121]. Preliminary reports assessed the value of prolonged oral azithromycin therapy in a total of 34 patients with BOS [122-124]; demonstrating an association with significant improvements in FEV1 for some of the patients. In a larger observational study, 24 out of 81 patients

showed an improvement in FEV1 [125]. Another study evaluating the use of anti-CD 52 antibody Alemtuzumab for BOS revealed stabilization in BOS grade, but not FEV1 in 7 out of 10 patients [126]. Furthermore, according to limited evidence it was suggested that high-dose inhaled glucocorticoids are not effective in slowing or preventing BOS development [127]. Finally, retransplantation has been considered as a treatment of BO. However, early experiences suggest that BO tends to recur in retransplant recipients in an accelerated fashion.

1.3. Apoptosis

All cells have a finite life span which is terminated by cell death that occurs either through passive necrotic processes or as result of an active process of programmed cell death, also known as "apoptosis"[128, 129]. Apoptosis is an important key factor in the maintenance of human embryonic development and adult tissue homeostasis [129]. Apoptosis is a complex and organized machinery that functions in eliminating damaged or unneeded cells in the body [128, 130]. Characteristics of cells undergoing apoptosis include: cell shrinkage, condensation, fragmentation of the nucleus and bubbling of the plasma membrane, known as "blebbing," and chromatin condensation and nucleosomal fragmentation [131]. Furthermore, resulting membrane-bound apoptotic bodies get consumed by either neighboring cells or by macrophages. In normal event, initiation of apoptosis occurs as a response to developmental stimuli such as a decrease in the local concentration of a particular tissue morphogen or growth factor. Other stimulating factors include: severe stress or damage to vital cellular components, which can result from ionizing radiation, heat shock, toxins, cell detachment from surrounding tissue, bacterial or

viral infection, and/or oncogenic signaling [132, 133]. Well functioning apoptotic pathways are essential for tissue homeostasis where dysregulation of it has been implicated in multiple diseases. Increase in apoptosis exacerbates many disorders such as: acquired immunodeficiency syndrome (AIDS), neurodegenerative disorders such as Alzheimer's disease and Huntington's disease, cardiac ischemia, and renal damage [133]. On the other hand, inadequate rate of apoptosis leads to development of cancer and autoimmune diseases [120]. Malfunction of apoptosis is a hallmark in cancer and essential in cancer development and tumor cell survival[134]. This suggests that targeting and manipulating apoptosis can serve as a therapeutic approach in treating cancer and other disorders [133].

Regulation of apoptosis is done via two main pathways: the intrinsic pathway, and the extrinsic pathway, both of which are anticancer therapeutic targets [135-137]. As the name implies, the intrinsic pathway is initiated from within the cell. This usually occurs as a response to cellular signaling due to DNA damage, defective cell cycle, detachment from the extracellular matrix (ECM), hypoxia, loss of cell survival factors, or other types of severe cell stress. Moreover, this pathway involves release of pro-apoptotic proteins that work on activating *c*ysteine-*asp*artic prote*ase* (caspase) enzymes. Activation of the caspase process ultimately triggers apoptosis [135, 138-140]. The intrinsic apoptotic pathway hinges on the balance of activity between pro-apoptotic and anti-apoptotic members of the Bcl-2 superfamily of proteins which work on regulating the permeability of the mitochondrial membrane. Some of the pro-apoptotic proteins include: BIK, BAD, and BIM; and anti-apoptotic include: Bcl-2, Bcl-X_L, and BCLW [138].

The extrinsic pathway starts outside the cell at the activation of specific pro-apoptotic receptors on the cell surface. This activation occurs by binding of specific molecules known as pro-apoptotic ligands which include: CD95L/Fas ligand (FasL), and Apo2 ligand/tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL), where these ligands bind to their cognate receptors CD95/Fas; and death receptor 4(DR4) and death receptor 5 (DR5), respectively [132, 135, 137, 141]. Unlike the intrinsic pathway, the extrinsic pathway triggers apoptosis independently of p53 protein (tumor suppressor protein 53) [142, 143]. Binding of the ligand to its receptor induces receptor clustering and recruitment of the adaptor protein Fas-associated death domain (FADD) and the initiator caspases 8 or 10 as procaspases forming a death-inducing signaling complex (DISC) [144-147]. The DISC formation results in bringing the procaspase molecules into close proximity to one another, leading them to be auto-catalytically processed and released into the cytoplasm where they activate effector caspases 3, 6, and/or 7; and thus, stimulating the intrinsic pathway [136, 148, 149]. The dimerization of caspase 8 is a key factor in its activation, and clustering of the receptors with associated DISC molecules enhance its activation [149]. Once DISC gets activated, the extrinsic pathway follows and adopts same machinery as the intrinsic pathway. Furthermore, it has been known that extrinsic pathway activation through binding of CD95L/FasL to CD95/Fas can result in two apoptotic programs, termed type I and type II. In the former type, cells are able to overcome the need for mitochondrial amplification of the death signal in CD95-mediated process by producing sufficient amounts of caspase 8 at the DISC which results in direct cleavage and activation of effector caspases and executes cell death [150]. Therefore, in type I cells bypass the mitochondrial involvement in CD95-

mediated apoptosis, expression of Bcl-2 or Bcl-X_L has no inhibitory effect on their apoptotic program. On the other hand, in type II cells active caspase 8 is produced at a minimal amount at the DISC and requires the mitochondrial amplification of the CD95 signal [150]. Amplification of this signal might be through the pro-apoptotic BH3 domain, which only contains the Bcl-2 family member, Bid [150]. Bid gets cleaved by caspase 8 resulting in its translocation to the mitochondria where it initiates the release of mitochondrial factors, leading to increase in cell death. Since type II cells depend on the apoptotic function of mitochondria, expression of Bcl-2/Bcl-X_L confers protection from apoptosis mediated by CD95 [150]. Differences between type I and type II cells remain unclear and need to be further studied and investigated.

1.3.1. Apoptosis and Fibrosis/Tissue Remodelling

Apoptosis of endothelial cells (EC) has been recognized as an early pathogenic event in fibrosis [151]. Increase in apoptotic EC has been associated with several fibrogenic disorders such as systemic sclerosis [152, 153], graft-versus-host disease [154, 155], and chronic rejection of solid allografts [156, 157]. The involvement of apoptotic EC was explained by its role in recruiting professional phagocytes such as macrophages [158]. As a result of apoptotic cell engulfment by the macrophages, transforming growth factor-beta1 (TGF-β1) gets produced [159]. The produced TGF-β1 stimulates myofibroblast differentiation and resistance to apoptosis in fibroblasts and myofibroblasts[57]. Furthermore, apoptotic endothelial cells have been suggested to have a direct impact in fibrogenesis by producing

paracrine mediators, such as TGF-β, connective tissue growth factor (CTGF), and perlecan (will be discussed in 1.4.4) that have been suggested to stimulate differentiation and resistance to apoptosis in fibroblasts [160, 161].

In the event of an injury, the process of wound healing is activated, such as endothelial damage; fibroblasts accumulate at the site of injury and differentiate into myofibroblasts, a fibroblast type characterized by presence of stress fiber and alpha-smooth-muscle actin (α-SMA)[162]. Normally, myofibroblasts undergo apoptosis once the healing process is terminated. Fibrosis follows the same pattern of wound healing; however, myofibroblasts develop resistance towards apoptosis which prevents their clearance, leads to accumulation of myofibroblasts and tissue contraction, which results in deformation and loss of function [163, 164]. Alteration in apoptosis' rate, whether it is in epithelial or endothelial cells, is a feature that has been implicated in several lung pathogenesis such as idiopathic pulmonary fibrosis (IPF) [165], acute respiratory distress syndrome (ARDS) [166], and bronchiolitis obliterans organizing pneumonia [167].

1.3.2 Apoptosis and Bronchiolitis Obliterans

In lung transplantation, the implication of apoptosis was investigated in ischemia-reperfusion injury which was associated with endothelial apoptosis [168-171] and in transbronchial biopsies obtained from patients undergoing acute or chronic lung allograft rejections have been associated with epithelial and macrophages apoptosis[172-174]. In BO after lung transplantation, it has been

mentioned that the main target of rejection is the bronchial epithelium [175], where apoptosis is suggested as the mode of cell death. Apoptotic cells and their role in allograft rejection and development of BO are still under investigation [176].

Previous studies demonstrated the importance of airway epithelial cells (AEC) as immunologic targets during the process of acute or chronic lung allograft rejection [177-179]. Activated epithelial cells result in production of various growth factors including: epidermal growth factor (EGF) [180], heparin binding EGF (HB-EGF) [179], basic fibroblast growth factor (bFGF) [179], granulocyte-monocyte colony-stimulating factor (GM-CSF) [181], insulin-like growth actor1 (IGF-1) [182], platelet-derived growth factor (PDGF), and TGF-β [179]. Studies have noted the involvement of these growth factors in inducing proliferation of fibroblasts and smooth muscle cells indicating their potential role in fibrogenic activity *in vivo* [182-189]. In addition, studies have reported elevated levels of PDGF, TGF-β, and IGF-1 during the development of BOS after lung transplantation [190-198]. However, cellular sources and stimuli for fibrogenic growth factor production during the process of BOS development remain unknown. Studies have reported development of anti-HLA class I antibodies as a predisposing factor in BOS development after lung transplantation [93]. In addition, the association of anti-HLA class I antibodies has been linked with the development of transplant atherosclerosis and graft loss after kidney and heart allograft transplantation [199, 200]. Since binding of anti-HLA induce proliferation and apoptosis of AEC, binding of anti-major histocompatibility complex (MHC) has been associated with apoptotic cell death of activated human T

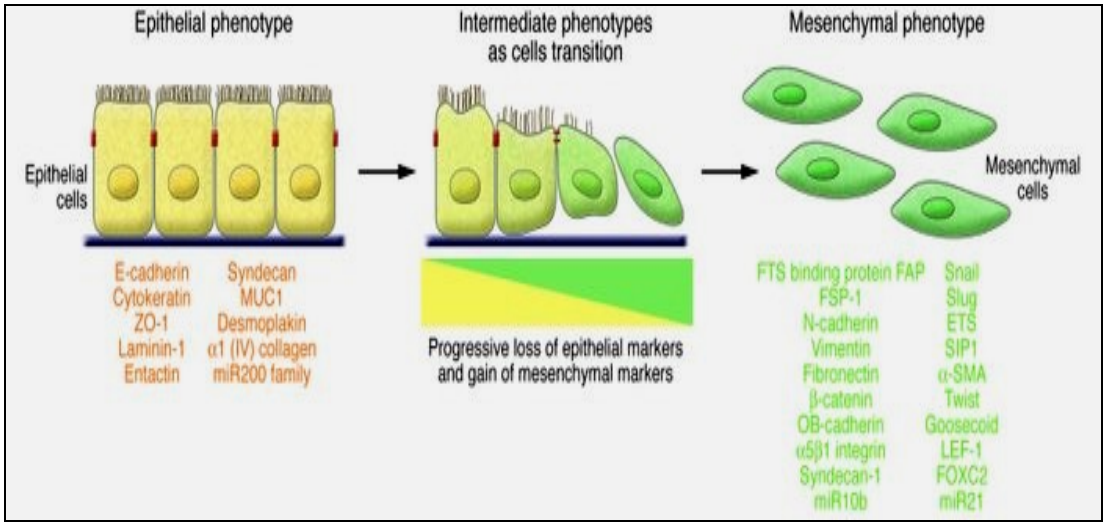
and B lymphocytes [201, 202], and of cardiovascular origin cells such as endothelial cells, smooth muscle cells, fibroblasts and monocytes [203]. The contribution of apoptosis has been noted in acute and chronic rejection of heart, lung, kidney, and liver allograft [172, 204-206]. Furthermore, studies have revealed increased levels of AEC apoptosis in lung allografts of patients with BOS [173]. In experimental lung transplantation (LT), an association between ischemic-reperfusion and endothelial cells was noted [169-171]. In one of our recent studies [207], we demonstrate the involvement of airway endothelial and epithelial apoptosis in the pathogenesis of BO, where the triggering factor of apoptosis initiation needs to be further investigated in order to help and improve the outcome of lung transplantation.

1.4 Epithelial-Mesenchymal-Transition (EMT)

The mystery behind the origin of mesenchymal cells that participate in tissue repair and pathological processes, tissue fibrosis, tumor invasiveness, and metastasis, is poorly understood. An important providing source that has been proposed for the generation of mesenchymal cells is epithelial-mesenchymal-transition (EMT). This process is defined as a transdifferentiating process where it allows an intact polarized epithelial cell, which has specific interaction with the basement membrane via its cell surface, to undergo multiple biochemical changes that enable it to assume properties of mesenchymal cells. The properties of mesenchymal cells include: enhanced

migratory capacity, invasiveness, elevated resistance to apoptosis, and increase the production of extracellular matrix (ECM) components [208]. Once the EMT process has been completed, it signals degradation of the underlying basement membrane, the newly formed mesenchymal cell migrates away from its original epithelial layer. Initiation and activation of the EMT process takes an orchestrated manner where number of complex molecular processes are involved in it. Such an organized process involves ordered steps: activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskelatal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs. The involved factors can be used as biomarkers to help in assisting the passage of a cell through an EMT (Figure 5) [209].

Figure 5: Epithelial-Mesenchymal-Transition:



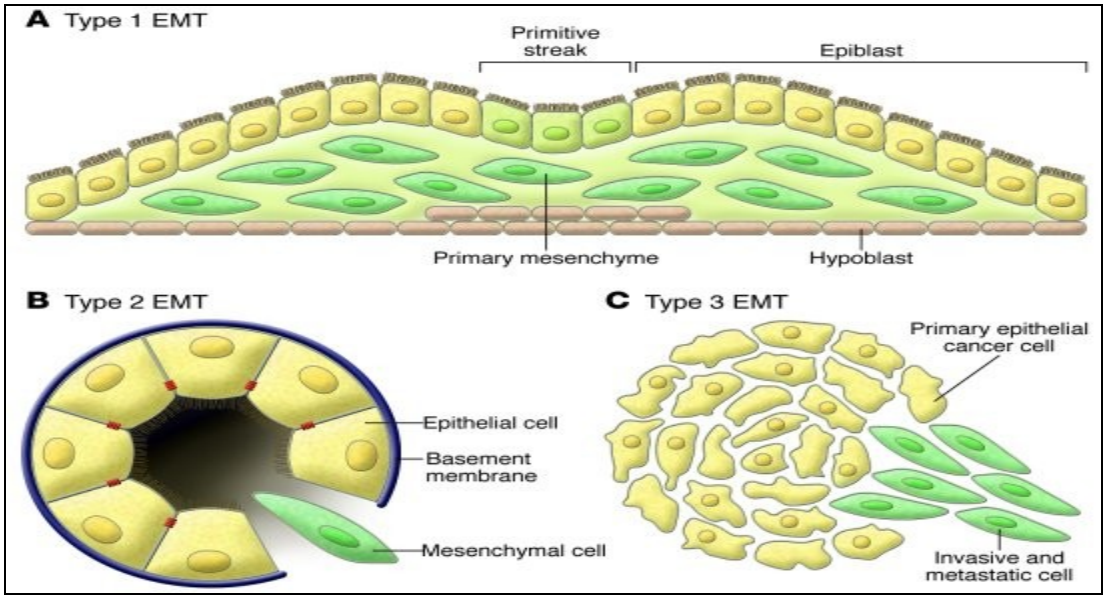
An EMT involves a functional transition of polarized epithelial cells into mobile and ECM component–secreting mesenchymal cells. The epithelial and mesenchymal cell markers commonly used by EMT researchers are listed. Colocalization of these two sets of distinct markers defines an intermediate phenotype of EMT, indicating cells that have passed only partly through an EMT. Detection of cells expressing both sets of markers makes it impossible to identify all mesenchymal cells that originate from the epithelia via EMT, as many mesenchymal cells likely shed all epithelial markers once a transition is completed. For this reason, most studies in mice use irreversible epithelial cell–lineage tagging to address the full range of EMT-induced changes. ZO-1, zona occludens 1; MUC1, mucin 1, cell surface associated; miR200, microRNA 200; SIP1, survival of motor neuron protein interacting protein 1; FOXC2, forkhead box C[209]. Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest **119**(6): 1420-1428

Based on previous studies done on developmental biology, during embryogenesis and organ development, certain epithelial cells appear to be plastic and thus able to move back and forth between the epithelial and mesenchymal states via the processes of EMT and MET (mesenchymal-epithelial-transition) [210]. This suggests that occurrence of such transdifferentiation allows conversion of epithelial cells to mesenchymal derivatives is needed during embryo development and adulthood. Activation of EMT has been also associated with tissue repair and

pathological stresses. This leads to recognizing EMT as a key player in dispersing cells in embryos, forming mesenchymal cells in injured tissues, and initiating the invasive and metastatic behavior of epithelial cancers (R. Kalluri) [209].

The epithelial-mesenchymal-transition (EMT) has been classified into three distinct biological classes leading to different functional consequences. The first class of EMTs, type I (Figure 6), represents the process that is associated with implantation, embryo formation, and organ development which leads to an organized generation of diverse cell types that share common mesenchymal phenotypes [211]. This class of EMTs is neither involved in fibrosis nor induces an invasive type which spreads out via the circulation system. In addition, the produced mesenchymal cells can switch back to epithelial cells through MET, leading to generation of secondary epithelial cells.

Figure 6: Different types of Epithelial-Mesenchymal-Transition:



(A) Type 1 EMT is associated with implantation and embryonic gastrulation and gives rise to the mesoderm and endoderm and to mobile neural crest cells. The primitive epithelium, specifically the epiblast, gives rise to primary mesenchyme via an EMT. This primary mesenchyme can be re-induced to form secondary epithelia by a MET. It is speculated that such secondary epithelia may further differentiate to form other types of epithelial tissues and undergo subsequent EMT to generate the cells of connective tissue, including astrocytes, adipocytes, chondrocytes, osteoblasts, and muscle cells. (B) EMTs are re-engaged in the context of inflammation and fibrosis and represent the type 2 EMTs. Unlike the type 1 EMT, the type 2 EMT is expressed over extended periods of time and can eventually destroy an affected organ if the primary inflammatory insult is not removed or attenuated. (C) Finally, the secondary epithelia associated with many organs can transform into cancer cells that later undergo the EMTs that enable invasion and metastasis, thereby representing type 3 EMTs.[209] Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest **119**(6): 1420-1428.

The second class of EMTs, type II (Figure 6), represents EMTs that are associated with wound healing, tissue generation and organ fibrosis. In this type of EMTs, the process starts out as a normal “repair” process that needs to generate

fibroblasts and other coupled cells in order to help in reconstructing the damaged area following an insult or inflammation. However, in contrast to type I EMTs, type II EMTs are activated as a response to inflammation and cease once the inflammation is attenuated, this is noted in wound healing and tissue repair. In the case of fibrosis, type II EMTs continue on as a response to ongoing inflammation, which leads to destruction of organs. In other words, tissue fibrosis is an unabated form of wound healing process due to persistent inflammation. The third class of EMTs (Figure 6) represents EMTs that occur in neoplastic cells that have already undergone through genetic and epigenetic changes that result in clonal outgrowth and localized tumors. These outcomes are due to alterations of oncogenes and tumor suppressor genes, thus the result is different from those of the other two types of EMTs.

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1.4.1 Implication of Epithelial-Mesenchymal-Transition in the pathologies of fibrosis

Since our work explore the implication of EMT in the formation and development of bronchiolitis obliterans (BO), we will be focusing on type II of EMTs which is associated with tissue regeneration and organ fibrosis. The organ fibrosis is triggered by inflammatory cells and resident fibroblasts that function to release a variety of inflammatory signals and extracellular matrix (ECM) components that include collagens, laminins, elastin, and tenacins [209]. The type II EMTs which leads to organ fibrosis has been associated with fibrosis occurring in kidney, liver, lung and intestine [212-215]. Such an association was proved by studies done on

transgenic mice that bear germ-line reporter genes whose expression was driven by specific promoters. Follow up of the expression of these reporters provided evidence for the involvement of epithelial cells as key promoter and generator of fibroblasts in organ fibrosis, via EMT [216-218]. Several biomarkers, such as fibroblast-specific protein 1(FSP-1), α-SMA and collagen I, are generated by the EMT process which leads to fibrosis of several organs [9, 10, 219]. In addition to previously mentioned biomarkers, other markers such as discodin domain receptor tyrosine kinase 2 (DDR2), vimentin, and desmin have been studied in identifying the epithelial cells of kidney, liver, lung and intestine that are mid-way through EMT associated with inflammation. What was noted is that cells at this stage not only show epithelial-specific morphology and molecular markers such as cytokeratin and E-cadherin, but also express mesenchymal markers FSP-1 and α-SMA. Possible explanation of such behavior is that these cells are likely to be at an intermediate phase of EMT, where epithelial markers are still expressed but mesenchymal ones are being acquired as well. This behavior serves as an early indication or prediction of epithelium being exposed to an inflammatory stress. Once these cells "leave their epithelial layer, negotiate their way through the underlying basement membrane, and accumulate in the interstitium of the tissue, they shed all of their epithelial markers and gain fibroblastic ones"[220].

1.4.2 Implication of Epithelial-Mesenchymal-Transition in lung

As mentioned before, EMT has been proposed as a possible contributor in the fibrosis of kidney, liver, and intestine [5-8]. This had lead to propose the possible involvement of EMT in the pathogenesis of lung fibrosis. The exact role of EMT as a

response to an injury and pathogenesis of fibrosis in the adult lung remains to be further studied. Evidence has suggested participation of EMT as a major source of pathogenic mesenchymal cells, such as myofibroblasts, that lead to the development of pulmonary fibrosis. EMT was identified in both the alveolar epithelium and airway epithelium. Alveolar epithelial cells have been proposed as a key pathogenic intermediary of idiopathic pulmonary fibrosis (IPF) [221-223]. The importance of alveolar epithelial cells rises due to its regulatory functions that involve: production and response to profibrotic mediators, regulation of fibroblasts' functions and differentiation through release of mediators, and modification of cell morphology and gene expression in response to injury [224-232]. Alveolar epithelial cells in IPF demonstrate the following features: morphological abnormality, pneumocytes hyperplasticity, and reactivity of elongated cells overlying the fibroblastic foci, which is presumed to be the site of active fibrogenesis [221, 233-235]. In addition, expression patterns of cytokeratin have been altered [236], and apoptosis of alveolar epithelial cells adjacent to fibroblastic foci has increased [237-239]. Upon activation of the alveolar epithelial cells in IPF synthesis of several procoagulant factors [229], and fibrogenic cytokines, such as PDGF [224], TGF-β [226-228], TNF-α [240], endothelin-1 [225], and CTGF [232] get produced which allows for a bidirectional signaling between alveolar epithelial cells and fibroblasts. The alveolar epithelial cells also stimulate production of matrix metalloproteinases (MMPs), which suggests contribution of alveolar epithelial cells in the extracellular matrix remodeling [241, 242].

Studies done on lung explants of transplanted mice models revealed that lung fibrosis can be initiated with epithelial injury and irregular repair mechanisms even in the absence of inflammation, and the presence of an intact epithelial layer has suppressed fibroblast proliferation and matrix deposition [243, 244]. Further confirmation was done by *in vivo* studies which provide evidence of the importance of EMT in fibrosis. These studies have used Cre-LOX system with β-galactosidase (β-gal) tagging, alveolar type II epithelium (AT2) has shown an expression of vimentin and undergo EMT when exposed to overexpression of TGF-β1 [215]. It has been noted that vimentin-positive cells within injured lungs were all β-gal positive, which suggests epithelial cells to be a reservoir of mesenchymal cells. Further reports done on AEC obtained from mice fibrotic lung overexressing insulin-like growth factor- binding protein-5 (IGFBP-5) coexpresses the expression of epithelial markers and α-SMA, suggesting EMT [245]. Airway epithelium has been investigated as potential contributor of intrapulmonary fibroblasts and myofibroblasts as a response to injury. Fibrotic obstruction of small and large airways is a key pathologic contributor in a variety of disorders, such as asthma [246], and obliterative bronchiolitis [247]. For instance, asthma is characterized with airway remodeling that can cause the disease and occur independently of inflammation [248, 249]. In recent studies, it has been suggested that abnormal epithelial-mesenchymal response to environmental challenges has a major role in the pathology of airways and physiology of asthma [250]. An increase in the deposition of collagen, fibronectin, and other ECM proteins was observed in asthma, which leads to subepithelial fibrosis and airway hyeperresponsiveness [251]. Possible explanation of the production of these

proteins is due to the presence of fibroblasts and myofibroblasts, where the number of these cells showed a correlation with the magnitude of subepithelial thickness [252].

1.4.3 Epithelial-Mesenchymal-Transition and Bronchiolitis Obliterans

Since understanding the pathogenesis of airway remodeling is needed for therapeutic development. One of the unresolved and poorly understood airway disorders is bronchiolitis obliterans (BO). BO is defined pathologically as airway response to chronic allograft rejection, and physiologically as bronchiolitis obliterans syndrome (BOS). This disorder is characterized by being an irreversible process which leads the patient to direct morbidity and mortality [47, 253]. Etiology behind BO is still poorly understood and still under constant investigation, but it is suggested to be a result of epithelial response upon an injury by an immunological or non-immunological events [254]. As mentioned earlier, remodeling and fibrotic obstruction of the small airways are key pathological factors in BO [34, 35]. Not only the precise mechanism of BO remains mysterious, but also the origin of fibroblasts responsible for airway fibrosis is unknown and serves as an important role in knowing the basic mechanisms. Up to the present date, only one study of its kind, Ward et al.[255], has suggested a link between lung transplant recipients (LTRs) and EMT. In this study it has been noted that airway epithelial cells obtained from stable LTRs have exhibited features of EMT. They were able to detect positive staining of fibroblast specific protein-1 (FSP-1), a marker of mesenchymal phenotype, in 15% of the sampled epithelium sampled. In addition, stimulation of obtained epithelium with

TGF-β caused cells to become more motile and penetrate collagen-coated filters. What is important of these results is the fact that these changes were noted prior to the development of detectable clinical deterioration of lung function. Therefore, this study suggests early detection and intervention of EMT in the LTRs airways can help in reducing airway remodeling and its severity. However, prevalence and mechanism of EMT in airway fibrosis and potential inhibition of it are being further investigated.

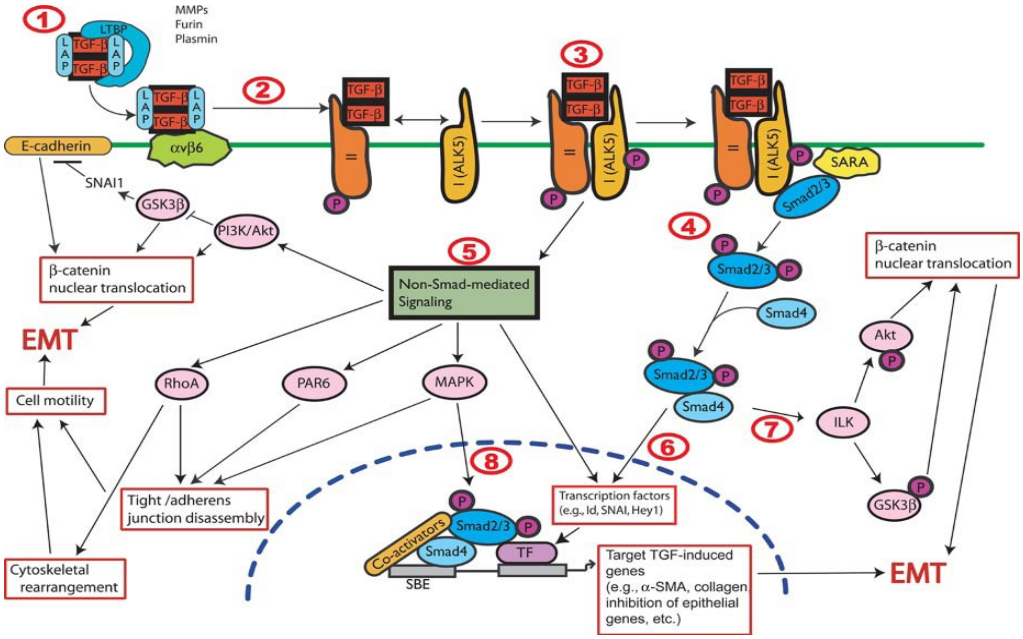
1.4.4 Mediators produced during apoptosis and Epithelial-Mesenchymal-Transition

Apoptosis, defined as programmed cell death, has been shown to contribute in human acute and chronic rejection of the human heart [22], lung [23], kidney [24], and liver [25]. The exact mechanism remains ambiguous to researchers. Upon completion of repair mechanism, apoptotic cells signal for phagocytes, such as macrophages, to assure engulfment of these cells [156, 158]. This results in production of mediators that work on activating other pathways. One of these mediators is transforming growth factor-beta (TGF-β) [159].

Transforming growth factor-beta is a critical cytokine because of its multifunctional actions that regulate tissue morphogenesis and differentiation by having direct influence on cell proliferation, differentiation, apoptosis, and ECM production [256]. In addition, TGF-β is a key inducer of EMT in development, carcinogenesis, and fibrosis with different isoforms and functions [257]. EMTs of different epithelial cells including renal proximal tubular, lens, and alveolar epithelial

cells are modulated by TGF-β [258-262], see figure 3 for TGF-β mechanism). In the tissue fibrosis TGF-β has been implicated as "master switch" in many tissues including lung [263]. In regard to fibrosis, upregulation of TGF-β in lungs of patients with idiopathic pulmonary fibrosis (IPF) has been observed, and expression of active TGF-β in lungs of rats induced a dramatic fibrotic response, whereas inability to respond to TGF-β provides protection from bleomycin-induced fibrosis [264]. In addition, TGF-β is a key inducer of EMT in development, carcinogenesis, and fibrosis with different isoforms and functions [257]. EMTs of different epithelial cells including renal proximal tubular, lens, and alveolar epithelial cells are modulated by TGF-β [258-261, 265], (see Figure 7 for TGF-β mechanism). Mentioned previously, studies have demonstrated the involvement of TGF-β *in vivo* (see 1.4.2) in inducing EMT in animal models. Therefore, these indications prove the involvement and importance of TGF-β in inducing EMT both *in vivo* and *in vitro* which lead to fibrosis of the lung.

Figure 7: Mechanisms of Transforming growth factor-beta1 in inducing Epithelial-Mesenchymal-Transition:



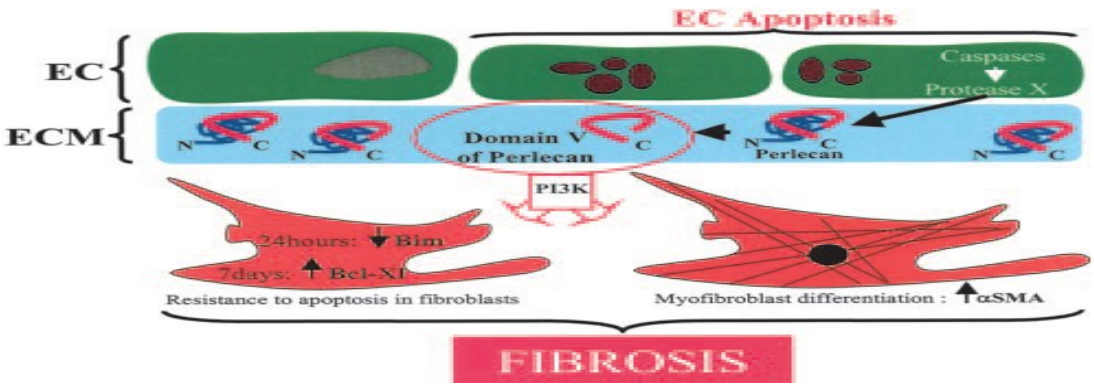
EMT can be induced by a wide array of stimuli and cytokines, TGF-β is considered to be a "master switch" of the process. The majority of TGF-β is present in the extracellular milieu in a latent form, kept inactive by the latency-associated peptide (LAP), and bound by the latent TGF binding protein (LTBP) (*1*). Upon release by LTBP, LAP-associated TGF is either freed through proteolysis by a variety of enzymes (e.g., plasmin) or is stabilized by membrane-bound integrins (e.g., αvβ6) and directly presented to TGF receptors (*2*). TGF-β dimers then associate with the type II TGF-β receptor that in turn associates with the type I TGF- β receptor (e.g., ALK-5) in a heterodimer (*3*). The receptor heterodimer becomes activated and initiates a variety of signaling pathways, resulting in both transcriptional and nongenomic signaling. Both Smad-mediated (Smad2 and Smad3) (*4*) and non-Smad-mediated (*5*) pathways are involved. Smad-mediated pathways result in activation of TGF-β-induced target genes (e.g., α-smooth muscle actin, collagen, plasminogen activator inhibitor-1, connective tissue growth factor, and others) as well as inhibition of epithelial genes (e.g., E-cadherin) (*6*), through activation/induction of and coassociation with a variety of transcription factors (including Snail1, Snail2, Notch, and others) and subsequent binding to Smad-binding elements. Smad-mediated signaling can also activate nongenomic signaling molecules, such as ILK (*7*), which leads to Akt and GSK3B activation and β-catenin nuclear translocation, contributing to EMT. Non-Smad-mediated pathways are numerous but include PI3K/Akt, RhoA, PAR6, and MAPK activation, leading to a host of cellular changes, including tight/adherens junction disassembly, cytoskeletal rearrangements, E-cadherin downregulation, β-catenin nuclear translocation, and EMT (*5*). Finally, non-Smad-mediated signaling pathways can interact with Smad-mediated genomic signaling through modulation and activation of transcription factors (e.g., through MAPK) (*8*).[265]

Apoptotic cells, such as apoptotic endothelial cells, produce mediators that are apoptosis pathway-dependent and can activate the EMT process. Connective tissue growth factor (CTGF) was identified as one mediators produced by apoptotic endothelial cells. Dr. Hebert and her team were able to identify CTGF as a protein with fibrogenic activity in medium conditioned by apoptotic endothelial cells (EC) using mass spectrometry and confirming their finding by Western blotting [151]. In their work, they were able to demonstrate that CTGF production occurs through a caspase-3-dependent pathway. Furthermore, they have indicated that the production or release of CTGF is independent of the apoptotic stimulus, and is specifically regulated at the execution phase of apoptosis. CTGF, a 37-kDa cysteine-rich peptide, is a member of the CCN family of matricellular proteins [266]. The N-terminal of this peptide promotes myofibroblast differentiation and collagen synthesis, whereas the C-terminal of it is implicated in proliferation and adhesion [267]. It has been noted previously that CTGF potentiates interactions between low levels of TGF-β1 and its cognate receptor [268]. "We consider the possibility of TGF-β1-dependent signaling in this fibrogenic loop, even in the absence of elevated amounts of TGF-β1. Blockage of TGF-β signaling with a pan-TGF-β-neutralizing antibody did not prevent myofibroblast differentiation induced by either SSC (conditioned apoptotic medium) or recombinant CTGF [151]. Studies have identified CTGF as a fibrotic marker in chronic renal and heart allograft rejection, diseases that are associated with sustained endothelial injury [269, 270]. Blockage of CTGF expression by using siRNAs (small interfering RNAs) showed to prevent fibrosis in a model of chronic renal allograft rejection [271]. These data prove the fact of recognizing CTGF as a key fibrotic

mediator in several fibrotic diseases. Pathways involved in the production of CTGF should be further investigated to provide a clear picture of its involvement.

In addition to CTGF production, apoptotic endothelial cells have been proposed to produce other bioactive mediators. The team of Dr. Hebert worked on fractionating the conditioned apoptotic medium (SSC4h), which yielded one bioactive fraction with a size of ~23 kDa and identical to the C-terminal domain V of perlecan. Perlecan is a 467 kDa proteoglycan composed of five distinct domains [272-274]. The most important element of perlecan is the C-terminal domain of perlecan (domain V), that contains an anchoring site for chondroitin sulfate and three laminin-type G modules separated by four EGF-like modules [275]. In their work they state that bioactive fraction of perlecan produced from conditioned apoptotic medium included only the C-terminal part. Furthermore, other fractions obtained from the apoptotic medium that were higher than 50kDa showed no bioactivity on vascular smooth muscle cells (VSMC), suggesting that native perlecan is not implicated in the bioactivity imposed by the conditioned apoptotic medium. They further suggest that during apoptosis some proteolytic enzymes are activated which results in liberating the bioactive fragment of perlecan (Figure 8).

Figure 8: Release of perlecan during endothelial cells apoptosis:



Apoptosis of EC triggers the release of soluble mediators, which include a C-terminal fragment of perlecan. This, in turn, activates PI3K in fibroblasts leading to resistance to apoptosis, sequentially regulated by modulation of Bim-EL and Bcl-xL protein levels, and to myofibroblast differentiation. However, chronic production of these mediators could lead to fibrosis [160]

Consistent with their results, they have demonstrated that the native form of perlecan was not able to inhibit apoptosis of VSMC, however, synthetic peptide containing the EGF-motif (present in domain V of perlecan) and chondroitin 4-sulfate (CS) did inhibit apoptosis of VSMC. Also, both the synthetic peptide and CS were able to induce sustained phosphorylation of ERK1/2and increase in Bcl-xl protein levels in VSMC. Hence, these results suggest that motifs of domain V of perlecan show anti-apoptotic activity in VSMC which results in proliferation and accumulation of fibroblasts and myofibroblasts, thus leads to development of fibrosis.

It is worth mentioning that previously mentioned fibrogenic factors and mediators produced at the event of apoptosis are responsible in activating pathways and cascades that lead to fibrosis. Some of these mediators work on initiating fibroblast formation, overexpressing already existing fibroblasts, or cause apoptotic resistance in them, all of which participate in the fibrosis and rejection of an organ.

Having a clear understating of the mechanisms in which these mediators participate in EMT, might help in solving the mystery behind organ rejection.

1.4.5. Signaling pathways involved in the Epithelial-Mesenchymal-Transition process

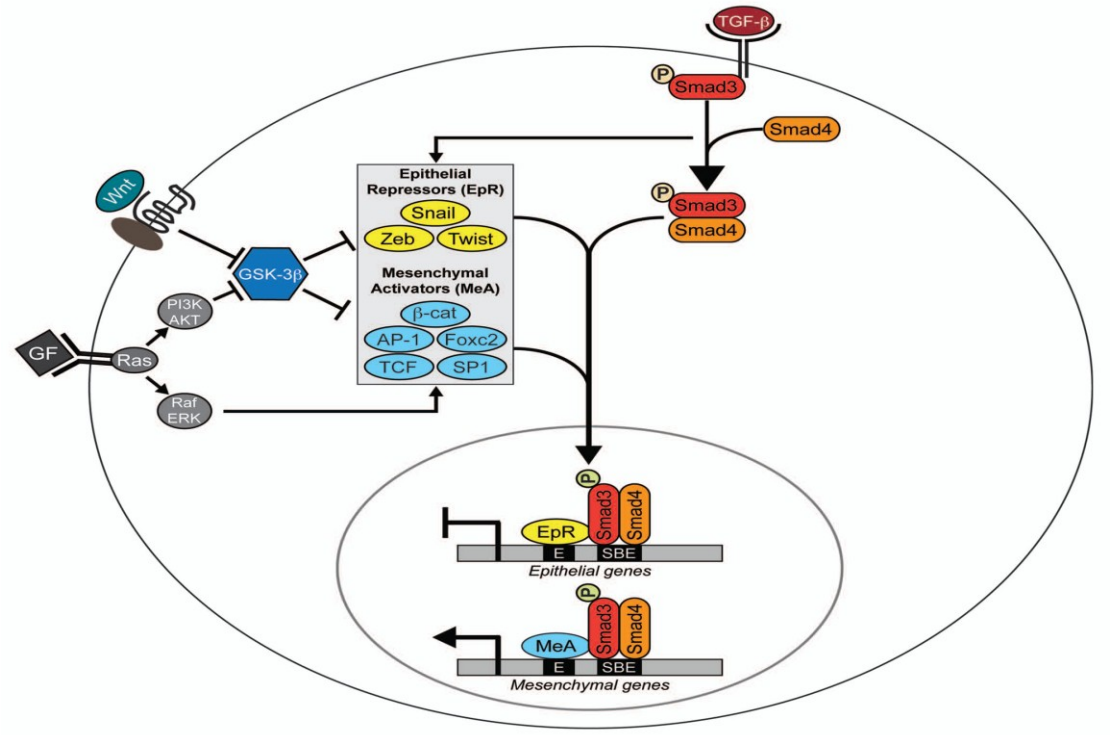
We have discussed previously possible mediators and fibrogenic factors that contribute in the EMT process. Here, we will discuss possible signaling pathways that have been involved or suggested to be implicated in EMT. Although treatment with TGF-β alone can induce EMT in certain types of cultured epithelial cells, other types seem to be resistant or moderately sensitive to TGF-β induced EMT [276]. Therefore it has been shown that TGF-β needs to cooperate with other signaling pathways, such as Wnt [277, 278], Hedgehog [279], oncogenic Ras/receptor tyrosine kinases [280-283], and Notch [284, 285], in order to induce complete EMT [257, 286, 287]. It has been noted that all of these pathways have been involved in stem cell renewal/proliferation [288, 289]. Three signaling pathways have been proposed to be involved in EMT: TGF-β/SMAD signaling, Wnt signaling, and Ras signaling.

It has been widely accepted that TGF-β has an essential role in inducing EMT during stages of embryogenesis, and in carcinoma progression and its invasive states [75, 76, 290]. The binding of TGF-β results in a complex formation of type I and type II receptors, the consequence of phosphorylation and activation of downstream effectors of the Smad family [291, 292]. More specifically, Smad2 and Smad3(R-Smad) get phosphorylated and bind with cytoplasmic Smad4 (co-Smad) to be

translocated to the nucleus. Translocation of the Smad complex regulates target genes through interaction with specific binding motifs in gene regulatory regions [293]. Smad signaling is an essential factor in TGF-β-induced EMT [294]. Studies have shown that epithelial renal tubular deficient in Smad3 failed to undergo EMT, and keratinocytes derived from $Smad3^{-/-}$ mice show reduction in the migration in response to TGF-β [295, 296]. Similar to Smad3, Smad4 is also essential for TGF-β-induced EMT. Studies, where Smad4 was blocked using RNA-interference, show preservation of E-cadherin expression after treatment with TGF-β [297-301], suppression of fibrotic type I collagen synthesis *in vitro*, and decrease in bone marrow metastasis *in vivo*. Furthermore, it has been noted that Smad4 is implicated in promoting tumor cell invasion in advanced pancreatic tumors [302]. These results suggest that the transcription factors Smad3 and Smad4 are important in TGF-β induced EMT (see Figure 9 for more details).

The second signaling pathway that is implicated in inducing EMT is the Wnt signaling pathway. This pathway is responsible in regulating stem-cell renewal and is implicated in inducing EMT in cancer. Unusual Wnt signaling through overexpression of Wnt ligands or silencing of Wnt inhibitors has been reported in different types of human cancer such as colon, breast, melanoma and prostate carcinomas, all of which were linked to EMT [303-308].

Figure 9: Transcriptional crosstalk between TGF-β, Wnt and Ras signaling pathways in EMT:



TGF-β binding to its receptor results in phosphorylation and nuclear translocation of Smad transcription factors, which achieve target gene specificity through interaction with transcriptional cofactors. EMT promoting transcription factors including epithelial repressors (EpR), such as Snail, Zeb and Twist, and mesenchymal activators (MeA), such as β-catenin (β-cat), AP-1, Foxc2, TCF and Sp1 interact with Smads, which results in the formation of EMT promoting Smad complexes (EPSC). These complexes drive EMT by repressing epithelial genes, such as E-cadherin, or activating mesenchymal genes, such as vimentin. Signals from Wnt and/or Ras pathways promote activation of Snail, Zeb, β-catenin and other EMT promoting transcription factors that can partner and form EPSC with Smads. Thus, the formation of EPSC represents a point of convergence between TGF-β, Wnt and Ras pathways. GSK-3β is a nodal protein, which negatively regulates stability of Snail and β-catenin. Activation of Wnt and Ras/PI3K/AKT pathways leads to inhibition of GSK-3β and thereby stabilization of Snail and β-catenin. TGF-β and Ras/Raf/ER K pathways also regulate EMT promoting transcription factors[309]

Finally, Ras signaling (figure 9) has been also proposed as a pathway involved in EMT. Activation of the Ras signaling cascades results from a stimulation of receptor tyrosine kinase (RTK) by a growth factor, such as epidermal growth factor (EGF) and fibroblast growth factor (FGF), which causes activation of PI3Kinase and Raf/ERK/MAPKinase pathways which regulate cell migration, proliferation, survival and cell cycle processes [310]. Ras signaling has been shown to cooperate with TGF-β in inducing EMT but exact mechanisms where this cooperation takes place remains poorly understood[311-313]. One of the transcriptional targets of Ras signaling is the high-mobility group A protein 2 (HMGA2) [314], which is induced by TGF-β and directly regulates expression of Twist and Snail, which are transcription factors that promote EMT [315, 316]. It was noted that Ras signaling is important in inducing EMT by the secreted interleukin-like EMT inducer (ILEI) [317]. The signaling of MAPK is essential for the acquisition of the EMT phenotype, the PI3Kinase signaling is required for the scattering phenotype. It was noted that inhibition of ERK-MAPK pathway restores the expression of E-cadherin in cells with moderate levels of Ras signaling [318].

1.4.6 Transcription factors involved in the Epithelial-Mesenchymal-Transition process

As mentioned before the EMT process involves both transcriptional inactivation of epithelial genes and activation of the mesenchymal ones. Some transcription factors in certain types of cells have the capability of inducing the whole

process of EMT by functioning both as repressors of epithelial genes and activators of mesenchymal genes. Other transcription factors are more specific in their genes targets, epithelial or mesenchymal genes [309]. Classification of transcription factors will be based on their transcriptional function, thus, two classes are suggested: 1.transcriptional repression of epithelial genes which involves the following transcription factors: Snail, Zeb, basic Helix-loop-Helix (bHLH) proteins family; and 2. transcriptional activation of mesenchymal genes which involves the transcription factors β-catenin, NFκB, activator protein-1 (AP-1) and Sp1.

One of the noted hallmarks of EMT is loss of E-cadherin expression. Studies done on E-cadherin, demonstrated that repression of E-cadherin expression during an EMT event is a consequence of a specific inhibitor binding to E-boxes sequences containing the core 5'-CACCTG-3' motif within the E-cadherin promoter[319, 320]. One of the transcription factors that was identified to repress E-cadherin by direct binding to the E-boxes is Snail. Studies have suggested Snail to be an "early switch" in activating EMT program, where it can induce EMT and overexpress the expression of other repressors [321, 322]. These findings were supported by data which shows Snail to be rapidly and transiently upregulated at the transcriptional level when EMT process is induced by TGF-β in namru mammary gland epithelial cells (NMuMG cells) [323]. This upregulation of Snail as response to TGF-β stimulation is mediated by interaction between Smads and HMGA2 [316]. Activation of the Ras signaling by growth factors cooperates with TGF-β to induce expression of Snail[324, 325]. At the post-translational level, Snail activity is regulated by glyocogen synthase kinase-3 beta (GSK-3β), a major Wnt signaling target, which works on phosphorylating Snail1

and leads to its nuclear export and degradation [326]. Evidence has shown that Wnt signaling has a direct influence in inducing the expression of Snail1 [327, 328]. Therefore, all these data support the idea of cooperation between TGF-β, Wnt and Ras to induce, activate and stabilize Snail during EMT.

Members of the bHLH family which includes: Twist, E47/TCF3 and TCF4/E2-2 function on inducing EMT as well as repressing the expression of E-cadherin [329, 330]. In breast epithelial cells Twist was shown to bind and repress the E-cadherin promoter [331]. However, Twist can act in inducing EMT through a mechanism that is different from Snail and Zeb proteins which does not involve direct binding of Twist to the E-cadherin promoter [332]. Twist presents metastatic properties to breast tumor cells and stem-like properties in epithelial cells [333, 334]. Furthermore, Twist1 and Twist 2 show a capacity for blocking oncogene-induced senescence by inhibiting p53-andRb-dependent pathways, and collaborate with Ras in inducing EMT [335]. Upregulation of Twist by TGF-β and PI3K pathways was noted in palate development [336]. In mammary epithelial cells, Twist is induced by Fibulin-5 during TGF-β induced EMT [337]. It has been noted that Twist was upregulated in mammary epithelial cells by Wnt signaling [338].

1.5 Hypothesis and rationale

In our study we hypothesize that ischemia-reperfusion injury, CMV infection, or other stressful events in the lung cause the endothelial cell to go through apoptosis, this results in proteolysis of the extracellular matrix and release of fibrogenic mediators. The released products, such as CTGF and perlecan for instance, stimulate the epithelial cells to go through transdifferential state where the process of epithelial-mesenchymal-transition is activated. This activation causes translocation of transcription factors (TF), such as Twist and Snail. The translocated TF work by repressing the expression of epithelial markers (E-cadherin and cytokeratin), and activating the expression of mesenchymal markers (CollagenI, α-SMA, and vimentin). This leads to formation of fibroblasts-like cells that will accumulate, resist apoptosis and cause obliteration of the airway and eventually leading to the development of BO in lung transplant recipients (see Figure 10).

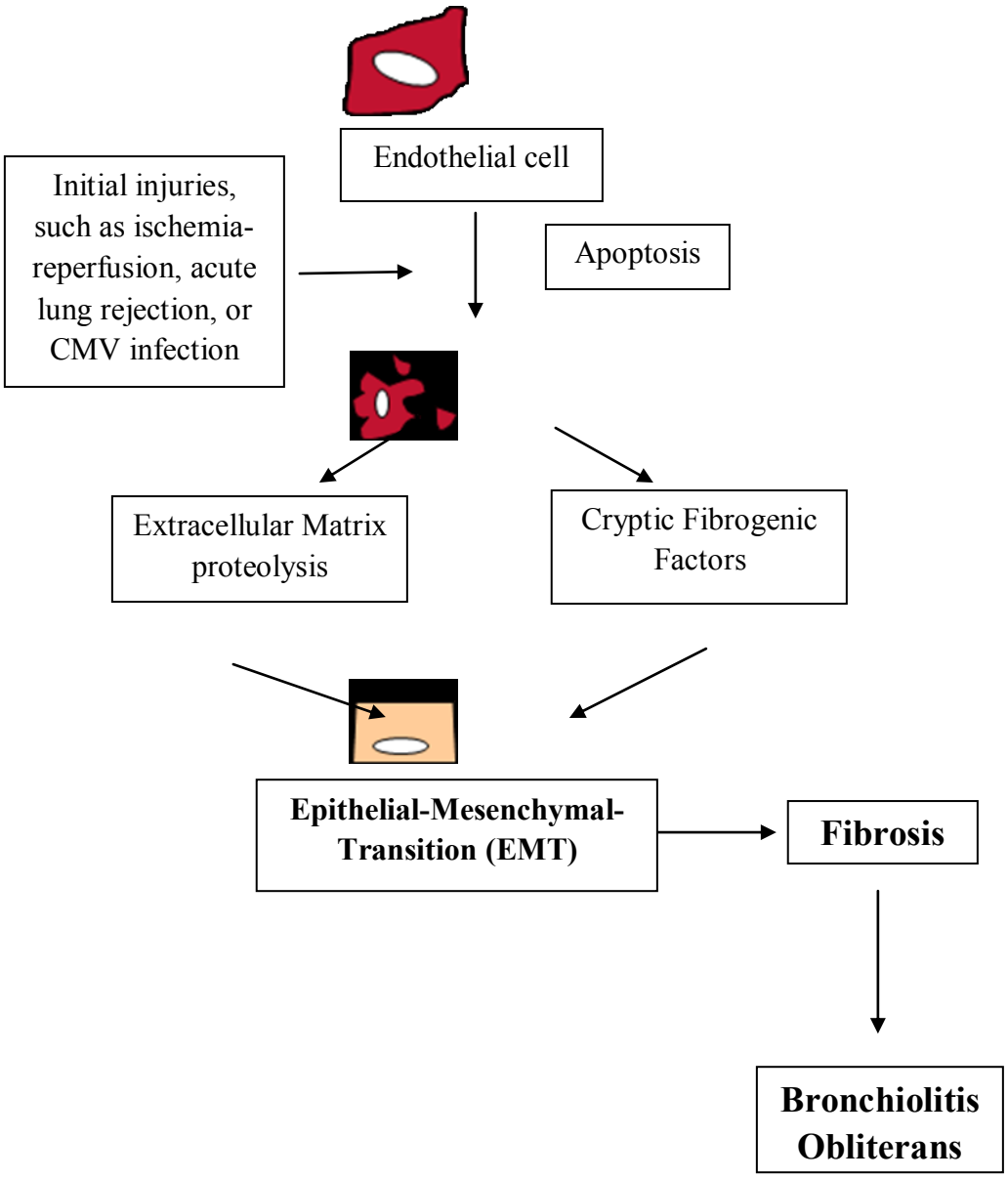


Figure 10: Proposed study hypothesis: Apoptotic endothelial cells cause release of fibrogenic mediators that lead to EMT activation causing formation of fibroblasts-like cells and leading to development of BO.

Chapter 2:

Epithelial- Mesenchymal- Transition in Lung Transplant Recipients: -Role in Bronchiolitis obliterans syndrome

Context of the study and contributing authors

For patients with end-stage lung diseases, lung transplantation is their only therapeutic option for them to survive basic activities. Chronic lung transplant rejection is considered one of the major challenges that lung transplant recipients (LTRs) have to face and overcome for the sake of their survival. The clinical definition of chronic lung transplant rejection refers to deterioration of the graft secondary to progressive airway disease with no other cause, also known as Bronchiolitis Obliterans Syndrome (BOS). Bronchiolitis Obliterans (BO) is referred to histological proof of fibrosis and tissue scarring that leads to airway remodelling in LTRs. Epithelial-Mesenchymal-Transition (EMT) is a process where epithelial cells go through transdifferentiation causing loss of epithelial properties and gain of mesenchymal ones. The EMT leads to fibrosis, and thus to the development of BO. The activation and induction of EMT is thought to be due to an activation of transcription factors (TF) that function primarily on repressing the expression of the E-cadherin gene and activating mesenchymal ones such as Vimentin. One of these transcription factors is Twist.

This chapter will go through the implications of epithelial-mesenchymal-transition in LTRs. Transbronchial biopsies (TBB) were studied retrospectively from LTRs. Immunohistochemistry (IHC) analyses were done on the obtained biopsies to verify the change in the expression level of both epithelial and mesenchymal markers, and indicate the occurrence of EMT in LTRs.

Dr Celine Bergeron is the principle contributor in the research hypothesis. She has also contributed in developing and expanding the ideas of this project. In addition, she has supervised the whole project and contributed to the final draft of the manuscript. Dr Charles Poirier is the director of the lung transplant program and head of the pulmonology department at the CHUM. He has helped in facilitating our access to the collected biopsies of lung transplant recipients at Notre-Dame Hospital part of the CHUM group. Mrs. Sawsan Al-Mot has a major contribution in the immunohistochemistry part which includes: procedure designing, optimization, and final analysis and scoring. Mr. Stanislaw Ptaszynski has contributed in measuring the percentage of collagen deposition of Van Giesen stainings. He also assisted in the statistical analysis of results. Finally, this project was funded by the CRCHUM fund to Dr Celine Bergeron, and Areej Al Rabea is the recipient of *Jean et Terry Lavoie-Dionne* award 2010.

Epithelial- Mesenchymal- Transition in Lung Transplant Recipients: -Role in Bronchiolitis Obliterans Syndrome

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Abstract

Background: Chronic lung transplant rejection, also known as Bronchiolitis Obliterans (BO), has been puzzling researchers for several years due to its complexity and implication in long term mortality in Lung Transplant Recipients (LTRs). Epithelial- Mesenchymal- Transition (EMT) is a proposed mechanism which participates in the pathogenesis of BO. In the EMT process, epithelial cells go through phenotypic transition to become mesenchymal cells and participate in airway remodelling through excessive production of fibroblast and myofibroblasts cells. Twist, a transcription factor (TF), is an initiator of the EMT mechanism, where it represses the expression of epithelial markers, and activates mesenchymal ones. **Aim:** To study and investigate *in situ* the presence of EMT in the small airways of LTRs. **Methods:** Immunohistochemical (IHC) staining and analysis were done on transbronchial biopsies (TBB) that were obtained retrospectively from LTRs who did or did not develop BO. Antibodies specific for E-cadherin, Vimentin, and Twist have been used in the analysis, where E-cadherin is an epithelial marker, Vimentin is a mesenchymal marker, and Twist is a transcription factor that is responsible in the induction of EMT. Furthermore, H&E, and Van Gieson staining were performed. All biopsy analyses were correlated with collected clinical data of the recruited patients. **Results:** Longitudinal data of IHC stainings done on transbronchial biopsies (TBB) taken on an average period of one year post lung transplant have demonstrated a decrease in the expression of E-cadherin in BO group with a mean intensity expression of 1.95 compared to the stable group that has a mean intensity expression of 2.82, and $p = 0.02$. Stainings of TBB also demonstrated an increase in the

expression of Vimentin in the BO group compared to the stable one with a mean intensity expression of 2.60 vs. 0.85, respectively, and p = 0.00004. The expression of Twist was also high in the BO group compared to the stable one with mean intensity expression of 3.1 vs. 2.0, respectively, and p = 0.0023. Correlations between EMT marker expressions revealed the following: -0.3448 between E-cadherin and Vimentin p = 0.366, -0.2248 between E-cadherin and Twist p = 0.180, and 0.2123 between Vimentin and Twist p = 0.213. Correlations between EMT markers expression and FEV1 loss were: -0.5558 with E-cadherin, 0.2663 with Vimentin, and 0.5770 with Twist whereas correlation with FEF25-75 loss were: -0.4027 with E-cadherin, 0.2476 with Vimentin, and 0.2042 with Twist. **Conclusion:** We demonstrate for the first time the occurrence of EMT in the small airways of LTRs. The occurrence is characterized by loss of epithelial marker expression, such as E-cadherin, and gain of mesenchymal marker expression, such as Vimentin. Induction of this process might be triggered by an increase in the expression level of Twist causing an induction of the EMT process. Our results suggest that EMT might play an important role in the initiation and formation of bronchiolitis obliterans.

Used abbreviations: BO: Bronchiolitis Obliterans, LTRs: Lung Transplant Recipients, EMT: Epithelial-Mesenchymal-Transition, TF: Transcription Factor, IHC: Immunohistochemistry, TBB: Transbronchial Biopsies.

Introduction

Lung transplant is the only therapeutic approach for patients with end-stage lung diseases. End-stage lung diseases include: Chronic Obstructive Pulmonary Disease (COPD), Cystic Fibrosis (CF), Idiopathic Pulmonary Fibrosis (IPF), and emphysema due to alpha-1 antitrypsin deficiency [2], along with other rare diseases. However, according to the Twenty-Sixth report of the International Society for Heart and Lung Transplantation, it states that the overall survival half-life is only 5.7 years. It is worth mentioning that long term survival of lung transplant is the worst compared to other solid organs due to the development of chronic lung transplant rejection. The chronic lung transplant rejection has two distinct definitions: Bronchiolitis Obliterans (BO), and Bronchiolitis Obliterans Syndrome (BOS). The definition of BOS refers to a clinical diagnosis indicating "graft deterioration secondary to progressive obstructive airway disease for which there is no other cause" [69, 71]. BOS classification is based on the percentage loss of FEV1 and FEF25-75, where an early BOS stage will demonstrate a stable FEV1 but a decline in FEF25-75 appears to be more sensitive in the detection of BOS [47]. In addition, the new and updated classification of BOS has a new category, called potential BOS, in an attempt to identify these patients at earlier stages. On the other hand histological definition of BO refers to proof of fibrosis presence, tissue scarring, and airway remodelling. Furthermore, airway remodelling, which leads to obstruction, is a result of activated fibroblast responses causing excessive deposition of extracellular matrix (ECM).

An aberrant repair process of the lung epithelium in response to repeated and/or sustained injury is the leading proposed concept explaining the fibro-

obliterative process observed in BO. The sources of injury in the transplanted lung are many, including ischemia, acute rejection, gastroesophageal reflux disease (GERD) and infections whether they are viral, bacterial or fungal [208, 339]. One (or more) of the above mentioned stimuli may lead to an aberrant repair process and ultimately contribute to the development of BO. Evidence from animal studies on airway obliteration, suggest that epithelial injury and failure to re-establish an intact epithelium might be a key factor in the pathogenesis [219]. Epithelial cells are well known for their adaptive responses toward stimuli that can range from repair, to necrosis and apoptosis. New evidence suggests an important role of epithelial-mesenchymal-transition (EMT) in the repairing process of the injured lung [215].

The EMT response is characterized by the loss of epithelial markers, such as cytokeratin and E-cadherin, and the acquirement of mesenchymal markers such as α-smooth muscle actin (α-SMA), collagen, fibronectin and Vimentin. Subsequently, EMT is also defined by increase in synthesis of other, non-structural proteins, such as matrix metalloproteinases (MMPs) which play an important role in the turnover and remodelling of the ECM [214]. Furthermore, during EMT there is a translocation of several transcription factors into the nucleus, namely Twist. This basic helix-loop-helix transcription factor has been reported to be involved, not only in lung fibrosis [340], but also in renal fibrosis [341], embryogenesis and carcinogenesis [342]. Once Twist is overly expressed during a stress, it functions by repressing the expression of E-cadherin proteins, and expressing mesenchymal ones such as Vimentin. These changes in protein expression lead to a sequential response by which epithelial cells become less adherent due to loss of E-cadherin-dependent contacts, and acquire the

phenotypic and functional features of fibroblasts and myofibroblasts, such as motility and ECM protein secretion. In a typical context this response would contribute to normal tissue repair, but chronic inflammation and repeated injury can overwhelm the global repair machinery and thereby contribute to fibrosis. Indeed, the EMT mechanism has been implicated in various fibrotic diseases of the kidney [219], lung [215], and liver [214]. Previous studies [343] have suggested that epithelial cells obtained from lung transplant recipients undergoing EMT cause release of MMPs and deposition of the ECM when targeted by a stimulus causing airway remodelling and thus leading to the development of BO. They have also worked on detecting the change in EMT marker expression in obtained sequential sections from LTRs and normal lung patients. Other group has demonstrated an increase in the expression of mesenchymal proteins by large airway bronchial epithelial cells in patients with BOS following lung transplantation [344]. One group [345] has also worked on TBB analysis in order to understand the cause of airway remodelling which is implicated in BOS. In their work they propose thickening of the reticular basement membrane (Rbm) as an informative parameter in detecting the development of BOS. The same group [345] have demonstrated that there is no correlation between inflammatory cell counts and the Rbm thickening. Also they demonstrated that there is no correlation between Rbm thickening and lung function. It is worth mentioning that very limited studies have considered the EMT to have a possible association with the development of BO.

In our study, we propose that expression of Twist as a response to stimuli found in the transplanted lung or in genetically predisposed LTRs, leads to repression

or decrease in the expression of E-cadherin, and increase in the expression of Vimentin. This inverse side of expression of both epithelial and mesenchymal markers leads to accumulation of fibroblasts and myofibroblasts, and thus results in airway remodelling which causes development of BO. To test our proposed hypothesis we have performed our study on transbronchial biopsies (TBB) that were obtained retrospectively from lung transplant recipients (LTRs) and tried to detect EMT presence and correlate their level of expression of EMT markers with collected data of recruited patients.

Methods

Study subjects

In this study we based our recruitment of subjects on lung transplant recipients (LTRs) that went through transplantation at Notre-Dame Hospital part of the University of Montreal Hospital Centre (CHUM). All recruited LTRs agreed to participate in the study either through a specific consent forms to access their past TBBs, or through the tissue bank consent form. Both consent forms have been approved by the local ethic committee. Based on patients' data that were obtained from the transplant database, the patients were divided into two groups: a stable group and a second group that eventually developed BOS (based on ISHLT classification of BOS). Paraffin embedded transbronchial biopsies (TBB) of the recruited patients were collected, along with their demographic data that was summarized in table 1. Each paraffin block contains multiple TBB ranging from 4 to 8 biopsies. The study

was conducted on lung transplant recipients (LTRs) with a total population of 19 patients, where 9 were stable and 10 developed BOS. Since each patient might have one or more blocks from TBB, the total number of TBB was 37 blocks for both groups where the stable group has 17 TBB blocks and the BOS group has 20 TBB blocks.

Histochemistry stainings of the transbronchial biopsies (TBB)

The TBB blocks (n=19 patients total of 37 blocks) were processed by the pathology department at Hotel-Dieu Hospital part of the CHUM. Processing of TTB included Van Gieson staining to assess in measuring total collagen deposition. Van Gieson (VG) staining was used to assist in measuring the thickness of collagen deposition in TBB. Measurements of collagen deposition were done using Image-Pro Analyzer provided by Olympus. Positive staining of VG reveal a dark pink color in the tissue of TTB, this color is selected for the entire slide to give total collagen deposition divided by total area x100 to give percentage of collagen deposition.

Immunohistochemisty (IHC) staining

We have performed immunohistochemistry staining to help in detecting the expression level of E-cadherin, Vimentin, and Twist in small airway epithelial cells on the obtained paraffin blocks of TBB. A modified immunoperoxidase method of immunohistochemistry was performed. Mouse monoclonal E-cadherin antibody

(Abcam, Canada) and rabbit polyclonal Twist (Abcam, Canada) stainings were done using heavy method antigen retrieval with ethylenediamine-tetraacetic acid (EDTA) buffer. Mouse monoclonal Vimentin antibody (Sigma, Canada) was done using a light method of antigen retrieval with Tris buffer. The compound 3,3'-Diaminobenzidine (DAB) was used in the IHC staining as a revealing method.

Immunohistochemistry scoring and statistical analysis

The area of positive bronchial epithelial staining was scored on a level of 0 to 4, where 0 = no expression, 1= low, 2= intermediate, 3= high, 4= very high. As one paraffin block contains multiple biopsies, the score represent the mean of biopsies observed in one block. The scoring of the positive area was done by two different, blinded readers to avoid bias in the scoring. The differences in the expression of EMT markers in stable and BO groups were analysed for each marker, and were assessed by a two-tailed Student-t-test. P value of ≤ 0.05 was considered statistically significant. Correlations (r =) were calculated between: measured expression of each marker, time of biopsies and EMT markers expression in both groups, percentage loss of FEV1 and expression of EMT markers (E-cadherin, Vimentin, and Twist), and percentage loss of FEF25-75 and EMT markers expression.

Statistical analysis

Collected data of the recruited patients that were analyzed in the statistical analysis included: percentage loss of forced expiratory volume in 1 second (FEV1), mid-expiratory flow rate (FEF 25-27), change in the expression of EMT markers, and percentage of collagen deposition. Differences in EMT marker expression in both groups were quantified for each marker and averaging their positive stainings. Results were presented as mean (with standard error of the mean, (SEM)). The significance difference between groups was assessed using paired two-tailed student's test (t-test) using Microsoft Excel. Differences with p value of ≤ 0.05 were considered significant. In addition, correlations between EMT markers expression, percentage loss of FEV1, percentage loss of FEF25-75, and percentage collagen deposition were assessed using Spearman test (r =) of correlation was assessed.

Results

Analysis of demographic data

Our demographic data of recruited patients (presented in Table IV) included a total number of 19 LTRs, where 9 were stable and 10 developed BOS. A total of 37 TBB biopsies were studied: 20 in the stable group, and 17 in BOS. Both groups were comparable in gender, age, transplant type, original disease, BOS grade, %FEV1 loss, and %FEF loss. From the demographic data it is noted that median age at transplant is 50 for BOS whereas the median in the stable group is 40. Original diseases which lead to lung transplantation included: Bronchiectasies, COPD, CF, and IPF, both

groups had the same original disease except for bronchiectasies which was not seen in the stable group. The BOS demonstrated a high percentage loss of both FEV1 and FEF 25-75 with medians of 40%, and 77%, respectively. The stable group demonstrated a low percentage loss (compared to BOS) of both FEV1 and FEF 25-75 with medians of 6%, and 27%, respectively.

Detection of Epithelial-Mesenchymal-Transition Expression

Staining of transbronchial biopsies (TBB) by immunohistochemistry procedure has assisted in detecting epithelial that were positive in expressing EMT marker: E-cadherin, Vimentin, and Twist. The expression of EMT markers was detected and analysed in both groups (Stable, and BOS). E-cadherin, an epithelial marker was highly expressed in the stable group where it had a mean intensity score of 2.82 compared to the BOS group with a mean intensity of 1.95 and a p value of 0.0195(see Figure 11). The signal was detected in the intact epithelium of TBB. Longitudinal analysis of E-cadherin expression demonstrates a significant decrease in the expression of E-cadherin at early stages following lung transplantation in the BOS group compared to the stable one (see Figure 12).

One the other hand, expression of Vimentin, a mesenchymal marker, was higher in the BO group with a mean intensity of 2.60 compared to that of the stable group which had a mean intensity of 0.85 and a p value of 0.00004 (see Figure 13). Positive staining of Vimentin was checked in the intact epithelium of TBB. Longitudinal data of Vimentin expression demonstrates a significant increase in the

expression of vimentin at early stages following lung transplantation in the BOS group compared to the stable one (see Figure 14).

Twist was highly expressed in the BO group with a mean intensity of 3.1 compared to the stable group which had a mean intensity of 2.0 and a p vale of 0.0023 (see Figure 15). Positive staining of Twist was checked in the intact epithelium and nuclei of epithelial cells of obtained TBB. Longitudinal data for Twist expression demonstrates a significant increase in the expression of Twist in the BOS group compared to the stable one (see Figure 16), also nuclear staining in the epithelium was detected as well.

Statistical analysis of gathered information

A negative correlation was revealed between the epithelial marker E-cadherin and the mesenchymal marker Vimentin ($r = -0.3448$ and $p = 0.0366$) and the EMT transcription factor Twist ($r = -0.2284$ and $p = 0.180$). As expected, a positive correlation was noted between Vimentin and Twist ($r = 0.2123$ and $p = 0.214$). Results regarding correlations between EMT markers and decline in FEV1% revealed the following: $r = -0.5558$ with E-cadherin and $p = 0.014$, $r = 0.2663$ with Vimentin and $p = 0.271$, and $r = 0.5770$ with Twist and $p = 0.815$. Correlations between the EMT markers and decline in FEF_{25-75}% revealed the following: $r = -0.4027$ with E-cadherin and $p = 0.087$, $r = 0.2476$ with Vimentin and $p = 0.307$, and $r = 0.2042$ with Twist and $p = 0.402$. E-cadherin did not demonstrate correlation with total collagen

deposition. However, a positive correlation was observed between collagen and Vimentin and twist. Correlations with percentage deposition of collagen revealed the following: r = -0.0133 with E-cadherin, r = 0.1383 with Vimentin, r = 0.3077 with Twist, r = 0.0738 with FEV1 loss, and r = 0.2712 with $FEF_{25-75\%}$ loss.

Discussion

Etiology behind the development of Bronchiolitis Obliterans in lung transplant recipients remains a mystery for researchers, and needs to be further investigated. Several hypotheses have been proposed to help in understating the reason behind it. From the previous definition of Bronchiolitis Obliterans which states histological presence of fibrosis, tissue scarring and airway remodelling, airway remodelling must be detected in patients who do actually develop BO. First, we have noticed that the level of E-cadherin expression in the stable group was higher than the BOS group, indicating that epithelial cells in TBB patients who developed BOS demonstrate loss in the epithelial properties. Second, in the BOS group we noticed a high expression level of the mesenchymal marker Vimentin compared to the stable one, thus, indicating that the epithelial cells in the BOS group gained mesenchymal properties due to a mechanism that was only activated in the BOS group. The points in which the markers start changing vary from 1-12 months depending on whether the EMT process gets activated or not.

Furthermore, when we found that the level of Twist expression was higher in the BOS group compared to the stable one. Twist is a transcription factor that

functions in repressing the expression of E-cadherin, and inducing the expression of mesenchymal markers such as Vimentin, therefore, it leads to activation of the EMT mechanism. The longitudinal analysis of the EMT markers suggest that Twist is early expressed, closely followed by an increase in Vimentin expression and finally the E-cadherin suppression appeared. Due to the fact that Twist was highly and early (as one month post transplantation) expressed in the BO group, suggests its implication in initiating EMT leading to BOS. These data support work done by others [343] where they have demonstrated a significant decrease of E-cadherin expression and increase of Vimentin expression in epithelium of explanted lung from patients with BOS compared to stable patients. However, we were able to demonstrate a significant increase in the expression of Twist which might have a link in the activation of EMT, where in the previously mentioned study they did not consider checking for transcription factor expression. In addition, our work studies change in the expression of EMT markers in the epithelial of small airways, whereas their work studies EMT expression in the airways.

The correlations suggest a strong connection between the expression of EMT markers and Twist where an increase in the expression of Twist is associated with an increased expression of Vimentin and a decreased expression of E-cadherin. These results emphasize on the role Twist plays in repressing the expression of E-cadherin, and activating the expression of Vimentin. A higher decline in either FEV1% or FEF25-75% is negatively correlated with the expression of E-cadherin, suggesting that low E-cadherin expression is associated to a grester decline in lung function. On the other hand, the decline in FEV1% and FEF25-75% correlated positively with the

expression of Vimentin. This suggests a possible impact of EMT formation on the clinical outcomes of LTRs. This is also supported by correlations between collagen deposition and EMT markers, FEV1% loss, and FEF25-75% loss. The increase in collagen deposition has a positive correlation with both Vimentin and Twist, which suggests that activation of the EMT pathway might be responsible for increasing the production of collagen through proteolysis of the ECM. Excessive collagen deposition is a feature of BOS in LTRs. One group has suggested Rbm thickening to be implicated in airway remodelling due to inflammation [345], which leads to the development of BOS. However, they demonstrated no correlations of Rbm with inflammation or with lung function. In our study, we were able to demonstrate correlations of induced EMT markers with collagen deposition, FEV1 % loss, and with FEF25-75 % loss. This emphasizes on EMT participation of ECM proteolysis and airway remodelling.

Conclusion

To conclude, we have found that changes in the expression level of EMT markers in the small airways can be detected at early stages and can be an indicator (or an alarm bell) for the development of BOS in lung transplant recipients. Several proposals were given to explain airway remodelling, a hallmark in BO development. In our study we were able to demonstrate that one possible way of remodelling is through a mechanism where epithelial cells loss their properties and gain mesenchymal ones, a process known as epithelial-mesenchymal-transtion. Several

stimuli can trigger the induction of this process, by translocating transcription factors that function primarily in inducing this process; one of these transcription factors is Twist. Translocation of this transcription factor induces EMT, causing remodelling of the airways, and thus leads to the development of BO in the recipients of lung transplant. Furthermore, association of clinical data with bench work reveals strong associations between EMT formation and decline in lung function. Our study proves the involvement of EMT in the pathogenesis of BO in small airways of lung transplant recipients.

Acknowledgment:

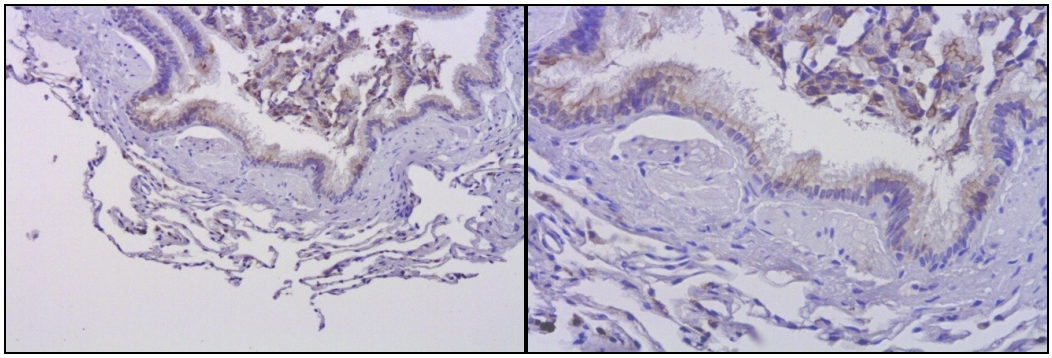
We thank the pathology department in their assistance in obtaining transbronchial biopsies, and Van Gieson (VG) staining on obtained biopsies.

Table IV: Demographic Data of Recruited patients:

	Gender	Age (at transplant)	Transplant type	Original disease	BOS grade	% of FEV loss	% of FEF 25-75 loss
BOS	M	53	Double	Bronchiectasies	BOS 2	35.00	80.16
	M	52	Double	Bronchiectasies	BOS 3	58.62	84.88
	M	56	Double	COPD	BOS 0-p	15.03	43.72
	M	54	Single	COPD	BOS 2	36.72	59.65
	M	55	Single	COPD	BOS 3	77.06	86.22
	F	36	Double	CF	BOS 2	44.22	77.60
	F	40	Double	CF	BOS 3	75.06	89.33
	M	47	Double	CF	BOS 0-p	11.81	31.49
	M	30	Double	CF	BOS 0-p	16.77	52.46
	F	47	Double	IPF	BOS 2	47.19	75.41
	Median (range)	**50 (30-56)**				**40 (11.81-77.06)**	**77 (31.49-89.33)**
Stable	M	56	Double	IPF		0.79	12.65
	M	51	Double	COPD		5.67	22.85
	F	44	Single	COPD		15.57	15.77
	F	33	Double	CF		29.27	31.86
	M	26	Double	CF	BOS 0-p	12.23	26.84
	M	40	Double	CF	BOS 0-p	6.17	33.56
	M	35	Double	CF	BOS 1	25.63	63.33
	F	23	Double	CF	BOS 0-p	1.53	46.26
	M	48	Double	CF		4.45	6.35
	Median (range)	**40 (23-56)**				**6 (0.79-29.27)**	**27 (6.35-63.33)**

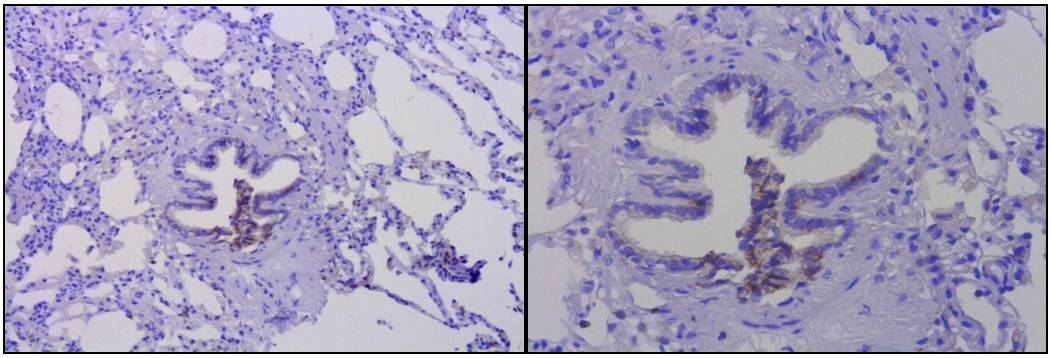
Figure 11: Immunohistochemistry staining of E-cadherin in TBB:

A) E-cadherin Expression in Stable group:



20X **40X**

B) E-cadherin Expression in BOS group

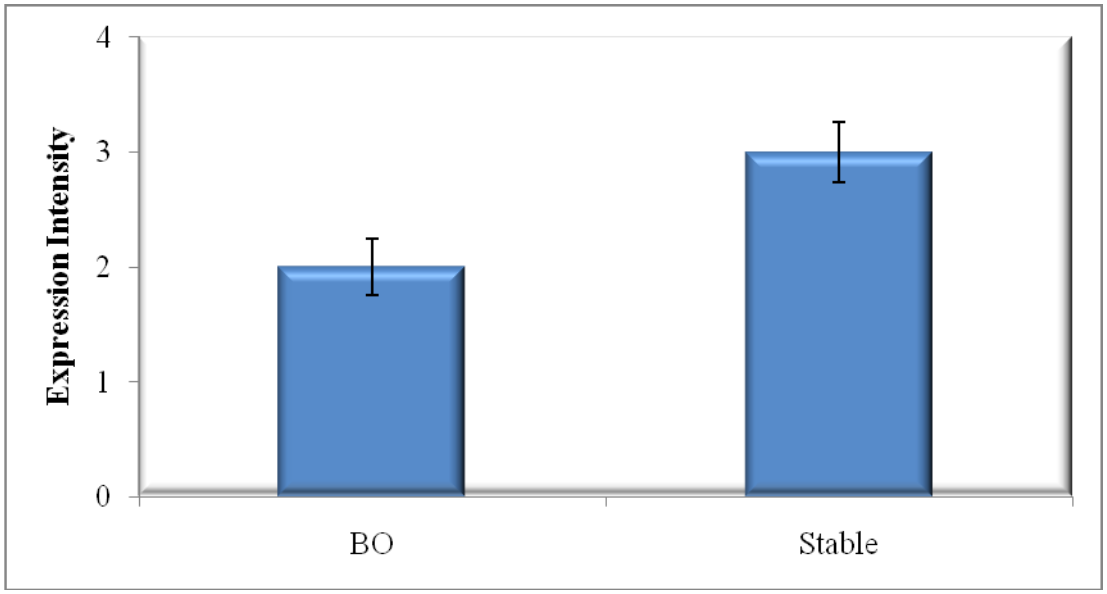


20X **40X**

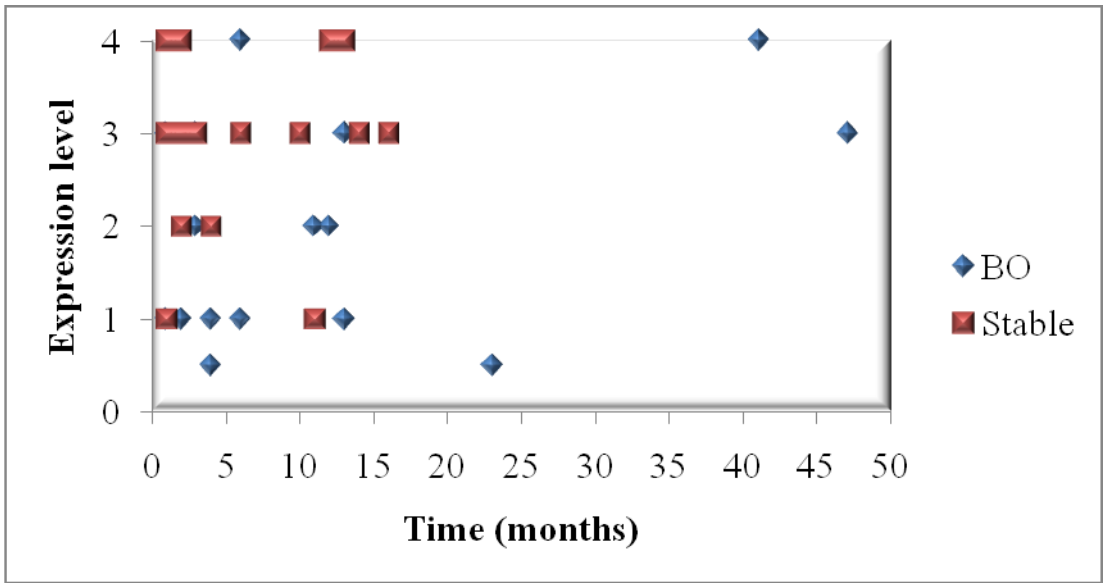
Immunohistochemistry images of E-cadherin for A) stable at 20X and 40X, B) and BOS groups at 20X and 40X

Figure 12: Quantification of E-cadherin signal in the epithelium of small airways in TBB:

A) Expression Intensity of E-cadherin in TBB



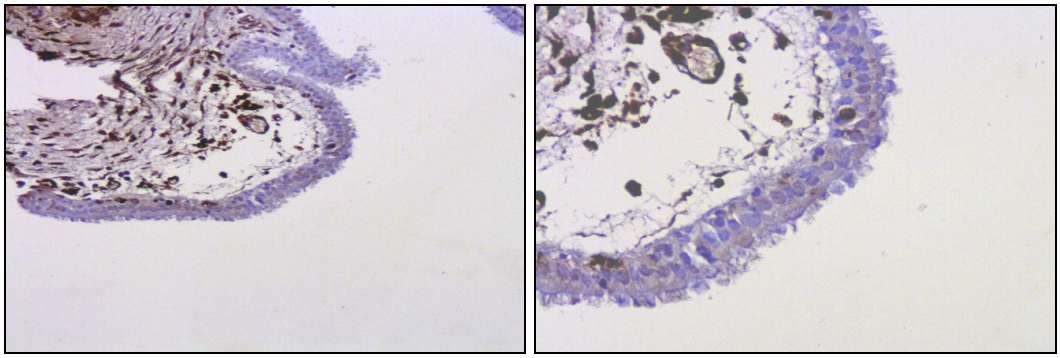
B) Longitudinal E-cadherin Expression Intensity in TBB



A) Level of E-cadherin expression in both stable and BO groups with means of 2.82 and 1.95, respectively and p=0.020. Add the SEM in graph. B) Longitudinal representation of E-cadherin expression in the epithelial with time after lung transplantation.

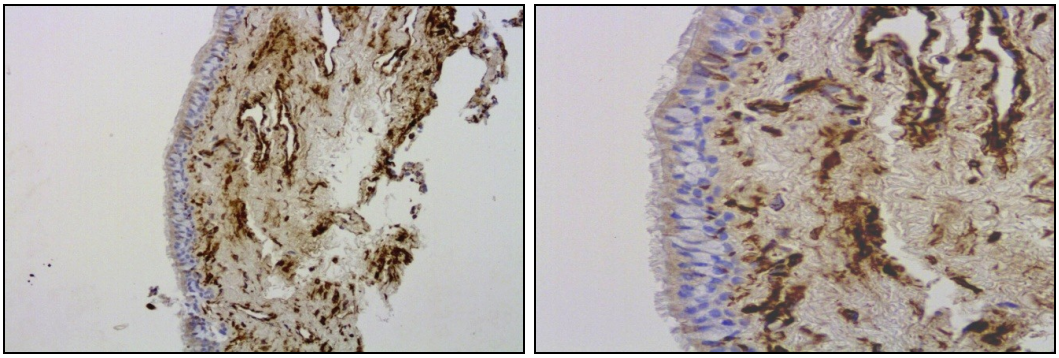
Figure 13: Immunohistochemistry staining of Vimentin in TBB:

A) Vimentin Expression in Stable group:



20X **40X**

B) Vimentin Expression in BO group

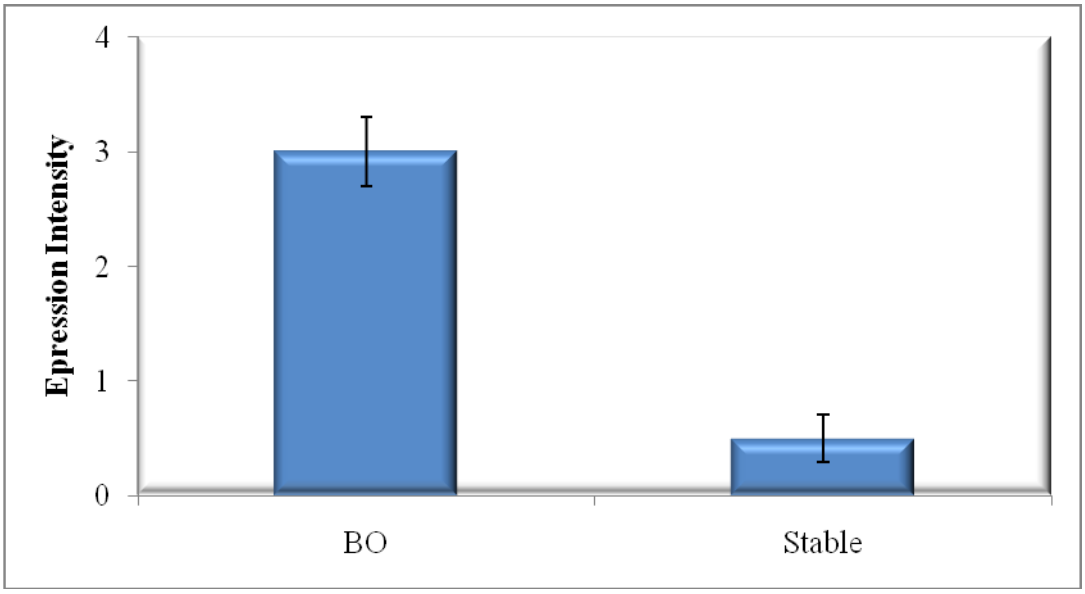


20X **40X**

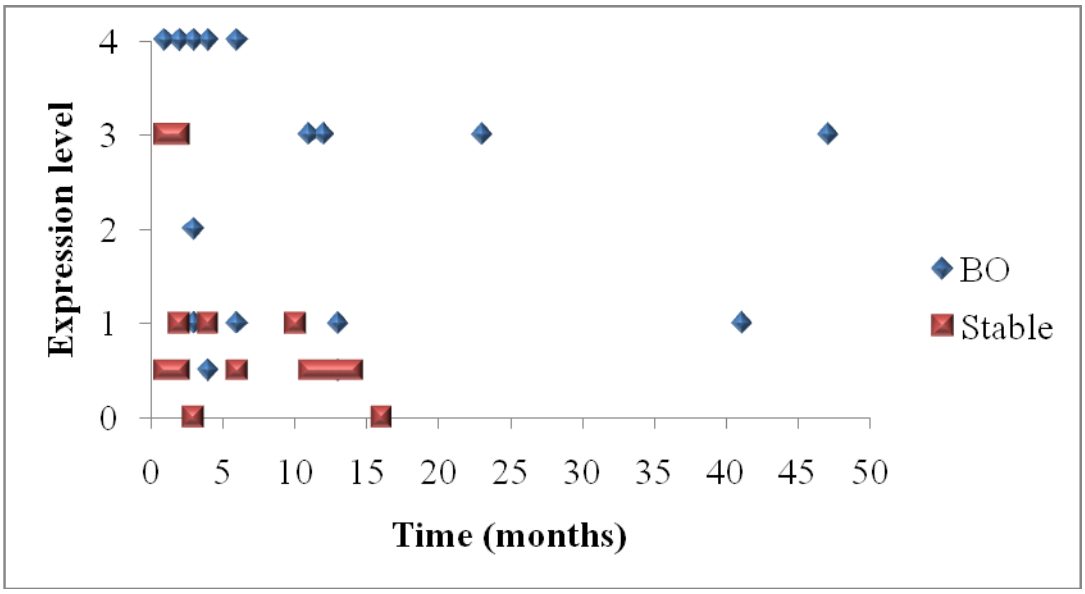
Immunohistochemistry images of Vimentin for A) stable at 20X and 40X, B) and BOS groups at 20X and 40X

Figure 14: Quantification of Vimentin signal in the epithelium of small airways in TBB:

A) Expression Intensity of Vimentin in TBB



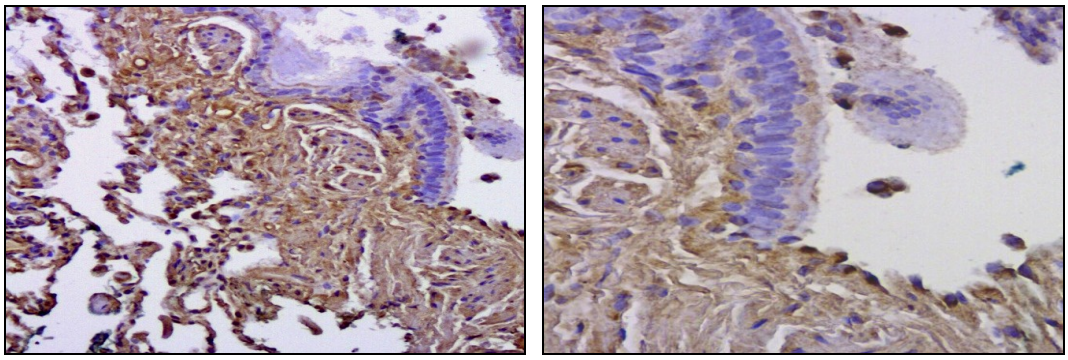
B) Longitudinal Vimentin Expression Intensity in TBB



A) Level of Vimentin expression in both stable and BO groups with means of 0.85 and 2.60, respectively and p=0.00004. B) Longitudinal representation of Vimentin expression in the epithelial with time after lung transplantation.

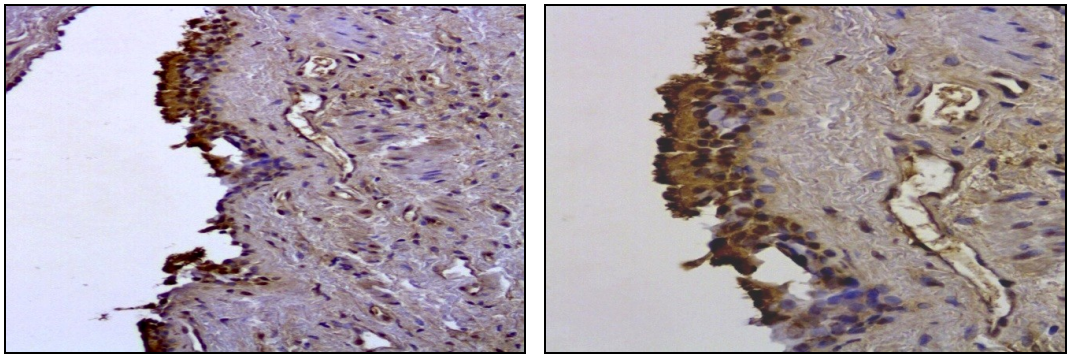
Figure 15: Immunohistochemistry staining of Twist in TBB:

A) Twist Expression in Stable group:



20X **40X**

B) Twist Expression in BO group

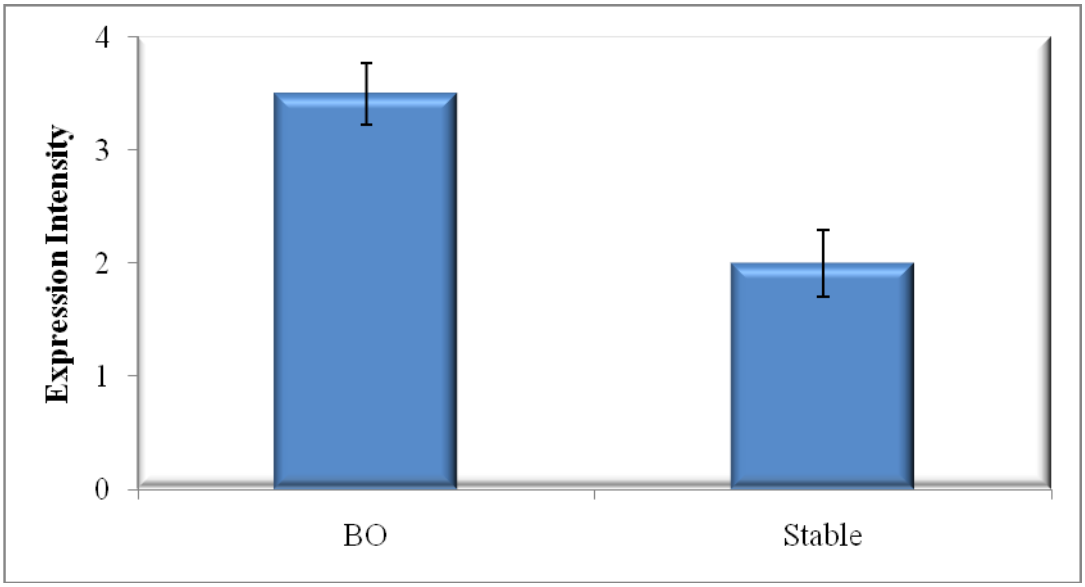


20X **40X**

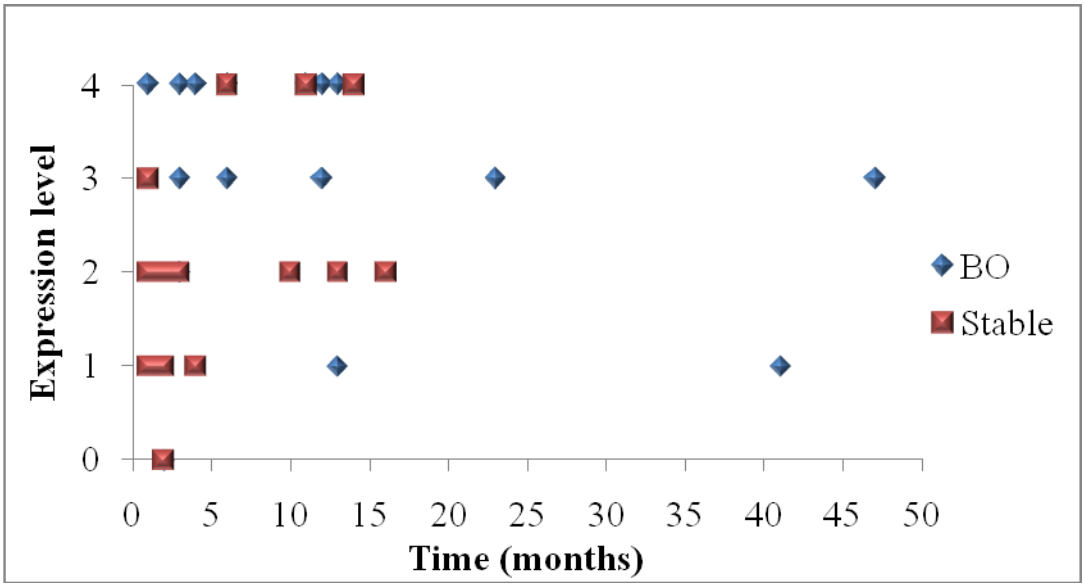
Immunohistochemistry images of Twist for A) stable at 20X and 40X, B) and BOS groups at 20X and 40X

Figure 16: Quantification of Twist signal in the epithelium of small airways in TBB:

A) Expression Intensity of Twist in TBB



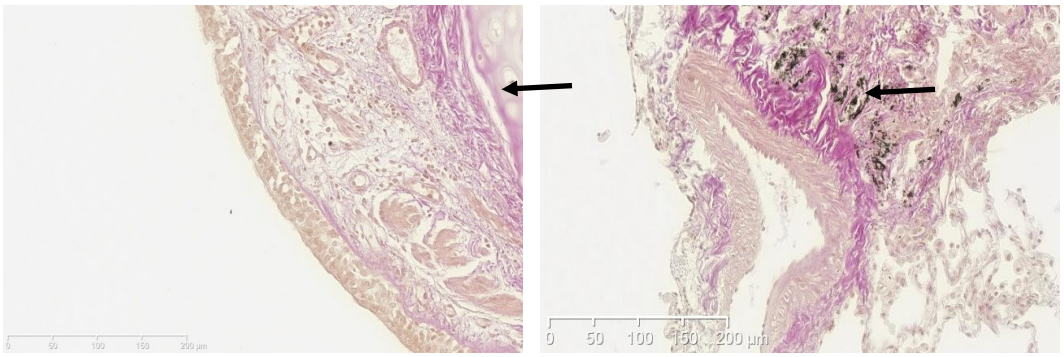
B) Longitudinal Twist Expression Intensity in TBB



A) Level of Twist expression in both stable and BO groups with means of 2.0 and 3.1, respectively and p=0.0023. B) Longitudinal representation of Twist expression in the epithelial with time after lung transplantation.

Figure 17: Van Gieson Stainings of obtained TBB:

A) Van Gieson staining in Stable group:



In Stable **In BO**

Van Gieson staining in obtained transbronchial biopsies (TBB). Black arrows indicate the staining for collagen in biopsies.

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Chapter 3:

Activation of Epithelial-Mesenchymal-Transition by Lung Apoptotic Endothelial Cells Mediators: Implication in Airway Remodeling and Development of Bronchiolitis Obliterans

Context of the study and contributing authors

Lung transplant is the only therapeutic option for unfortunate patients with end-stage lung diseases. For lung transplant recipients (LTRs), chronic rejection is a major obstacle for their long term survival. Chronic lung transplant rejection is defined clinically as deterioration of the graft secondary to progressive airway disease with no other cause, also known as Bronchiolitis Obliterans Syndrome (BOS). Bronchiolitis Obliterans (BO) is referred to histological proof of fibrosis and tissue scarring that leads to airway remodelling in LTRs. Programmed cell death or apoptosis is usually activated to maintain homeostasis and balance following a stimulus or a stress. Deregulated apoptosis causes imbalance of produced mediators, where these mediators can cause induction of Epithelial-Mesenchymal-Transition (EMT). This process is a transdifferentiating process where epithelial cells lose their epithelial properties and gain mesenchymal ones. The induction of EMT is thought to be due to an activation of transcription factors (TF) such as Snail and Twist. Activated TFs function primarily on repressing the expression of epithelial markers such as E-cadherin, and inducing the expression of mesenchymal markers such as collagen, alpha smooth muscle actin (α-SMA), and Vimentin. Studies have shown EMT to be implicated in the pathogenesis of several fibrotic disorders, and suggested EMT to be possibly implicated in airway remodeling which leads to the development of BO.

This chapter will go through the involvement of mediators produced by apoptotic endothelial cells in inducing epithelial-mesenchymal-transition *in vitro*. Apoptotic (SSC4h) and non-apoptotic (SSC4h-ZVAD) media were conditioned and

produced from Human MicroVascular Endothelial Cells from Lungs (HMVEC). Released mediators in both produced media were detected by using Enzyme-linked immunosorbent assay (ELISA) to help in detecting level of connective tissue growth factor (CTGF) and transforming growth factor-beta (TGF-β). Immunobloting assay was done as well to detect Perlecan in produced media. Detection of gene level expression was done using quantitive- polymerase chain reaction (Q-PCR) to check for the expression of EMT markers (E-cadherin, Collagen, and alpha-smooth muscle actin (α-SMA)). Finally, detection of protein expression of EMT markers was done using immunofluorescence.

Dr Celine Bergeron is the principle contributor to the research hypothesis. She has also contributed in developing and expanding the ideas of this project. In addition, she has supervised the whole project and contributed to the final draft of the manuscript. Dr. Marie-Josee Hebert is Holder of the Shire Chair in Nephrology and Renal Transplantation and Regeneration at the Université de Montréal. Dr. Hebert and her team were generous in providing guidance, instructions, and products to help in setting the bases of this project, especially in the area of apoptotic endothelial cells. Mr. Stanislaw Ptaszynski is a research assistant at Dr. Celine Bergeron's lab, who had a major contribution in this project and helped in PCR procedures and final analysis. Finally, this project was funded by the CRCHUM fund to Dr Celine Bergeron, and Areej AL Rabea is a recipient for the *Jean et Terry Dionne Award* 2010.

Activation of Epithelial-Mesenchymal-Transition by Lung Apoptotic Endothelial Cells Mediators: Implication in Airway Remodeling and Development of Bronchiolitis Obliterans

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Abstract

Background: For several years researchers have been puzzled by the complexity of chronic lung transplant rejection, also known as bronchiolitis obliterans (BO), due to its implications in long term morbidity and mortality in Lung Transplant Recipients (LTRs). Endothelial cells going through apoptosis, as result of a stimulus or stress, lead to imbalanced production of mediators in several fibrotic disorders. Studies have demonstrated that production of such mediators is a key player in inducing and activating the process of Epithelial-Mesenchymal-Transition (EMT). In this process, epithelial cells lose their properties and gain mesenchymal ones, therefore, becoming more mobile and invasive. Translocation of transcription factors (TF), such as Twist and Snail, is suggested to participate in the EMT process by repressing the expression of epithelial markers such as E-cadherin, and inducing the expression of mesenchymal markers such as Collagen and alpha-smooth muscle actin (α-SMA). Aim: Study and investigate *in vitro* mediators produced by apoptotic endothelial cells, and detect mRNA and protein expression of EMT markers in the small airway epithelial cells (SAEC). **Methods:** Apoptotic (SSC4h) and non-apoptotic (SSC4h-ZVAD) media were produced from Human Microvascular Endothelial Cells (HMVEC). ELISA was used to detect and measure released mediators such as connective tissue growth factor (CTGF) and transforming growth factor-beta (TGF-β), in conditioned media. On the other hand, immunoblotting was used to detect perlecan in both media. Small Airway Epithelial Cells (SAEC) were stimulated with the following conditions: RPMI, 10 ng/ml TGF-β, 20 ng/ml CTGF, SSC4h, SSC4h +neutralizing anti-CTGF (SSC4h+Ab-CTGF), and SSC4h-ZVAD. The extracted

mRNA was followed by reverse transcription and Quantitive-polymerase chain reaction (Q-PCR) to detect the level of mRNA expression of E-cadherin (an epithelial marker), Collagen IA1 and α-SMA (mesenchymal markers). SAEC stimulated for 48 hours were then used for immunofluorescence procedure to detect the level of protein expression, where antibodies specific for epithelial markers (Cytokeratin and E-cadherin), mesenchymal markers (Collagen I, α-SMA, and Vimentin), and for transcription factors (Twist and Snail) were used to assist in detecting the protein level. **Results:** CTGF-ELISA done on conditioned media revealed a high level of CTGF in SSC4h compared to SSC4h-ZVAD, 62.70vs.15.63 pg/ml, respectively. However, TGF-beta-ELISA done on conditioned media has failed in detecting TGF-beta in both media. Immunoblotting against Perlecan on extracted proteins from both media showed low or no presence of Perlecan, a 23kDa fragment, in proteins extracted from SSC4h-ZVAD medium, but was highly visible in proteins extracted from SCC4h with 9 fold increase of density unit compared to SSC4h-ZVAD. Therefore Perlecan is produced by endothelial cells that go through apoptosis. Results of mRNA expression of stimulated SAEC revealed the following: decrease of E-cadherin expression in SAEC stimulated with SSC4h compared to cells stimulated with both SSC+Ab and SSC4h-ZVAD, increase in the expression of collagen IA1 in cells stimulated with TGF-β, CTGF, SSC4h, and SSC4h+Ab-CTGF compared to the baseline (RPMI), and SSC4h-ZVAD. Finally, an increase in the expression of α-SMA was detected in SAEC stimulated with TGF-β, CTGF, SSC4h, SSC4+Ab-CTGF compared to the baseline (RPMI), and SSC4h-ZVAD. Immunofluorescence procedures of SAEC stimulated with SSC4h revealed the following: decrease in the intensity/area of the epithelial markers E-cadherin and cytokeratin; increase in the

intensity/area of mesenchymal markers collagen, SMA, and Vimentin; and increase in the intensity/area of both Snail and Twist. **Conclusion:** Our results demonstrate that apoptosis of lung endothelial cells produced mediators that work and stimulate the initiation of the EMT process in SAEC. Activation of such a process causing airway remodeling can initiate the development of bronchiolitis obliterans in LTRs.

Used abbreviations: BO: Bronchilitis Obliterans, LTRs: Lung Transplant Recipients, EMT: epithelial-mesenchymal-transition, HMVEC-L: Human Microvascular Endothelial Cells-Lungs, SACE: Small Airway Epithelial Cells, TF: transcription factor, TGF-β: transforming growth factor-beta, CTGF: connective tissue growth factor, SSC4h: conditioned apoptotic medium 4hrs, and SSC4h-ZVAD: conditioned non-apoptotic medium.

Introduction

Lung transplant is considered to be the only therapeutic option for patients with end-stage lung diseases. Despite the acceptable 1-year survival rate of 73%, the 5-year survival rate still remains poor at 45% [2].Even with the enhancements that have been done to increase the success of lung transplantation, patients may have to face several obstacles in order to survive post lung transplant. One of the major hurdles is chronic lung transplant rejection which is defined histologically as bronchiolitis obliterans (BO), and clinically as bronchiolitis obliterans syndrome (BOS) through diagnosis and grading via spirometry [47]. In the 2010 report of the registry of the International Society for Heart and Lung transplantation, it is stated that development of BO remains one of the major risk factors post lung transplant with percentage of 36.9 and 54.1 within 5-year and 10-year post lung transplant, respectively [2]. Bronchiolitis obliterans remains as one of the major causes of long term morbidity and mortality in lung transplant recipients (LTRs).

Epithelial-Mesenchymal-Transition (EMT) is a transdifferentiation process, where the epithelial cells go through morphological changes that lead them to lose their epithelial properties and gain mesenchymal ones. The acquired properties allow for production of metalloproteinases (MMPs) and deposition of extracellular matrix (ECM)[208]. One study has suggested that thickening of the reticular basement membrane by collagen deposition might be a possible explanation for airway remodeling in LTRs. [345]. This group has demonstrated the existence of Rbm

thickening with median time post-transplant of 6 months, suggesting that change in the ECM, collagen deposition, and thickening of Rbm might be an informative parameter in detecting airway remodeling. The importance of EMT rises due to its implication in several pathologies such as chronic inflammation, fibrosis, and the development of cancer [208, 280, 346, 347], where it functions mainly in generating mesencymal cells that participate in the pathogenesis of a disease. According to other studies, the EMT process has been implicated in fibrotic diseases of the kidney [219], lung [215], and liver [214].

Studies on fibrosis have noted apoptosis of endothelial cells (ECs) as an early pathogenic event. Fibrogenic disorders such as systemic sclerosis [152, 153] , graft versus-host disease [154, 155], and chronic rejection of kidney allograft [156, 157] , have been associated with deregulated and increased apoptotic EC. Some studies indicate that apoptotic EC favors recruitment of professional phagocytes [155, 157], which results in inducing the production of transforming growth factor-beta1 (TGF-β1) [158] . In previous studies it has been mentioned that the C-terminal fragment of perlecan (LG3), that resulted from translocation of cathepsin L from apoptotic EC, induces Bcl-xl upregulation and resistance to apoptosis in fibroblasts, mesenchymal stem cells and smooth muscle cell, all of which are crucial in tissue or vascular remodeling and repair [156, 159-161, 275, 348].

The EMT process involves two major steps: inactivation or repression of the epithelial genes and activation of the mesenchymal ones. Both steps are regulated by transcription factors (TF) that work on either repressing epithelial genes, or activating mesenchymal genes, some TF can function in both directions. The hallmark of EMT is loss of E-cadherin expression as consequence of an inhibitor binding at the E-box sequences of the E-cadherin promoter [319, 349] One of TF that work on repressing E-cadherin by binding to its promoter is Snail (Snail1), which is capable of direct binding to the E-boxes of E-cadherin's promoter [320, 350, 351]. Another transcriptional repressor of the E-cadherin expression is Twist, a member of the basic helix-loop-helix (bHLH) family [321, 329]. Studies have suggested that Twist might work on repressing E-cadherin through a different mechanism that does not necessarily involve direct binding of Twist to the E-caderin promoter [330] .

In our study, we hypothesized that stress or stimulus causes apoptosis of endothelial cells, and leads to production of paracrine mediators. We aimed to characterize mediators released by apoptotic EC, where these released mediators work on translocating TF such as Snail and Twist, therefore, inducing the EMT process. Activation of EMT leads to fibrosis, tissue scarring, and possibly airway remodeling which might be a possible contributor in the development of bronchiolitis obliterans in the recipients of lung transplantation.

Methods

Cell culture

Human Lung MicroVascular Endothelial Cells (HMVEC-L), and Human Small Airway Epithelial Cells (SAEC) were obtained from Clonetics Lonza. HMVEC-Ls were used at passages 4-7, seeded on gelatinized dishes throughout the procedure to ensure good attachment of the cells, and grown in EBM-2 medium (Clonetics Lonza, Canada). On the other hand SAECs were used at passages 2-10, and grown in SABM medium (Clonetics Lonza, Canada). Both media come with their supplementing products. Three different lots (n=3) of HMVEC were used in the production of media, whereas four different lots (n=4) of SAEC were used for stimulation.

Screening for apoptosis in HMVEC-Ls with fluorescence microscopy

Procedures for identifying the apoptotic HMVEC-L were kindly provided by Dr. Marie-Josée Hebert and her team. In this part of the experiment, fluorescence microscopy was done on unfixed adherent endothelial cells that were stained with Hoechst 33342 (2´-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2, 5´-bi-1H-benzimidazole (HT)) (Invitrogen, Canada) and propidium iodide (PI) (Invitrogen, Canada). In summary, endothelial cells were seeded on a 24-well polycarbonate culture plates. Once the endothelial cells have reached confluence, they were divided into three groups: no pre-incubation, pre-incubation with Dimethyl sulfoxide (DMSO, Sigma, Canada) only, and pre-incubation with DMSO+ ZVAD-FMK (R&D, Canada), a pan caspase inhibitor that prevents endothelial apoptosis. The time of

incubation was 2 hours. Afterwards, all three groups of cells were washed with PBS and then were serum-starved for 4 hours with serum free medium RPMI (Invitrogen, Canada). HT was added for a final concentration of 1 μg/ml for 10 min at 37°C with 5% CO_2/95% air, cells were then washed with 1x PBS. PI was added with a final concentration of 5μg/ml prior to fluorescence microscopy analysis. Percentage of normal, apoptotic, and necrotic endothelial cells were estimated by an investigator blinded to the experimental conditions.

Viable cells are characterized by normal nuclear and cytoplasmic morphology and should show blue staining. Apoptotic cells are characterized by cell shrinkage, nuclear condensation, and preservation of plasma membrane integrity. When the chromatin are condensed it has enhanced HT fluorescence staining (bright blue staining), integrity of the cell membrane prevents PI staining. Characteristics of late apoptosis (also known as secondary necrosis) are similar to the ones of apoptosis, in addition to PI staining due to loss of cell membrane integrity. Finally, primary necrosis show characteristics of increase in the cell size, absence of chromatin condensation, and cell membrane disruption.

Production of conditioned apoptotic (SSC4h) and non-apoptotic media (SSC4h-ZVAD)

In order to produce enough amounts of both apoptotic and non-apoptotic media used as a negative control, HMVEC were seeded on a gelatinized T75. Once the endothelial cells have reached confluence they were either pre-incubated with DMSO, DMSO+ZVAD-FMK or not. For the production apoptotic medium,

endothelial cells did not need to be pre-incubated and were stimulated with serum-free medium (RPMI) from 4 to 24 hours at 37°C with 5% CO_2/95% air, to produced the serum starved conditioned medium SSC. On the other hand, production of non-apoptotic medium was done by pre-incubating the endothelial cells with 100μM of dissolved ZVAD-FMK in DMSO at 37°C with 5% CO_2/95% air. Afterwards, cells were washed with 1x PBS and then stimulated with serum-free medium (RPMI) from 4 to 24 hours at 37°C with 5% CO_2/95% air, to produce the non-apoptotic conditioned SSC-ZVAD medium. Time-course serum starvation demonstrated that the optimal period for producing both media was 4 hours, leading to production of SSC4h and SSC4h-ZVAD, for apoptotic and non-apoptotic media, respectively. At the concentration used, DMSO was not found to induce cell toxicity. Staining using HT and PI were used to calculate percentage of apoptosis and necrosis. The percentage of apoptosis vs. necrosis in SS4h, SSC4h+DMSO, and SSC4h-ZVAD was as follow: 7.4% vs. 0.36%, 4.34% vs. 2.69%, and 1.76% vs. 0.07%, respectively. The harvested media were then stored at -20°C for further experimental procedures.

Detection of CTGF and TGF-beta levels in produced media

The media were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) specific for human connective tissue growth factor (CTGF) and transforming growth factor-beta (TGF-β), where ELISA kits were obtained from Peprotech (Canada), and R&D (Canada), for detecting CTGF, and TGF-beta, respectively. Procedure on the usage of ELISA was provided by manufacturers.

Immunoblotting

Proteins were extracted from both SSC4h and SSC4h-ZVAD media. In order to compare the level of protein expression, 700µl from each medium was obtained. Afterwards, sodium deoxycholate 2% was added to each sample with 1:100 dilution for 30 min on ice, TCA was added for an overnight incubation, centrifuged at 1400 r.p.m for 10 min, washed with cold acetone and solubilized in Laemmli sample buffer, followed by western blotting against perlecan. The antibody for western blotting is rabbit-polyclonal α-Perlecan (Santa Cruz, USA) with a dilution of 1:250 in TBS-5% milk, at 4°C overnight. Prior to blotting with desired antibody, membranes were stained with Ponceau S Red as a way of protein detection.

Stimulation of Small Airway Epithelial Cells (SAECs)

Epithelial cells were cultured on 6-well plates and 8-chambers lab-teks, for RNA extraction and immunofluorescence procedures, respectively. Once SAEC seeded on 6-well plates reached confluence, each well was stimulated for 24 hours, for RNA extraction, by one of the following conditions: RPMI, hrTGF-β, hrCTGF, SSC4h, SSC4h +Ab-CTGF, and SSC4h-ZVAD. Where RPMI is serum-free medium, hrTGF-β is human recombinant transforming growth factor beta, hrCTGF is human recombinant connective tissue growth factor, SSC4h is conditioned apoptotic medium, SSC4h+Ab-CTGF is conditioned apoptotic medium with human CTGF neutralizing antibody (ProSci, Canada), and SSC4h-ZVAD is conditioned non-

apoptotic medium. The negative controls were: SSC4h+Ab-CTGF and SSC4h-ZVAD, whereas the positive controls were: TGF-β and CTGF, all were used in order to assess and compare the biological effects of conditioned SSC4h. Epithelial cells cultured on 8 chamber lab-teks were also stimulated by previously mentioned conditions, where each lab-tek was stimulated by one condition of the mentioned conditions for 48 hours to be used in immunofluorescence analysis.

RNA Extraction/ Reverse Transcription-Polymerase Reaction (RT-PCR)/ Quantitative-PCR (Q-PCR)

RNA extraction of SAEC was done using RNeasy mini kit from Qiagen and procedure was provided by manufacturer. According to the manufacturer, extraction of RNA using RNeasy technology is done based on the selective binding of silica-based membrane and the use of microspin speed. Furthermore, ethanol is used as well in the procedure to help in ensuring proper binding conditions of RNA to the RNeasy mini spin column. Resulted high-quality RNA is then eluted in RNAse-free water.

Following RNA purification, reverse transcription-polymerase chain reaction (RT-PCR) was performed to obtain complementary DNA (cDNA). In this procedure, synthesis of cDNA from a single-stranded RNA was done using a DNA polymerase reverse transcriptase (SuperScriptII, Invitrogen, Canada).

The amplified cDNA was then used in real-time-PCR, also known as Quantitive-PCR (Q-PCR). This procedure was done using RotorGene program "Three Step with Melt". The process starts out by activating the enzyme with 10 min hold at 95°C. Then the amplification process is done in three steps: Step 1: Hold at 95°C for 10 min, and 0 sec; Step 2: hold at 59°C for 15 sec; Step 3: hold at 72°C for 20 sec, with a total of 40 cycles. The sequences of used primers provided by Invitrogen are as follow: CollagenIA1-Forward: CTGGAAGAGTGGAGAGTACTG, Collagen IA1-Reverse: ATGTACCAGTTCTTCTGGGC, E-cadherin-Forward: GCTGGAGATTAATCCGGACA, E-cadherin-Reverse: ACCTGAGGCTTTGGATTCCT, GAPDH-Forward: CTCTCTGCTCCTCCTGTTCGAC,GAPDH-Reverse: TGAGCGATGTGGCTCGGCT, α-SMA-Forward: GCTGTTTTCCCATCCATTGT, α-SMA-Reverse: TTGTGATGATGCCATGTTCT.

Immunofluorescence

Immunofluorescence procedure was used in order to detect the protein expression level of epithelial and mesenchymal markers; as well as the protein level of transcription factors. In this procedure, stimulated lab-teks (mentioned previously) were blocked with 5% BSA in PBS for 1hr, and then incubated for 1hr with primary antibodies against the following human antigens: mouse-monoclonal α-SMA (Sigma, Canada), mouse-monoclonal Collagen I (Sigma, Canada), mouse-monoclonal Cytokeratin (ZYMED laboratories, Canada), mouse-monoclonal E-cadherin (Abcam,

Canada), rabbit-polyclonal Snail (Abcam, Canada), rabbit-polyconal Twist (Abcam, Canada), and mouse-monoclonal Vimentin (Sigma, Canada). Following incubation with primary antibodies, the lab-teks were then incubated for 45 min with either goat anti-mouse, or goat anti-rabbit antibodies that are marked with Alexa 488 (Invitrogen, Canada). Images of immunofluorescence were captured by Olympus fluorescence microscope and then analyzed by Image-Pro Analyzer provided by Olympus to help in obtaining needed measurements for further analysis. For each stimulation a duplicate of captured images were obtained for all lots (for n = 3) of used SAEC. Fluorescent intensity associated with immunoreactivity was determined in all stimulated cells was determined by calculating average raw pixel intensity in captured area of staining. For each captured image a mean of intensity and mean of area were obtained from Image-Pro Analyzer statistical analysis. Obtained mean intensity was divided by obtained mean area to give a score of mean Intensity/Area. A mean score of intensity/area of duplicate images obtained for each type of stimulation were calculated and used in the final calculation of mean intensity/score for all three lots.

Statistical analysis

Calculated data for: CTGF concentration, Perlecan intensity expression, mRNA expression of EMT markers, proteins fluorescence intensity of EMT markers were all expressed a mean (with standard error of the mean SEM)). The significance difference between groups was assessed using paired two-tailed student's test (t-test) using Microsoft Excel. Differences with p value of ≤ 0.05 were considered significant (represented by the symbol *).

Results

Produced Mediators by Apoptotic Endothelial Cells

Human lung apoptotic endothelial cells (SSC4h) produce a high level of CTGF when compared to non apoptotic media (SSC4h-ZVAD), 62.70pg/ml vs.15.63 pg/ml (Figure 18A). Surprisingly, TGF-b was not detected in both media (data not shown). Results obtained from immunoblotting against perlecan done on proteins extracted from both media reported an elevated signal of perlecan in SSC4h that was 9 folds higher than SSC4h-ZVAD (Figure 18B). This indicates that the pathway of apoptosis is a critical factor in the production of CTGF and perlecan as apoptotic mediators.

Expression Level of Epithelial-Mesenchymal-Transition Genes

Analysis of RNA expression from stimulated SAEC using Q-PCR helps in understanding the level of expression for epithelial (E-cadherin) and mesenchymal (collagenIA1 and α-SMA) markers. Figure 19 represents the level of E-cadherin expression in stimulated SAEC. Although the expression of E-cadherin in cells stimulated with SSC4h (1.36 fold of expression) was not lower than the RPMI baseline expression. However; it has been noted that the expression of E-cadherin in cells stimulated with SSC4h was slightly lower than cells stimulated with TGF-β (1.53 fold), CTGF (1.45 fold), SSC4h+Ab-CTGF (1.61 fold), and SSC4-ZVAD (1.56 fold). Figure 20 represents the level of collagenI expression in stimulated SAEC. It has been noted a high expression level of collagenI in cells stimulated with TGF-

β (8.17 fold), CTGF (1.87 fold), SSC4h (1.78 fold), and SSC4+Ab-CTGF (2.08) compared to those stimulated with RPMI (1.00) and SSC4h-ZVAD (0.89). The expression level of collagenI was significant in cells stimulated with baseline RPMI and SSC4-ZVAD when compared to TGF-β with $p \leq 0.05$ (represented by * in the Figure). Figure 21 is a representation of the gene expression of alpha-smooth muscle actin (α-SMA), where a pattern of expression similar to collagen IA1 was noted in stimulated SAEC. Stimulated SAEC demonstrated increase in the expression of α-SMA in cells stimulated with TGF-β (1.24 fold), CTGF (1.97 fold), SSC4h (1.94 fold), SSC4h+Ab-CTGF (1.86) compared to those stimulated with RPMI and SSC4h-ZVAD (0.40 fold).

Expression Level of Epithelial-Mesenchymal-Transition Proteins

Immunofluorescene staining using antibodies specific to epithelial and mesenchymal markers and transcription factors has helped in detecting the level of protein expression. Analysis of the captured fluorescence images helped in determining the level of intensity of each marker per captured area, where the level of protein expression is presented by intensity/area. Figures 22A and 22B represent the expression of epithelial markers, E-cadherin and cytokeratin, in stimulated SAEC. It was noted that the intensity/area score in cells stimulated with SSC4h demonstrated a decrease in the expression of epithelial markers, with a score of intensity/area of 18.27 in cytokeratin and 343.33 in E-cadherin, compared to the intensity of the baseline (RPMI) which had an intensity/area of 46.08 in cytokeratin and 378.07 in E-

cadherin. Cells stimulated with SSC4h-ZVAD had a high expression of the epithelial marker E-cadherin with an intensity/area of 377.97 compared to those stimulated with SSC4h. The level of epithelial markers expression in cells stimulated with TGF-b, CTGF, SSC4h+Ab-CTGF demonstrate similar results to those stimulated with SSC4h. Figures 23A and 23B represent the immunofluorescence results of the mesenchymal markers collagen, a-SMA, and Vimentin in stimulated SAEC. These data demonstrate a high level of expression of the mesenchymal markers in cells stimulated with SSC4h compared to those stimulated with RPMI and ZVAD with intensity levels of with collagen having an intensity of 217.69 intensity/area, α-SMA of 115.99 intensity/area, and Vimentin of 174.19 intensity/area. Figures 24A and 24B represent the level of expression for the transcription factors Snail and Twist, where Snail has an expression level of 94.92 intensity/area in cells stimulated with SSC4h compared to RPMI which had an expression of 62.59 intensity/area. Twist had an expression level of 54.58 intensity/area in cells stimulated with SSC4h compared to RPMI which had an expression of 30.89 intensity/area.

Discussion

Etiologies behind the development of chronic lung transplant rejection, or bronchiolitis obliterans (BO), remain poorly understood due to the involvement of several, complex and fine pathways in it. Studies suggested that a hallmark of BO development is the destruction of the epithelial which lead to luminal obliteration

[175, 179, 332, 352]. One mechanism where epithelial cells go through morphological changes is Epithelial-Mesenchymal-Transition (EMT), a transdifferential process, in which the epithelial cells lose their epithelial properties and gain mesenchymal ones. The acquired properties allow for production of metalloproteinases (MMPs) and deposition of extracellular matrix (ECM)[346]. EMT has been implicated in the pathogenesis of chronic inflammation, fibrosis, and the development of cancer [2, 208, 280, 346].

Studies done on fibrogenic disorders such as systemic sclerosis [152, 214], graft versus-host disease [153, 154], and chronic rejection of kidney allograft [155, 156], suggest apoptosis of endothelial cells (EC) as a key initiator of disorder. It is favorable that apoptotic endothelial cells work on producing and releasing mediators through a complex and fine pathway, such as proteolysis of the extracellular matrix (ECM). In the study done by Hebert and her team [348], it is suggested that generation of bioactive mediators by apoptotic cells is done through an "energy-efficient" ECM proteolysis. Since the ECM is a good reservoir of growth factors and cryptic bioactive factors [353-355] to maintain cell survival. In their previous work [348], one bioactive mediator produced by apoptotic EC is a C-terminal fragment of the domain V of perlecan, which is suggested to be released through activation of a caspase-dependent pathway [356].

In our study we were able to induce apoptosis in lung HMVEC as well as inhibit apoptosis through pre-incubation with ZVAD-FMK, which is a caspase inhibitor. Analysis on conditioned apoptotic (SSC4h) and non-apoptotic (SSC4h-ZVAD) media suggest that only the apoptotic medium was capable of producing

mediators such as CTGF and perlecan as a consequence of apoptosis, possibly through proteolysis of the ECM. These mediators were not or barely detected in SSC4h-ZVAD, this indicates that inhibition of apoptosis in HMVEC prevents proteolysis of ECM, thus inhibits the production of major mediators. Furthermore, TGF-β was not detected in both media which suggests that apoptosis of EC does not produce TGF-β in the mix of both conditioned media. Detection of CTGF and perlecan supports that work done by others [151], who were able to produce CTGF and perlecan through conditioning apoptotic endothelial media. Also, they were not able to detect TGF-β in their conditioned media which also supports our results of failing to detect TGF-β in our media.

Since the EMT process involves repression of the epithelial genes and activation of the mesenchymal ones, studying the level of genes expression of stimulated cells with SSC4h is an essential step in detecting the occurrence of the EMT process. This process is critically regulated by transcription factors, such as Twist and Snail, which function in an organized matter by binding to the E-cadherin promoter [320, 349], repressing its expression, and activating the expression of mesenchymal markers. Although the mRNA expression of E-cadherin in small airway epithelial cells stimulated with SSC4h was slightly higher but not statistically different to the baseline expression. A similar pattern was observed for all the conditions with the exception of TGF-beta showing a significant increase in E-cadherin mRNA expression (1,53X). We propose that 24 hours stimulation is not enough to induce a significant change in E-cadherin mRNA expression.

A significant increase in the mRNA expression of collagenIA1 was observed in SAEC stimulated with SSC4h (1.78 fold), as well as in the positive controls TGF-β (8.17 fold), CTGF (1.87 fold), and SSC4+Ab-CTGF (2.08) when compared to the negative controls such as baseline RPMI (1.00) and SSC4h-ZVAD (0.89). This suggests that mediators in SSC4h work on inducing the mRNA expression of collagenIA1 in a similar manner to CTGF and to a lesser extent when compared to TGF-b. Total inhibition of CTGF in SSC4h (SSC4h+Ab-CTGF) did induce the mRNA expression of collagenIA1 as well which suggests involvement of other mediators in the apoptotic mix that work on inducing the EMT markers. In addition, these results suggest that mediators produced by SSC4h conditioned medium play a crucial role in activating the expression of mesenchymal markers, and inhibition of apoptosis, which results in inhibiting the production of these mediators, might prevent induction of EMT. The mRNA expression of α-SMA in stimulated SAEC demonstrated similar pattern of expression to those of collagenIA1. An increase in the mRNA expression of α-SMA was observed in SAEC stimulated with SSC4h (1.94 fold) similar to the positive control CTGF (1.97 fold) and higher that the other positive control TGF-β (1.24 fold) when compared to baseline expression. The increased expression with the SSC4h was not inhibited by neutralization of CTGF. No stimulation of the expression was detected with the negative control non apoptotic media.

We completed our mRNA expression analysis by microscopic analysis of stimulated SAEC. We first noted that the morphology of SAEC did change when

stimulated for 48 hours with conditioned apoptotic medium where SAEC take a fibroblastic appearance as noted by the elongation of cells and ramification formation. We noted that protein expression of epithelial markers, E-cadherin and Cytokeratin, was low in SAEC stimulated with SSC4h compared to those stimulated with RPMI, SSC4h+Ab-CTGF, and SSC4h-ZVAD. TGF-beta did not succeed to decrease the epithelial markers. On the other hand, the expression of mesenchymal markers, such as collagen, α-SMA, and Vimentin demonstrate a high level of protein expression when compared to those stimulated RPMI, SSC4h+Ab-CTGF, and SSC4h-ZVAD. Increase in the expression of Twist and Snail was noted in SAEC stimulated with SSC4h when compared to baseline RPMI, and in SAEC stimulated with SSC4h+Ab-CTGF, and SSC4h-ZVAD. At the protein level, the neutralization of CTGF in the conditioned apoptotic media succeeds to inhibit the EMT effect of SSC4h. Furthermore, SAEC stimulated with TGF-β and CTGF demonstrate similar reaction to that of SAEC stimulated with SSC4h where decrease in epithelial markers and increase in the mesenchymal ones are observed as results of the stimulation. This suggests involvement of released mediators by apoptotic endothelial cells to be responsible in inducing EMT, and inhibition of their release might prevent fibrosis.

Our results support others work [343] where they have worked on inducing EMT in epithelial cells obtained from lung transplant recipients. In their studies, they have stimulated EMT using TNF-α and TGF-β, and checked their effect in driving the fibrosis process and deposition of ECM. They were able to detect decrease in the expression of E-cadherin an epithelial marker, and note an increase in the expression of Vimentin and α-SMA in patients who developed BOS compared to the controls

and stable LTRs. In their studies they also confirm the implication of EMT as a mechanism driving the development of BO. This confirms our results where released mediators through ECM proteolysis have direct consequences in inducing EMT and thus causes airway remodeling which leads to BO development. .

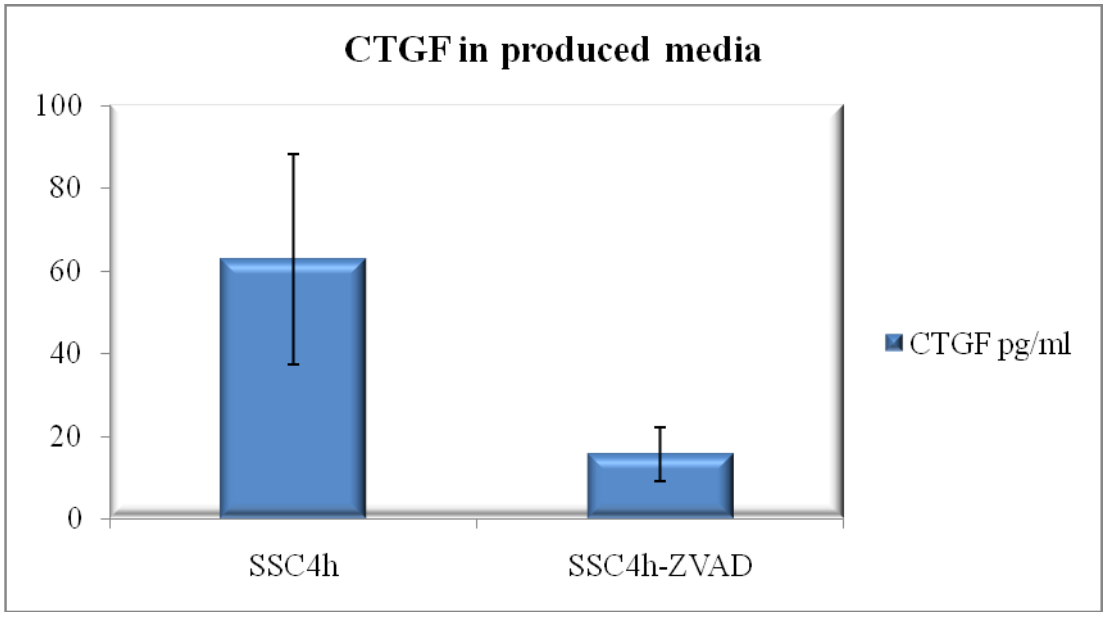
Furthermore, SAEC stimulated with TGF-β and CTGF demonstrate similar reaction to that of SAEC stimulated with SSC4h where decrease in epithelial markers and increase in the mesenchymal ones are observed as results of the stimulation.

Conclusion

Stimulation of apoptotic mediators of lung EC in activating EMT might help in having a better understanding of the involvement of EMT in several pathologies. It might also help in revealing possible connection between EMT and airway remodeling which is a key feature in lung diseases such as BO. Early detection of apoptotic EC might help in treating the disorder at an early stage which leads to higher chances of survival.

Figure 18: Mediators produced in apoptotic (SSC4h) and non-apoptotic media (SSC4h-ZVAD):

A) Level of connective tissue growth factor (CTGF) in produced media:



B) Perlecan detection in produced media:

~23 kDa

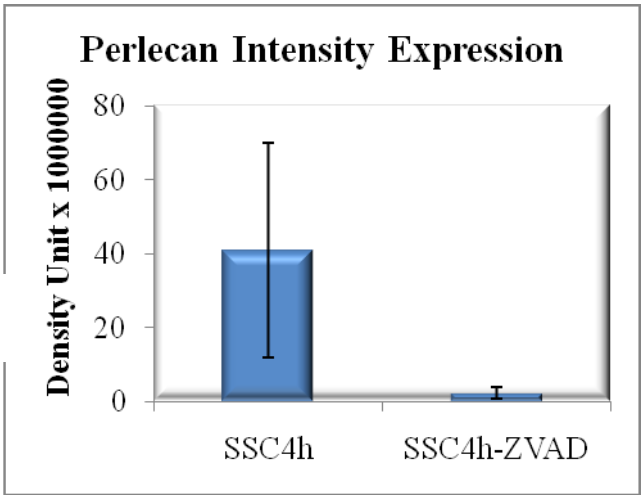


Figure 18: A) ELISA detection shows a high CTGF level in SSC4h (62.70pg/ml) compared to SSC4h-ZVAD (15.63 pg/ml). Mean ± SEM, n=3. B) Immunoblotting against Perlecan shows an expression of 9 folds higher in SSC4h medium (~23kDa highlighted with black rectangular) compared to SSC4h-ZVAD medium. Mean ± SEM, n=2.

Figure 19: Level of E-cadherin Expression in stimulated Small Airway Epithelial Cells (SAEC):

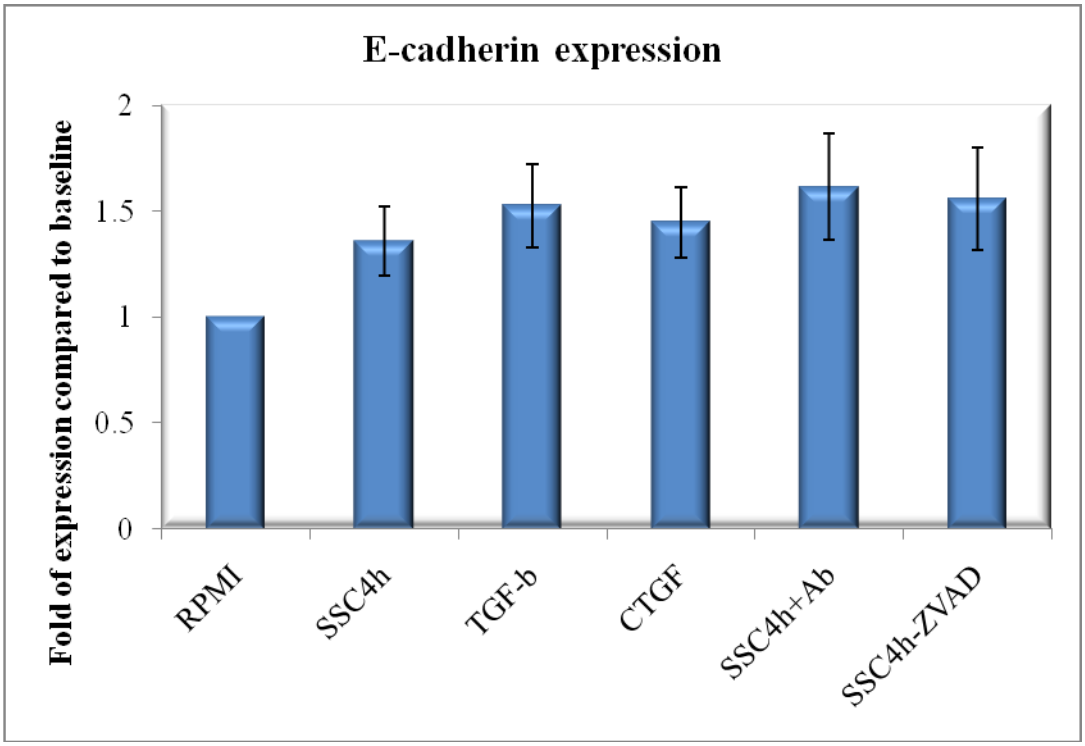


Figure 19: Level of E-cadherin mRNA expression of stimulated SAEC for 24hrs with: RPMI, apoptotic medium SSC4h, 10ng/ml TGF-β, 20 ng/ml CTGF, apoptotic medium SSC4h + neutralizing anti-CTGF, and non-apoptotic medium SSC4h-ZVAD. Mean ± SEM, paired t-test n=4, (*) statistically significant $p \leq 0.05$.

Figure 20: Level of Collagen IA1expression in stimulated Small Airway Epithelial Cells (SAEC):

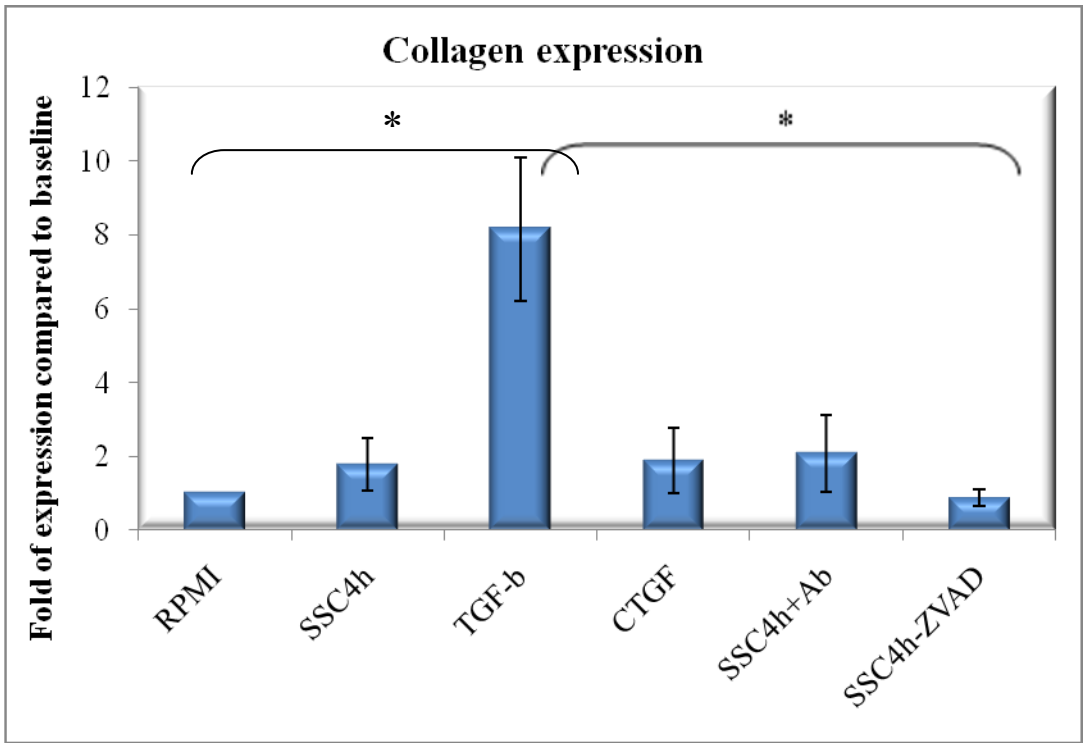


Figure 20: Level of CollagenIA1 mRNA expression of stimulated SAEC for 24hrs with: RPMI, apoptotic medium SSC4h, 10 ng/ml TGF-β, 20 ng/ml CTGF, apoptotic medium SSC4h + neutralizing anti-CTGF, and non-apoptotic medium SSC4h-ZVAD. Mean ± SEM, paired t-test n=4, (*) statistically significant $p \leq 0.05$.

Figure 21: Level of alpha Smooth Muscle Actin (α-SMA) expression in stimulated Small Airway Epithelial Cells (SAEC):

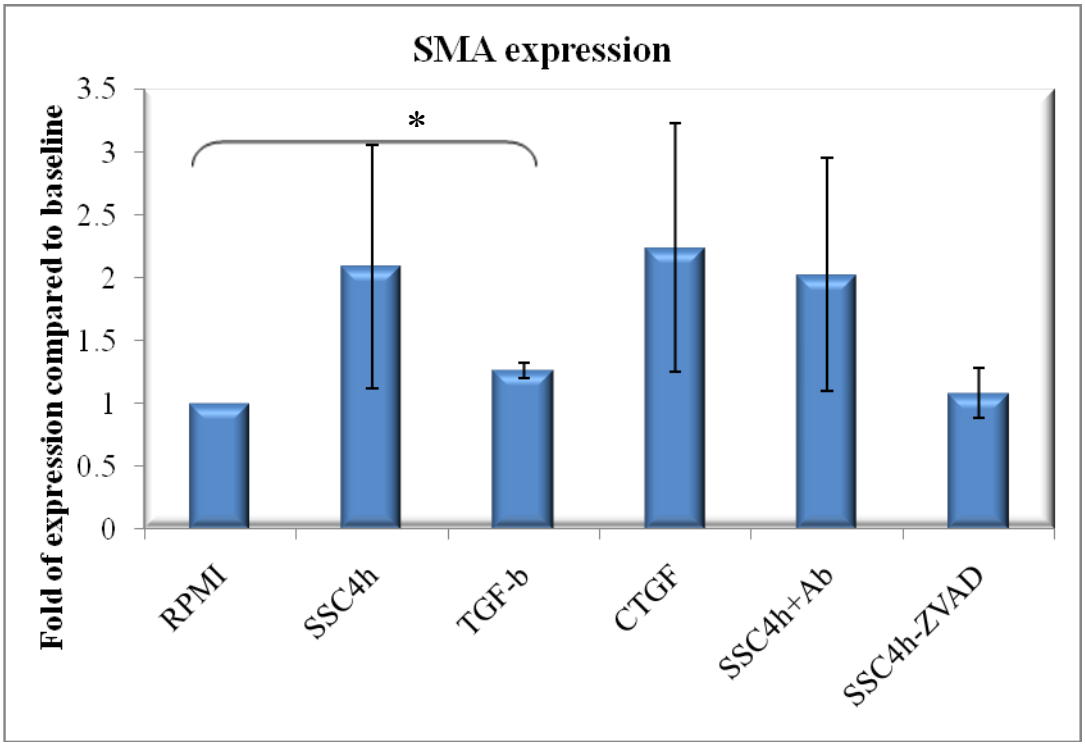
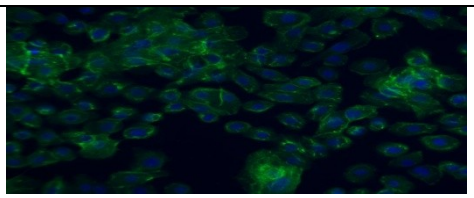
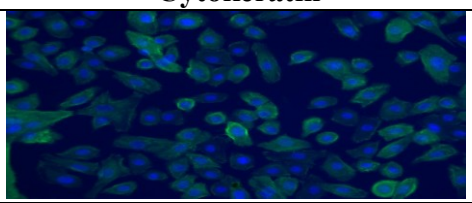
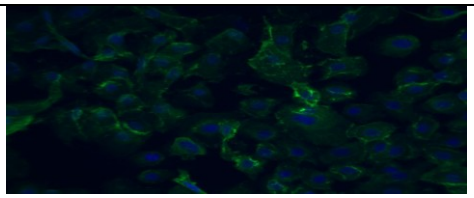
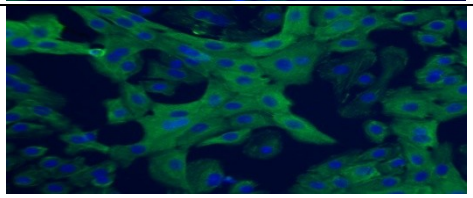
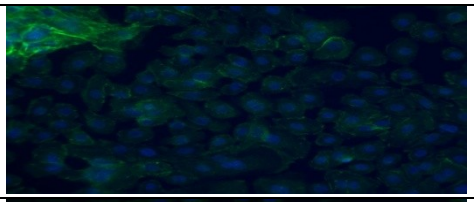
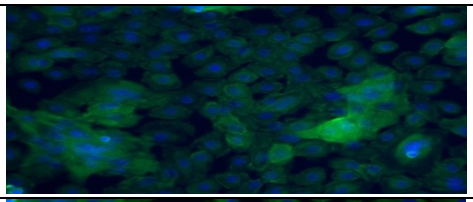
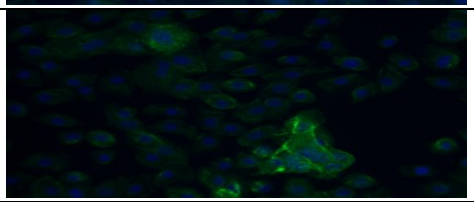
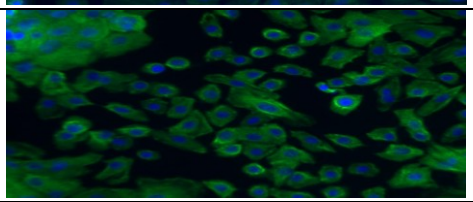
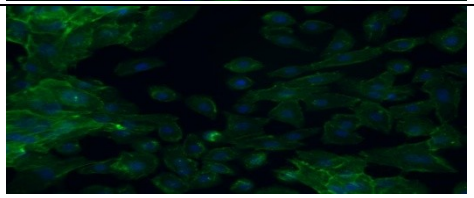
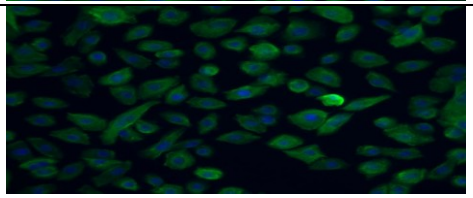
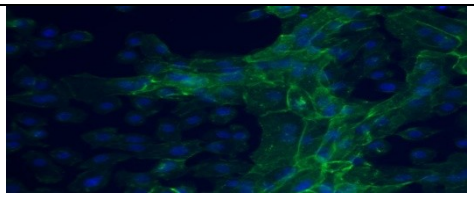
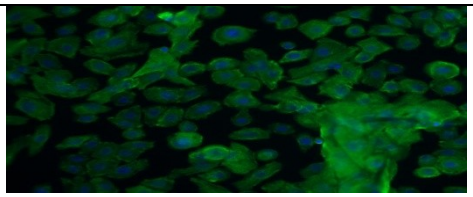


Figure 21: Level of alpha Smooth Muscle Actin (α-SMA) mRNA expression of stimulated SAEC for 24hrs with: RPMI, apoptotic medium SSC4h, 10 ng/ml TGF-β, 20 ng/ml CTGF, apoptotic medium SSC4h + neutralizing anti-CTGF, and non-apoptotic medium SSC4h-ZVAD. Mean ± SEM, paired t-test n=4, (*) statistically significant $p \leq 0.05$.

Figure 22: Immunofluorescence of Epithelial markers:

A)

	E-cadherin	Cytokeratin
SSC4h		
RPMI		
TGF-β		
CTGF		
SSC4h +Ab-CTGF		
SSC4h-ZVAD		

B) Fluorescence intensity of epithelial markers:

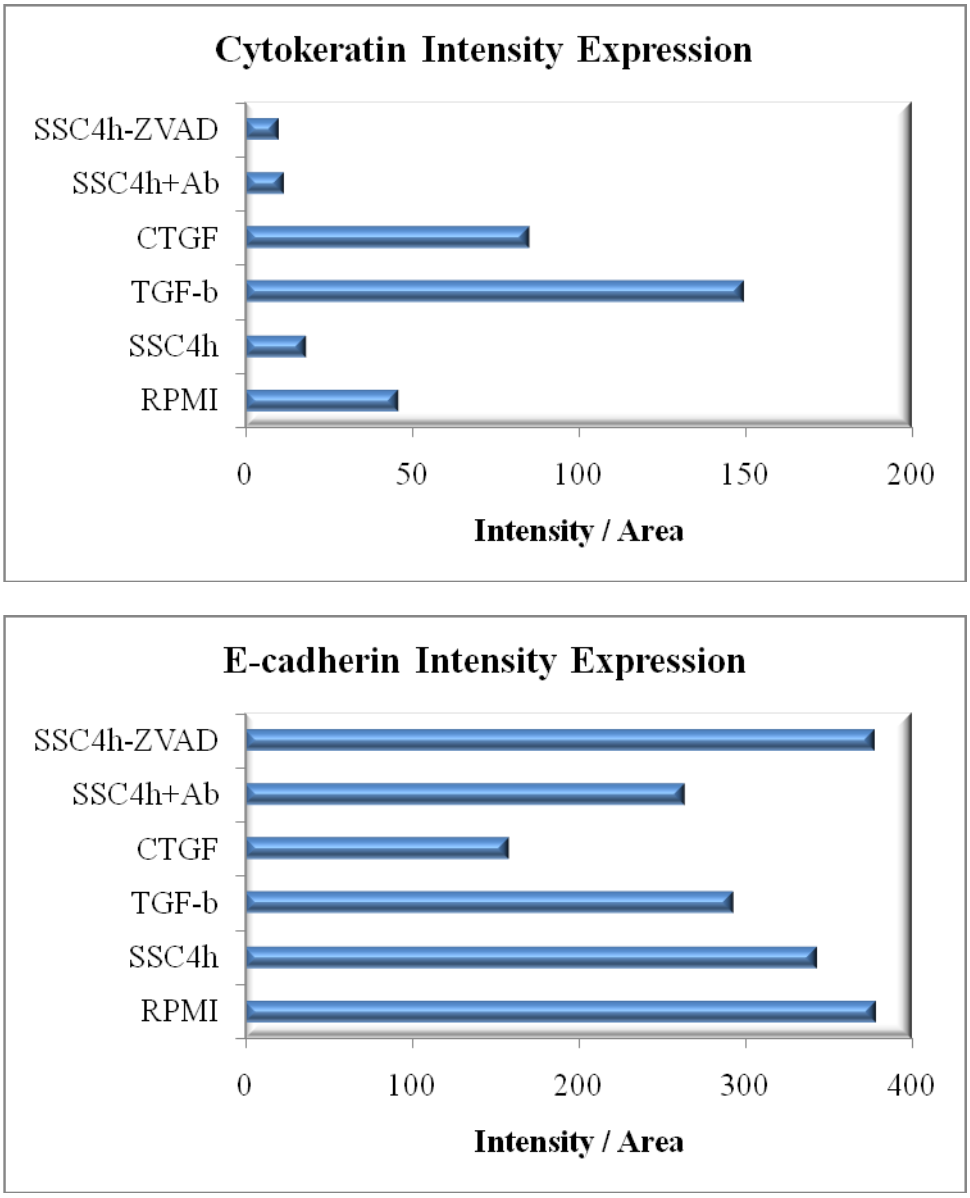
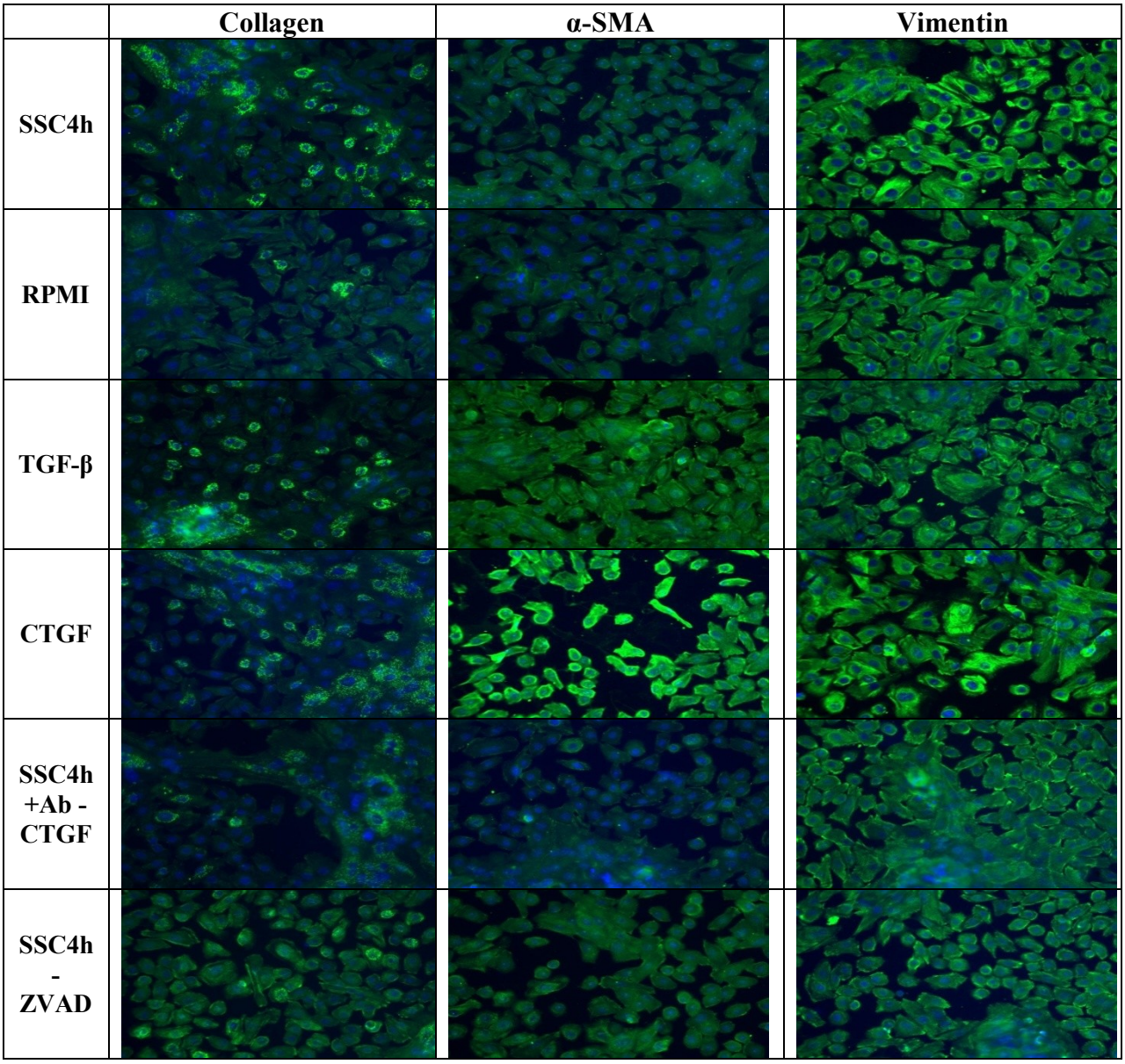


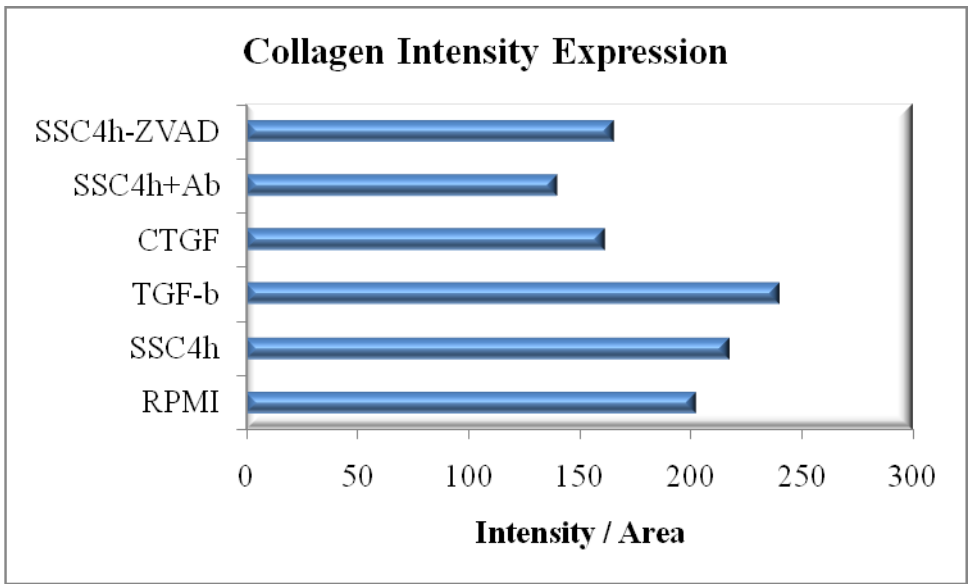
Figure 22: A) Protein level expression was done using immunofluorscence on non-stimulated (SABM) and stimulated SAEC for 48hrs with: RPMI, apoptotic medium SSC4h, 10 ng/ml TGF-β, 20 ng/ml CTGF, apoptotic medium SSC4h + neutralizing anti-CTGF, and non-apoptotic medium SSC4h-ZVAD, n=3. Primary antibodies specific for epithelial markers (E-cadherin and Cytokeratin) were used. B) Representation of fluorescence signaling by Intensity/ Area.

Figure 23: Immunofluorescence of Mesenchymal markers:

A)



B) Fluorescence intensity of mesenchymal markers:



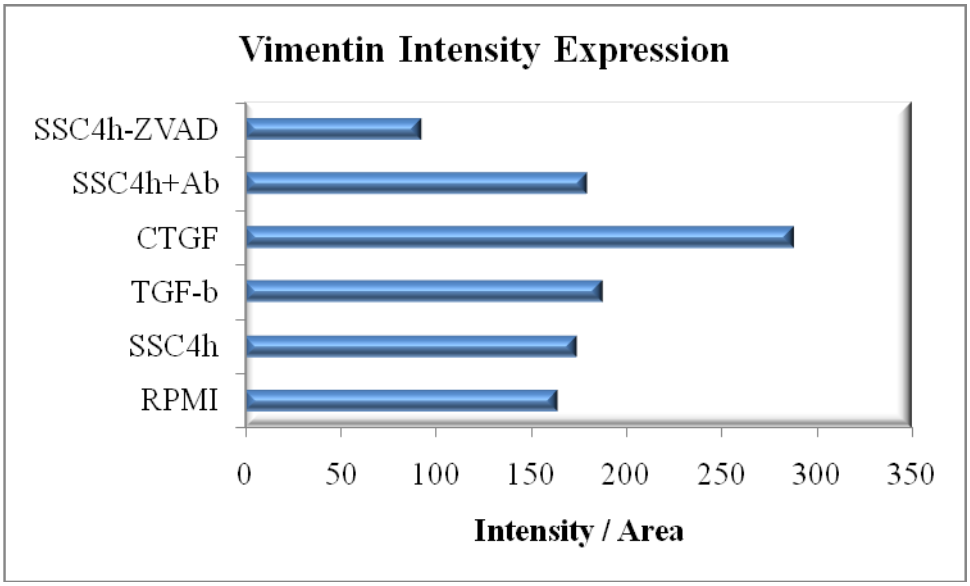
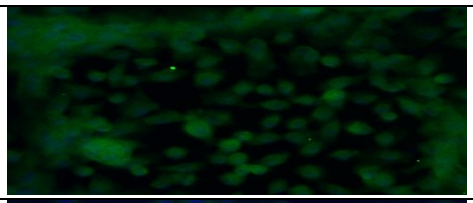
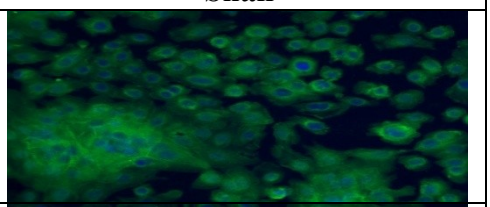
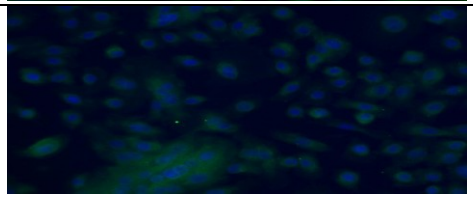
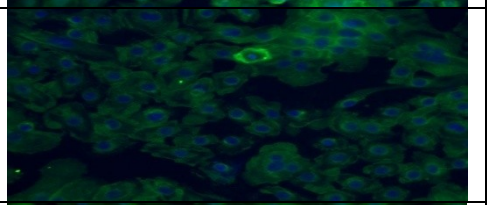
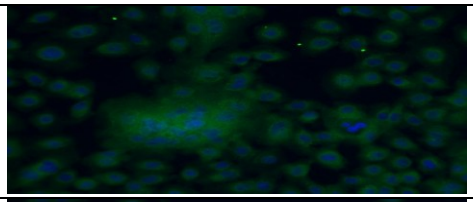
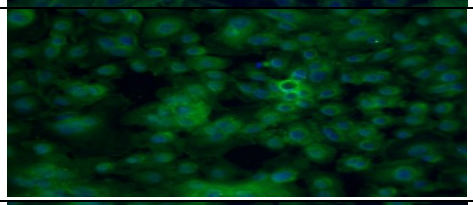
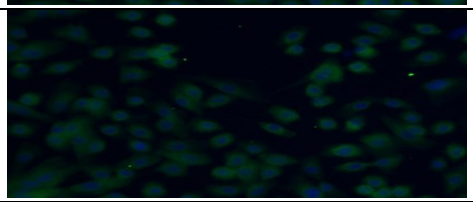
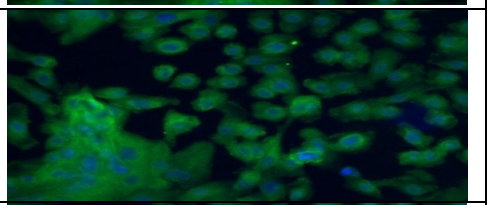
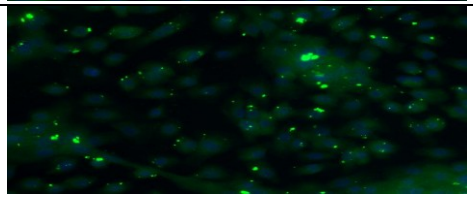
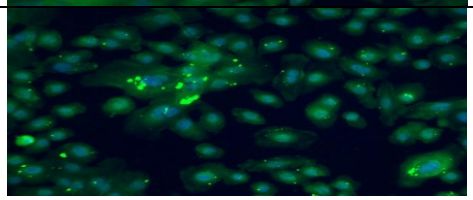
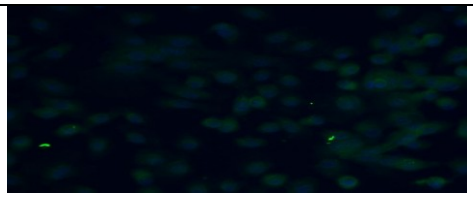
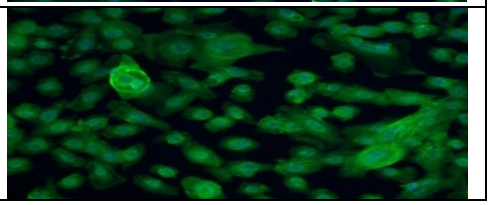
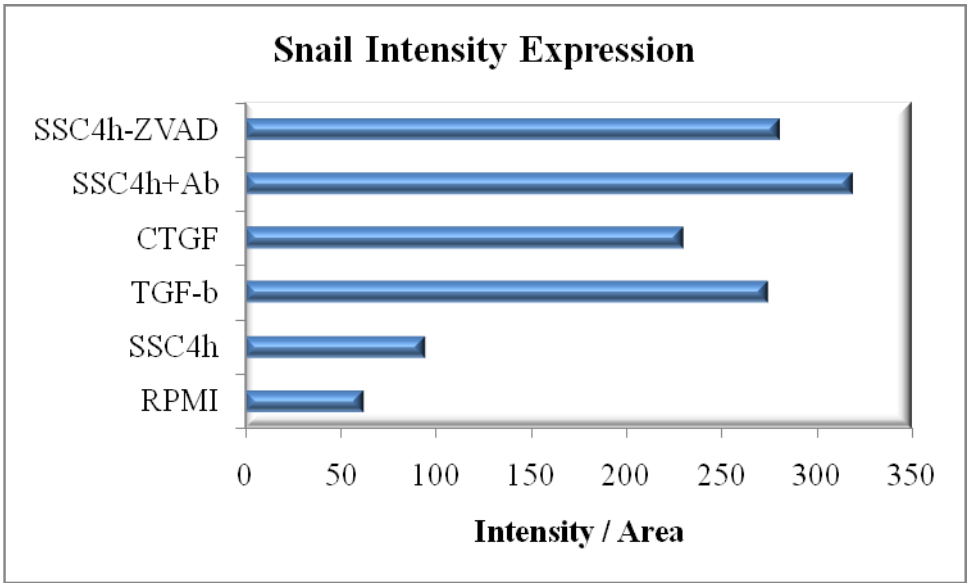


Figure 23: A) Protein level expression was done using immunofluorscence on non-stimulated (SABM) and stimulated SAEC for 48hrs with: RPMI, apoptotic medium SSC4h, 10 ng/ml TGF-β, 20 ng/ml CTGF, apoptotic medium SSC4h + neutralizing anti-CTGF, and non-apoptotic medium SSC4h-ZVAD, n=3. Primary antibodies specific for mesenchymal markers (Collagen, α-SMA, and Vimentin) were used. B) Representation of fluorescence signaling by Intensity/ Area.

Figure 24: Immunofluorscence of Transcription Factors:

	Twist	Snail
SSC4h		
RPMI		
TGF-β		
CTGF		
SSC4h +Ab-CTGF		
SSC4h-ZVAD		

B) Fluorescence intensity of transcription factors:



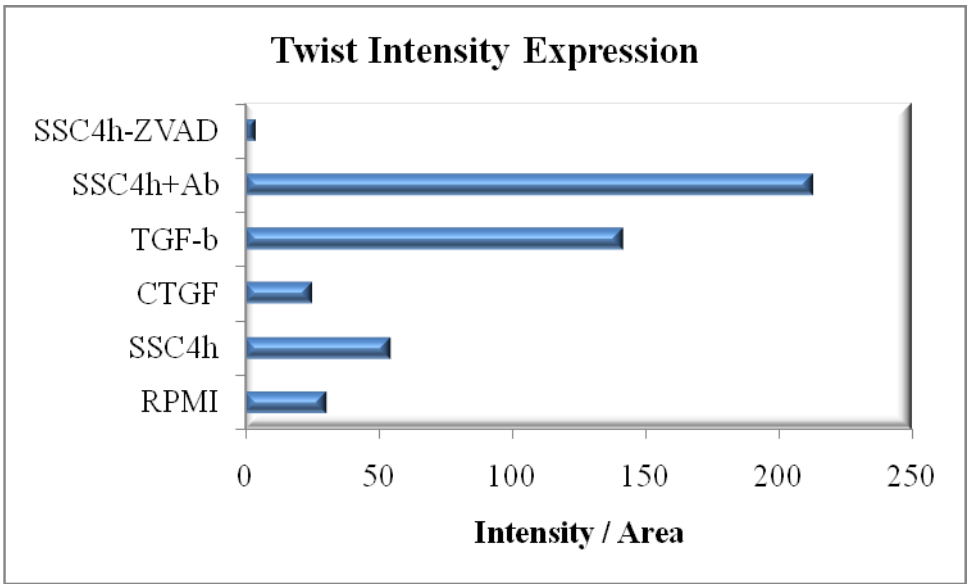


Figure 24: A)) Protein level expression was done using immunofluorscence on non-stimulated (SABM) and stimulated SAEC for 48hrs with: RPMI, apoptotic medium SSC4h, 10 ng/ml TGF-β, 20 ng/ml CTGF, apoptotic medium SSC4h + neutralizing anti-CTGF, and non-apoptotic medium SSC4h-ZVAD, n=3. Primary antibodies specific for transcription factors (Twist and Snail) were used. B) Representation of fluorescence signaling by Intensity/ Area.

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Chapter 4:

Discussion

In the present study, we propose that injured endothelial cells, due to ischemia-reperfusion or other stimulus, lead to proteolysis of the extracellular matrix (ECM) and production of cryptic fibrogenic factors in which they work on inducing and activating remodeling and fibrotic processes. One of these processes is epithelial-mesenchymal-transition (EMT). There are different types of EMT, where this study focuses on the second type (type II in figure6) which is usually involved in tissue generation and wound healing. The activation of this type of EMT has been implicated in the fibrogenesis of several disorders [209, 210, 212-215]. In addition, the altered EMT process works on producing and generating excessive mesenchymal cells in an uncontrolled mater that participates in airway remodeling. This has allowed proposing EMT as a possible key factor and contributor in the progressive irreversible airway remodeling in bronchiolitis obliterans. Studying the EMT process and its implication in the current study was done in two parts. The first part involves detection of EMT occurrence *in situ* in transbronchial biopsies (TBB) obtained retrospectively from lung transplant recipients (LTRs) following lung transplantation. The second part of the study involves induction and stimulation of EMT process *in vitro* in small airway epithelial cells (SAEC) through mediators produced by conditioned apoptotic medium (SSC4h) from endothelial cells of the lung.

4.1. Epithelial-Mesenchymal-Transition in situ

The second chapter of this study sheds light on the detection of epithelial-mesenchymal-transition in transbronchial biopsies (TBB) obtained from lung transplant recipients (LTRs). Identification of EMT is proposed to be an early indicator or an alarming factor in the development of bronchiolitis obliterans syndrome (BOS) following lung transplantation (LT) by causing airway remodeling. Obtained results of EMT expression were also correlated with gathered clinical data, showing interesting correlation with decline in lung function. Other studies have also worked on associating bench work done on TBB with clinical data. One study [345] suggested airway remodeling to be resulted from thickening of reticular basement membrane (Rbm) as a result of inflammation in LTRs. Proposing the thickening of Rbm as an explanation of the airway remodeling which is seen in LTRs.

4.1.1. Detection of Epithelial-Mesenchymal-Transition

Airway epithelial cells serve at the main targets of injury, and repeated assaults might trigger activation of certain process. According to previous studies, epithelial-mesenchymal-transition is considered as a defense option for epithelial cells exposed to chronic or repeated injury. The EMT process is characterized by loss of E-cadherin, an epithelial marker, and gain of mesenchymal proteins, such as S100A4 and Vimentin, which in turn leads eventually to generation of myofibroblastic markers, such alpha-smooth muscle actin (α-SMA) and the ED-A splice variant of

fibronectin (ED-A FN) [357]. Furthermore, studies have demonstrated that increase in the expression of Twist, a transcription factor (TF), has been involved in inducing the EMT process. Twist results in loss of the E-cadherin mediated cell-cell adhesion, activation of mesenchymal markers, and induction of cell motility, all of this emphasizes on the involvement of Twist in inducing the EMT process [330]. Based on these gathered data, we expected that induced EMT process in TBB reveals an increase in the expression of both Twist and Vimentin (a mesenchymal marker) and decrease in the expression of E-cadherin (epithelial marker).

The demographic data of the recruited patients did not show any significant differences in regard of the age range, median age, and original diseases. However significant differences were observed, for the range of FEV1% loss, median of FEV1% loss, range of FEF25-75 % loss, and median of FEF25-75% loss as it is directly related to the BOS diagnosis. The recruited patients were divided into two groups: a stable one with 9 patients, and a BOS group with 10 patients. Total number of included TBB was 37 biopsies, where 17 biopsies were in the stable group, and 20 biopsies were in the BOS ones. The obtained biopsies were then stained using immunohistochemistry procedure with antibodies specific for E-cadherin, Vimentin, and Twist to detect the expression and occurrence of EMT process in TBB.

Loss of E-cadherin expression is noted as a hallmark of induced EMT. In the current study we were able to detect decrease in the expression of E-cadherin in the airway epithelium, where It was noted that E-cadherin was significantly more expressed in the stable group when compared to the BOS. We were able to detect change in the expression of E-cadherin as early as 1 month after LT. These results

indicate that the BOS group demonstrates a loss or repression of the E-cadherin expression compared to the stable group. This might be an early sign of EMT induction and occurrence in obtained biopsies following LT. Our study might not be the first one in studying changes of EMT markers expression since there are other studies that have worked on detecting these changes for studying the development of asthma [358] and BOS (or BO) [343, 344]. However, we are the first to report these changes of EMT markers expression in the small airways or in TBB obtained from LTRs.

Gain of mesenchymal markers expression, such as Vimentin, is considered as the following step after loss of E-cadherin expression in the EMT process. The Vimentin marker was significantly more expressed in epithelium and its cytoplasm in the BOS group compared to the stable one. We were able to note this increase of Vimentin expression as early as one month post LT. These results indicating an induced expression of Vimentin in the BOS group supports and demonstrates gain in the expression of the mesenchymal marker Vimentin compared to the stable group. This suggests that high expression of Vimentin is a characterization of BOS and its development.

According to previous studies, Twist is a transcription factor that has been implicated in inducing the EMT process. Expression of Twist was also checked in the stained biopsies for Twist. . The transcription factor Twist was significantly more expressed in epithelial, cilia of the epithelial cells, cytoplasm, and nuclei in the BOS group compared to the stable one. We were able to note this increase of Twist expression as early as one month post LT. High expression of Twist in the epithelial

and specially its expression as a nuclear staining suggesting translocation of this protein into the nucleus to help in inducing the EMT process. The transcription factor Twist functions in inducing EMT by direct binding to the promoter of E-cadherin and causing repression of it [331]. It has been mentioned that Twist can induce EMT through a mechanism that does not necessarily involve direct binding to E-cadherin promoter and thus it can induce mesenchymal expression without the need of repressing E-cadherin expression [332]. Based on these data, high expression of Twist is a key factor in inducing EMT process in patients who develop BOS. Twist can induce EMT by repressing the expression of E-cadherin and inducing the expression of Vimentin as early as one month. This suggests a high expression of Twist might be an important indicator alarming for BOS development in LTRs.

In addition to detection of change in the expression of EMT markers, we have also checked for possible correlation and association between expressed EMT markers relative to each other. The expression of E-cadherin marker revealed negative correlations with both Vimentin and Twist expressions, however; the expressions of both Vimentin and Twist showed a positive correlation. This demonstrates that decrease in the expression of E-cadherin correlates with an increase in the expression of both Vimentin and Twist, whereas increase in the expression of Vimentin correlates with the expression of Twist. This supports suggestions of Twist having a critical role regarding expressed markers of EMT. Induced EMT process results in an excessive generation of mesenchymal cells; where these cells participate in airway remodeling, and lead to BOS development.

4.1.2. Correlations of Clinical Data with Epithelial-Mesenchymal-Transition

Since we suggest that EMT is involved with the airway remodeling which results in BO development, we proposed the possibility of EMT having a clinical impact on lung function of LTRs. The activated EMT not only stimulates the generation of mesenchymal cells, but also causes release of mediators the further stimulate production and proliferation of mesenchymal cell. Excessive production of mesenchymal cells might play an important role in narrowing the airways which influences the lung function. Some of the gathered information about the recruited patients is their lung function tests (FEV1 and FEF25-75) done on a regular basis post lung transplantation procedure, and their BOS grade if any. Expressions of EMT markers have been correlated with decline in lung function represented as FEV1% loss and FEF25-75% loss. The E-cadherin expression demonstrated negative correlations with percentage loss of FEV1 and FEF25-75, whereas both expressions of Vimentin and Twist were positively correlated with the percentage loss of FEV1 and FEF25-75. This suggests that airway remodeling in LTRs caused by induced EMT leads to decrease in lung function and thus leads to decrease in the survival of patients.

4.1.3. Collagen Deposition Association with Epithelial-Mesenchymal-Transition

The extracellular matrix (ECM) is composed of several complex macromolecules that include: collagen, elastin, fibronectin, tenascin, and proteoglycan. One of the major ECM proteins is collagen. Previous studies have

demonstrated positive correlation between collagen deposition and asthma, a disorder characterized by having airway remodeling [359, 360]. This has allowed us to propose a possible correlation between collagen deposition and with the expression of EMT markers, FEV1% loss, and FEF25-75% loss. We were able to detect appositive correlations between collagen deposition and the expressions of both Vimentin and Twist. Also positive correlations were detected with collagen deposition and percentage loss of bothe FEV1 and FEF25-75. However, collagen deposition demonstrated negative correlation with E-cadherin expression. The positive correlations between collagen deposition and expressions of Vimentin and Twist suggest possible implication of expressed markers in the increase of collagen deposition. These results support the idea of ECM proteolysis and release of collagen during generation of mesenchymal cells from induced EMT process. Furthermore, correlations between collagen deposition and loss of FEV1 and FEF25-75 suggest the possible impact of collagen deposition on lung function by causing narrowing and remodeling of the airways. Therefore, induced EMT plays a major role in the development of BOS.

4.2. Epithelial-Mesenchymal-Transition in vitro by Apoptotic Endothelial Cells

Previous studies have suggested that apoptotic endothelial cells (EC) can release mediators that play an important and direct role in fibrogenesis by regulating resistance to apoptosis and differentiation of fibroblasts [160, 161]. In the present study, we work on producing conditioned apoptotic (SSC4h), and non-apoptotic (SSC4h-ZVAD) media from Human Microvascular Endothelial cells-Lung

(HMVEC-L). These media were then analyzed to detect possible produced and released mediators in them. The media were then used *in vitro* to induce the EMT process in human small airway epithelial cells (SAEC).

4.2.1. Produced Mediators from Apoptotic Endothelial Cells

At the activation of apoptotic pathways whether it was intrinsic and/or extrinsic pathways, a group of intracellular cysteine enzymes named caspases are activated and work on destroying essential cellular proteins and eventually lead to cell death. During apoptosis there are two tiers of caspase activation: an initiator caspases (caspases 2, 8, 9, and 10) that are activated through apoptosis-signaling pathways and activate the effector caspases (caspases 3, 6, and 7) which, in an expanding cascade, carry out apoptosis [136, 148, 151].

A group of professional phagocytes, such as macrophages [156, 158], are recruited by endothelial cells (EC) apoptosis. This recruitment causes macrophages to produce an increased amount of transforming growth factor-beta1 (TGF-β1) [159]. Release of such mediator has been suggested to be involved and contribute in fibrogenesis through causing differentiation of myofibroblast and resistance to apoptosis in fibroblasts/myofibroblasts [162]. However, it has been proposed the possibility of other mediators to contribute in the fibrogenesis process. One of these proposed mediators is connective tissue growth factor (CTGF) which works in a TGF-β1-independent manner [361, 362].

In this part of the study, we have worked on conditioning an apoptotic (SSC) and non-apoptotic (SSC-ZVAD) media from HMVEC, where serum starvation stimulation with 4 hours demonstrated to have an optimal percentage of apoptosis vs. necrosis Analysis of the harvested media using ELISA assay specific for TGF-β did not detect any expression of TGF-β in both media. However, when ELISA assay specific for CTGF was used it revealed a high concentration of CTGF in SSC4h compared to SSC4h-ZVAD, 62.70 pg/ml vs.15.63 pg/ml, respectively. Furthermore, immunoblotting against perlecan done on proteins extracted from both media revealed an elevated signal of perlecan in SSC4h that was 9 fold higher than the one in SSC4h-ZVAD. From these results several points can be drawn. One of these points is to prove release of cryptic fibrogenic mediators through apoptosis of endothelial cells. However, endothelial cells which go through apoptosis do not produce TGF-β as mediator. Moreover, inhibition of the caspase cascades does in fact inhibit the release of certain mediators that are known to be involved in fibrogenesis, such as CTGF and perlecan. This also suggests the possibility that prevention of apoptosis either through clinical trigger control or by using inhibition of caspase cascade as therapeutic method in targeting fibrogenic mediators might help in preventing fibrosis caused by apoptotic mediators. The involvement of TGF-b, CTGF, and perlecan in the fibrogenesis of airways remodeling have been reported in several studies done in studying different pulmonary disorders. However there is no study that demonstrates the involvement of perlecan produced from apoptotic endothelial cells as a key factor in airway remodeling of BOS following lung transplantation.

4.2.2. Change in Epithelial-Mesenchymal-Transition Gene expression

In this part of the study, produced conditioned media were used to stimulate induction of EMT process in small airway epithelial cells (SAEC). In addition to the conditioned media (SSC4h), positive and negative control conditions were tested as well. Detections for mRNA expressions of the following EMT markers, E-cadherin, CollagenIA1, and α-SMA, were done on stimulated SAEC.

E-cadherin is an epithelial marker and an important structural protein that functions in cell adhesion to ensure cells within tissue are bound together. Since the EMT process involves repression of E-cadherin expression, and activation of mesenchymal expression, therefore; E-cadherin mRNA expression should be identified and verified in stimulated epithelial cells that are going through EMT. It was noted that mRNA expression of E-cadherin in SAEC stimulated with SSC4h was slightly higher but not statistically different from the baseline expression. A similar pattern was observed for all the other conditions with the exception of TGF-β which revealed an increase in the expression of E-cadherin mRNA (1.53 fold). This demonstrates that produced mediators in SSC4h did not repress the expression of mRNA E-cadherin. Also, it should be noted that these stimulations were done in a period of 24 hours which might not be enough to repress the mRNA expression of E-cadherin.

In addition to detection of mRNA expression of the epithelial marker E-cadherin, mRNA expression of mesenchymal markers such as CollagenIA1 and α-SMA were also measured in stimulated SAEC. An increase in the expression of collagenIA1 mRNA was observed in SAEC stimulated with SSC4h, as well as in the

positive controls TGF-β, and CTGF, when compared to the baseline, and to the negative control SSC4-ZVAD. SAEC that were stimulated with SSC4h+Ab-CTGF demonstrated an increase in the expression of mRNA CollagenIA1 similar to those stimulated with SSC4h. Similar pattern of α-SMA mRNA expression was observed in stimulated SAEC. An increase in mRNA expression of α-SMA in SAEC stimulated with SSC4h, CTGF, TGF-β was observed when compared to the baseline RPMI and to stimulation with the non-apoptotic medium (SSC4h-ZVAD). We noted that SAEC stimulated with SSC4h+Ab-CTGF demonstrated an increase in the mRNA expression of α-SMA. These obtained data indicate and support the idea of released mediators in having an important role in generating mesenchymal cells. However, each mediator seems to enhance different mesenchymal markers, and seems to enhance mesenchymal expression in an independent manner from each other. In addition, inhibition of CTGF in SSC4h by neutralizing antibody did not inhibit the induction of neither collagenIA1 nor α-SMA, suggesting the involvement of other produced mediators in the conditioned medium that can induce the EMT process even at absence of CTGF activity.

In this part of the study we have worked on inducing the EMT process in small airway epithelial cells (SAEC) through apoptotic endothelial cells. In addition to production of conditioned apoptotic endothelilal cells medium (SSC4h), a conditioned non-apoptotic medium was also produced through inhibting the activation of caspases cascade using ZVAD-FMK (a caspase cascade inhibitor). This part works on detecting possible mediators released by apoptotic endothelial cells and the impact these released mediators has in inducing the EMT process.

4.2.3. Change in Epithelial-Mesenchymal-Transition Proteins Expression

In this part we checked for the expression of EMT markers at the protein level using indirect imuunofluorescence microscopy and using antibodies against epithelial and mesenchymal markers and against transcription factors. Stimulation of SAEC was done using previously mentioned conditions for 48 hours to detect the level of protein expression. Change in the morphology of stimulated SAEC was taken into consideration and whether epithelial cells have preserved their morphology or had fibroblast-like ones. Fibroblast cells are characterized by having an elongated shape with branched cytoplasm surrounding an elliptical, speckled nucleus having two nucleoli. Fibroblasts are capable of producing collagens, glycosaminoglycans, reticular and elastic fibers, where glycoproteins are found in the ECM. Generation of fibroblasts is usually stimulated during tissue damage which induces mitosis of fibroblasts[363]. In contrast to epithelial cells, fibroblasts do not form a flat monolayer, and are not restricted by polarized attachement to a basal lamina. They are also capable of migration in contrast to epithelial cells. Fibroblasts are like other cells of connective tissue are derived from primitive mesenchyme. Therefore, fibroblasts express vimentin as an intermediate filament protein, which is recognized as a marker to distinguish mesodermal origin[363]. However, detection of vimentin a mesodermal marker might not necessarily be specific as epithelial cells cultured in vitro on adherent substratum may also express vimentin after a certain period of time [363]. Indeed, our some of our SAEC stimulated with SSC4h demonstrated changes

in their morphologic features where they more elongated shape resembling features more of fibroblasts than those of the epithelial cells.

Following stimulation, SAEC were analyzed for proteins expressions of E-cadherin, cytokertin, collagenI, α -SMA, Vimentin, Twist, and Snail. E-cadherin and cytokeratin are epithelial markers, whereas collagenI, α-SMA, and Vimentin are mesenchymal markers. Twist and Snail are transcription factors that induce the EMT process. We used software the helped in analyzing captured images of our immunofluorescence results in order to avoid individual interpretation bias where we have used the mean area of captured image and mean intensity of the captured area to obtain a mean intensity/area score for each marker. The score helps in determining the protein expression of each marker (total of seven markers), each stimulation (total of stimulating conditions) and allow for comparing how all markers get expressed depending on a type of stimulation, as well as how each marker expression varies depending on the condition of stimulation.

The score of intensity of epithelial proteins such cytokeratin and E-cadherin were detected in stimulated SAEC. For cytokeratin intensity SAEC stimulated with SSSC4h had the lowest score of intensity compared to the rest of stimulations, even when it was compared to the positive controls TGF-β and CTGF. For E-cadherin intensity score was low in SAEC stimulated with SSC4, TGF-b, CTGF, and SSC4h+Ab-CTGF when compared to baseline RPMI and SSC4h-ZVAD. The obtained results demonstrate the ability of produced mediators in repressing the protein expression of E-cadherin but not cytokeratin, however; stimulation with SSC4h was able to repress the expression of both proteins.

The mesenchymal protein expressions such as collagen I, α-SMA, and Vimentin were measured in stimulated SAEC. High protein expression of collagen I was seen in SAEC stimulated with SSC4h and TGF-β when compared to those stimulated with baseline RPMI, CTGF, SSC4h+Ab-CTGF, and SSC4-ZVAD. This demonstrates the ability of SSC4h's produced mediators and TGF-β in inducing the protein expression of collagenI, and CTGF failed to induce the protein expression of collagenI. High protein expression of α-SMA was noted in SAEC stimulated with SSC4h and CTGF compared to those stimulated with baseline RPMI, TGF-β, SSC4+Ab-CTGF,and SSC4h-ZVAD. This demonstrates the ability of SSC4h's produced mediators and CTGF in inducing the protein expression of collagenI, which TGF-βhas failed to induce. In addition, inhibition of CTGF through neutralizing its activity revealed decrease in the expression of α-SMA suggesting CTGF to be a key inducer of α-SMA protein expression. Furthermore, high protein expression of Vimentin was noted in SAEC stimulated with SSC4h, TGF-β, CTGF, and SSC4h+Ab-CTGF, when compared to baseline RPMI and SSC4h-ZVAD. This reveals the implication of mediators produced by SSC4h in addition to TGF-β and CTGF in inducing the protein expression of Vimentin. In addition, SAEC stimulated with SSC4h-ZVAD had a low expression of Vimentin confirming on the important role produced mediators have in inducing the expression of Vimentin.

To sum up, stimulation of SAEC with SSC4h demonstrated a high level of mesenchymal protein expression compared to baseline SAEC, or stimulated with SSC4h+Ab-CTGF, and SSC4h-ZVAD.. This indicates that mediators produced during apoptosis play an important role in inducing EMT process, and this can be

proven by stimulation done using the non-apoptotic medium (SSC4h-ZVAD) where release of these mediators is inhibited and thus does not induce EMT. Stimulation of SAEC with TGF-β and CTGF revealed similar results to those obtained in SSC4h stimulation, which emphasize on their role and involvement of inducing EMT and could be possible targets in inhibiting EMT process.

Protein expressions of transcription factors, such as Snail and Twist, have been detected in addition to detection of epithelial and mesenchymal markers. The reason behind detecting the expression of these transcription factors is because these transcription factors participate in inducing EMT process by repressing the expression of E-cadherin through direct or indirect binding to its promoter. Therefore, detection of transcription factors might serve as the missing piece in understanding the mechanism behind EMT induction. From our study, we have observed that stimulated SAEC with SSC4h demonstrated a high intensity of Snail and Twist expressions compared to SAEC that were incubated with either baseline RPMI, or SSC4h-ZVAD.. These results prove that expression of Twist is highly enhanced by mediators produced by the conditioned apoptotic medium, where inhibition of their production in the non-apoptotic did not express Twist as much as the apoptotic medium did. Stimulated SAEC with TGF-β and CTGF demonstrated enhanced expression of Snail and Twist compared to those that were stimulated with baseline RPMI. This emphasizes on the role of these mediators have in expressing these transcription factors.

Chapter 5:

Conclusion

In our study we were able to confirm the implication of epithelial-mesenchymal-transition as a contributing mechanism behind the development of bronchiolitis obliterans syndrome. We have demonstrated the occurrence of EMT *in situ*, which was done on transbronchial biopsies (TBB) obtained from lung transplant recipients (LTRs), and have stimulated its induction *in vitro* through stimulating small airway epithelial cells by apoptotic lung endothelial cells.

In the *in situ* part of our study, we were able to detect the change in expression of EMT markers, and transcription factors that are known to induce EMT. Hallmark of EMT initiation is a decrease in the expression of E-cadherin, an epithelial marker, was noted in LTRs group that developed BOS compared to the stable group of LTRs. However, the BOS group demonstrated a high expression of Vimentin which is a mesenchymal marker. Twist, a transcription factor that is known to induce EMT by repressing E-cadherin and expressing mesenchymal expression, was highly expressed in BOS group. All this suggests EMT as a source for mesenchymal cells generation which is induced through transcription factors, such as Twist, in which they initiate the machinery by repressing epithelial expression and activating mesenchymal ones.

In the *in vitro* part of our study, we were able to condition apoptotic (SSC4h) and non-apoptotic (SSC4h-ZVAD) media from endothelial cells and confirm release of fibrogenic mediators by the apoptotic medium. Released mediators from SSC4h included: CTGF and perlecan, where these mediators have been confirmed to be implicated in the fibrogenesis of several disorders. Stimulation of SAEC using SSC4h demonstrated at the mRNA level an increase in the expression of collagenIA1 and α-SMA (mesenchymal markers), and no change in the expression of the epithelial

marker E-cadherin. In addition, stimulated SAEC with SSC4h for 48 hours demonstrated a decrease in protein expression levels of the epithelial markers E-cadherin and cytokeratin; an increase in the expression of the mesenchymal markers collagenI, α-SMA, and Vimentin; and an increase in the expression of the transcription factors Snail and Twist. This proves that mediators released by apoptotic endothelial cells are key factors in inducing EMT and might be considered as a diagnostic tool or therapeutic targets in the future.

From our study, we can confirm that induced EMT process can be associated with causing airway remodeling and narrowing through continuous generation of mesenchylmal cells. Early detection of EMT in LTRs might be an indicator of BOS development and might help in the treatment procedure.

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