university of groningen / faculty of science and engineering

Mechanisms of action of mesenchymal stem cell therapy in lung emphysema

Bachelor's thesis

Mireille C. Schipper

University of Groningen

BSc Biology, major Biomedical Sciences
Supervisor: Prof. dr. H.I. Heijink
June 24, 2017

Abstract

Chronic obstructive pulmonary disease (COPD), including chronic bronchitis and emphysema, is a progressive respiratory condition and a leading cause of mortality and morbidity worldwide. Emphysema is characterized by loss of alveolar structure and destruction of the extracellular matrix, resulting in irreversible enlargement of alveolar spaces. Although various treatment options for COPD exist, none has been found to repair or reverse emphysematous destruction of the lung. Mesenchymal stem/stromal cells (MSCs) have however been identified as a novel possible strategy for the treatment of emphysema in COPD patients. Although MSC therapy in animal studies has showed promising results concerning reparation of alveolar epithelial damage, no beneficial effects of MSCs in human clinical trials have been observed yet. Therefore, this review provides an overview of our current knowledge of the molecular pathogenesis of emphysema and aims to investigate the characteristics and mechanisms of action of MSCs necessary in order to restore or regenerate alveolar epithelial damage in emphysema. In addition, this review attempts to explore the reasons behind the lack of significant results of MSC therapy in COPD patients, in contrast to promising animal studies.

Key words: Mesenchymal stem cells, emphysema, therapy, COPD

Table of contents

Introduction

Chronic obstructive pulmonary disease (COPD) is the most frequent chronic respiratory disease and a leading cause of mortality and morbidity worldwide. COPD is characterized by a progressive, poorly reversible loss of lung function, and an abnormal inflammatory response of the lungs to noxious gases and particles (Guan *et al.,* 2013). Tobacco smoking is the most common risk factor associated with COPD. However, an estimated 25-45% of patients with COPD have never smoked (Guan *et al.,* 2013). Exposure to indoor and outdoor air pollution may also play a role in the development of COPD in some individuals. Other possible risk factors include genetic predisposition and long-standing asthma (Wecht *et al.,* 2016).

The pathogenesis of COPD is a complex process and includes both chronic obstructive bronchitis and pulmonary emphysema. Pulmonary emphysema is a key pathological change in COPD and is characterized by destruction of terminal bronchioles and alveolar walls, resulting in an irreversible enlargement of alveolar spaces (Akram et al., 2012). Normally functioning lungs are elastic, efficiently expanding and recoiling as air passes freely through the bronchus reaching the alveoli, where oxygen is moved into the blood and carbon dioxide is filtered out. In emphysema, collapse of the alveoli contributes to reduced lung elastic recoil, which decreases the driving force of air from the lungs. Furthermore, alveolar break down in emphysema results in a disruption of the gas exchange taking place in the alveoli (Jones et al., 2016). In severe cases of emphysema, coughing and breathlessness (dyspnea) occur, which severely affect the quality and productivity of a patient's life (Taraseviciene-Stewart and Voelkel, 2008). While there are several treatment options for COPD, none of these treatments have been found to repair or reverse the damage done to the lungs in emphysema (Wecht and Rojas, 2016). Therefore, there is a pressing need to find innovative treatment options for patients with emphysema.

Stem cell therapy is one of the novel approaches believed to have the potential to reverse a lung disease or halt its further progression. A stem cell is defined as an undifferentiated cell with three primary functions: clonality, self-renewal and differentiation into different types of cells and tissues (Wecht and Rojas, 2016). Development of cell therapies for lung diseases has rapidly progressed over the past 10 years (Weiss, 2014). Specifically, mesenchymal stem cells (MSCs), a population of non-haematopoietic multipotent stromal cells, have attracted the attention of scientists and clinicians for their potential use in treatment of lung diseases, including emphysema, because of their ability to migrate to the site of injury and initiate tissue repair. Furthermore, MSCs have been shown to exhibit anti-inflammatory and protective abilities, which could assist in the reparation or regeneration of destroyed lung tissue caused by emphysema. Huh *et al.* were the first to demonstrate the reparative effects of MSCs in a smoke-induced rat model of emphysema. Female rats treated with MSCs after cigarette smoke exposure exhibited restored alveolar architecture 2 months after MSC treatment (Huh *et al.,* 2011). In addition, Zhen and colleagues demonstrated that systemic administration of bone-marrow derived MSCs improved emphysematous changes in irradiation and papain-induced experimental mouse models (Zhen *et al.,* 2008).
Donation of MSCs to various animal models with emphysema has thus shown very promising results, indicating the first steps of alveolar regeneration. In addition, a placebo-controlled,

randomized trial of MSCs in COPD revealed that MSC administration appears to be safe in patients with moderate to severe COPD (Weiss *et al.,* 2013). On the other hand, no beneficial effects on the emphysematous characteristics in the lungs of the COPD patients after MSC administration were observed in this clinical trial (Weiss *et al.,* 2013). Therefore, there are still many unanswered questions concerning the characteristics of MSCs and the mechanisms of actions of these cells necessary in lung repair and regeneration in emphysema. This review discusses the molecular pathogenesis of emphysema and aims to investigate which mechanisms of action of MSC therapy are relevant in order to repair or regenerate the alveolar epithelial damage caused by emphysema. In addition, this review attempts to explore the reasons behind the lack of significant results of MSC therapy in COPD patients, in contrast to promising animal studies.

1. Molecular pathogenesis of emphysema

Pulmonary emphysema is defined as airspace enlargement as a result of alveolar breakdown in the adult lung (Figure 1). Human emphysema is originally described by Ruysch in Amsterdam at the end of the 17th century, and in the 19th century by a French physician named Laennec. Laennec noted "marked variations in the size of air vesicles, which might be smaller than a millet seed or as a large as a cherry stone or haricot. Vesicles of the latter size were produced by the coalescence of adjacent air spaces following rupture of the alveolar walls" (Snider *et al.*, 1985; Laennec, 1819; Laennec, 1834). Since these original descriptions, the pathogenesis of emphysema is an arena of ongoing, active research, and new developments continue to arise.

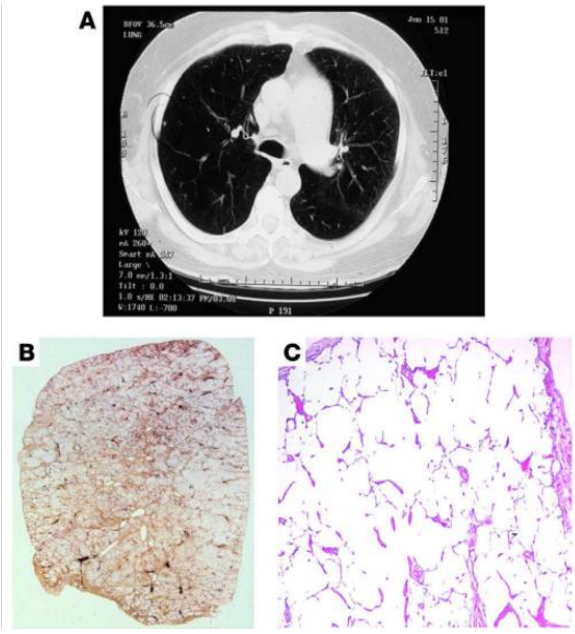

Figure 1. Human Emphysema
A) Chest CT scan of a 56 year-old man with COPD demonstrating loss of the lung parenchyma and paucity of lung vessels. **B)** Whole lung section demonstrating "holes", i.e. emphysema. **C)** Histology of end-stage emphysematous lung. Hematoxylin and eosin staining; magnification x40. Adapted from: Taraseviciene-Steawart and Voelkel., 2008.

Emphysema is a complex disease, as various pathological processes occur simultaneously. These processes work individually or in concert and are often interrelated, but all eventually result in the loss of alveolar septal cells and airspace enlargement (Taraseviciene-Stewart and Voelkel., 2008). In this chapter, an overview of our current knowledge of the molecular pathogenesis of emphysema, according to recent literature, is given.

1.1 Chronic inflammation and development of emphysema

Chronic inflammation can be considered as one of the key aspects in the development of emphysema. Inflammation is defined as the presence of inflammatory cells and altered levels of mediators of inflammation in the parenchyma. Cigarette smoke exposure, the foremost risk factor for COPD development,

has been shown to directly activate macrophages, a type of phagocytic white blood cell. Macrophage activation results in the release of inflammatory substances that mediate alveolar wall destruction and contribute to the establishment of emphysema. For instance, one of the effects of macrophage activation is the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). In addition, the release of chemokines such as interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), and leukotrine B4 attracts additional immune and inflammatory cells to the lungs, including T-cell and neutrophils. T-cells are a type of lymphocyte that play a central role in cell-mediated immunity. Neutrophils are the most abundant type of granulocytes. Cigarette-smoke activated neutrophils have been shown to advance alveolar destruction via the release of oxidants and proteases. Activated T-cells, mainly CD8+ T-cells, are able to release cytotoxic perforins such as granzyme B and TNF-α, directly leading to cell death and apoptosis of alveolar epithelial cells.

Besides activating macrophages, cigarette smoke can activate epithelial cells in the lung to secrete a variety of inflammatory mediators, thereby supporting the inflammatory processes that contribute to the development of emphysema (Jin *et al.,* 2014). Inflammation in emphysema is thus a synergy of multiple immune cells activated by cigarette-smoke, which are able to release various pro-inflammatory substances that mediate alveolar wall destruction and contribute to development of emphysema.

1.2 Protease/antiprotease imbalance in emphysema

In the lung, a delicate balance between protease and antiprotease activity is required for appropriate lung maintenance. Proteases can enzymatically degrade lung extracellular matrix (ECM) proteins, and antiproteases protect against their destruction (Jin *et al.,* 2014; Taraseviciene-Stewart and Voelkel, 2008). The ECM is a highly dynamic three-dimensional network of non-cellular components present within all tissues and organs. The ECM consists of various ECM proteins, such as collagens, elastin, fibronectin, laminin and proteoglycans (PGs). The ECM provides structural support and affects cell shape and function. Especially in the alveolar compartment, the ECM forms a strong yet expansile framework that supports the alveolar epithelial-capillary interface (Straaten van *et al.,* 1999). A derangement of the protease/antiprotease balance can result in increased alveolar destruction caused by break down of the ECM proteins in the alveoli, inappropriate repair of the lung, and eventually the development of emphysema (Taraseviciene-Stewart and Voelkel, 2008; Jin *et al.,* 2014).

Cigarette smoke and the associated inflammatory processes have been shown to both increase protease production and protease release from inflammatory cells and structural cells. Macrophages and neutrophils are the two main sources of proteases in the lungs, and many studies have shown correlations between the degree of neutrophil and macrophage inflammation and the severity of airflow obstruction (Sharafkhaneh *et al.,* 2008; Saetta *et al.,* 2001).

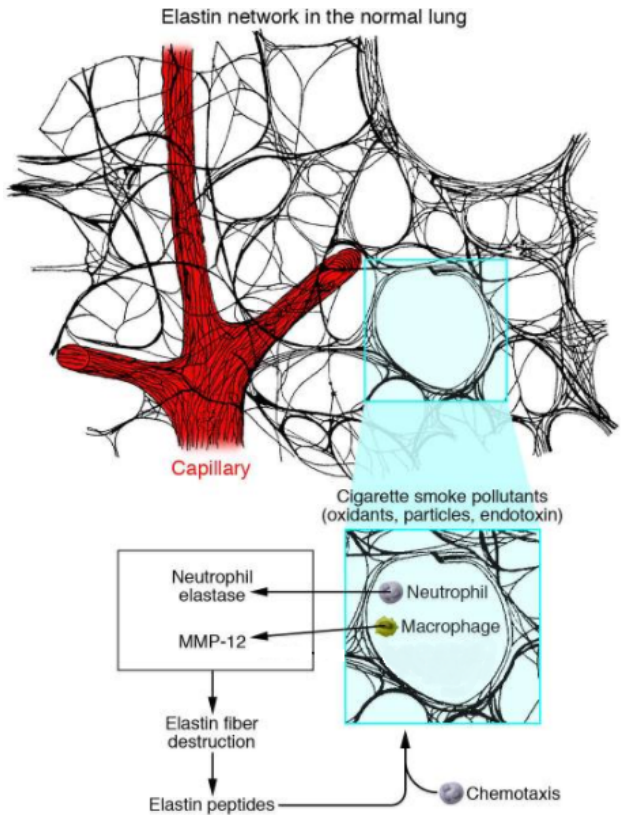

Figure 2. Proteolytic destruction of the elastin fiber network in the lung by cigarette smoke activated immune cells
This schematic illustrates the elastin fiber network in the lung. Neutrophil elastase and matrix metalloproteinase-12 (MMP-12), released by activated neutrophils and macrophages, can degradate the elastin network in the lung. Subsequently, the resulting elastin peptides are chemotactic and can attract additional inflammatory cells to the lung, generating a vicious cycle.
Adapted from Taraseviciene-Stewart and Voelkel, 2008.

A group of proteases known to play a vital role in emphysema are the matrix metalloproteinases (MMP's), a group of calcium-dependent zinc-containing endopeptidases, capable of degrading all kinds of ECM proteins. Especially MMP-12 (an elastase), MMP-8 (a collegenase) and MMP-9 (a gelatinase) have been shown to influence the development of emphysema (Inamdar and Inamdar, 2013). For example, MMP-12 knockout mice showed resistance to development of emphysema after exposure to cigarette smoke (Hautemaki *et al*., 1997). In addition to the MMP's, the serine protease neutrophil-elastase and lysosomal proteases cathepsins S,L and G, are other proteases released by inflammatory cells upon exposure to cigarette smoke, that may play an important role in the development of emphysema (Sharafkhaneh *et al.,* 2008).

Neutrophil-elastase and MMP-12 are both types of elastases, and are able to enzymatically destroy the elastin scaffold of the alveolar spaces. Elastin is a highly elastic protein in the connective tissue of the lung and is essential for the elasticity and extensibility of lung tissue (Taraseviciene-Stewart and Voelkel, 2008). Multiple studies have shown a decrease in elastin in the alveoli and small airways in COPD patients of varying severity (Eurlings *et al.,* 2014; Merrilees *et al.,* 2008). Break down of the elastin network contributes to the alveolar breakdown in emphysema. Moreover, the resulting elastin fragments are in turn chemotactic and attract even more inflammatory cells to sites of injury, reinforcing the joint role of inflammatory cells and proteases (Figure 2) (Sharafkhaneh *et al.,* 2008; Taraseviciene-Stewart and Voelkel, 2008).

Besides up-regulation of proteases, cigarette smoke reduces the activity of antiproteases, such as α1-antitrypsin (AAT) (Jin *et al.,* 2014). Normally, AAT inhibits neutrophil elastase and therefore protects against the destruction of the elastin network in the lung. For example, AAT protects against experimental emphysema in rodents. A decrease in AAT as a result of exposure to cigarette smoke further stimulates the degradation of the ECM scaffold in the alveoli. Another group of antiproteases are the tissue inhibitors of metalloproteinases (TIMPS). TIMPS are the endogenous inhibitors of MMPs. Alveolar macrophages from COPD patients release less TIMP's in vitro compared to macrophages from smokers without COPD

and non-smokers. Thus, cigarette smoke induces release of MMPs from macrophages, without inducing an increase in TIMPs, which inhibit MMPs, leading to a strengthening of the protease/ antiprotease imbalance and destruction of ECM proteins (Pons *et al.,* 2005).
For example, besides a decrease in elastin, van Straaten *et al.* found a diminished staining of the interstitial PGs, decorin and biglycan, in the peribronchiolar area, in lung tissue from patients with severe emphysema, compared with lung tissue from control patients (Straaten van *et al.* 1999). Interstitial PGs are able to interact with fibrillar collagens and fibronectin, and are known to stabilize the fibrillar collagen matrix in vivo (Danielson *et al.,* 1997). The observed alterations in interstitial PGs might therefore affect the structural collagen network of the lung tissue and thereby the respiratory function of COPD patients (Straaten van *et al.,* 1999).

Examples of the involved proteases and antiproteases illustrate how complex proteolytic lung destruction may be, particularly given the large number of involved enzymes as well as their targets – many structural proteins. Overall cigarette smoke results in a disruption in the delicate balance of the protease/antiprotease activity, which contributes to alveolar wall destruction and airspace enlargement via degradation of ECM proteins.

1.3 Oxidative stress in emphysema

Several types of reactive species are generated in the body as a result of metabolic reactions in the mitochondria, in the form of free radicals, or non-free radicals. These species may be either oxygen or nitrogen derived, and are called (pro)-oxidants (Irshad and Chaudhuri, 2002). These oxidants attack macromolecules including proteins and DNA, causing cellular and tissue damage. Fortunately, to counter the effect of these destructive oxidants, the body has another category of compounds called antioxidants (Irshad and Chaudhuri, 2002).

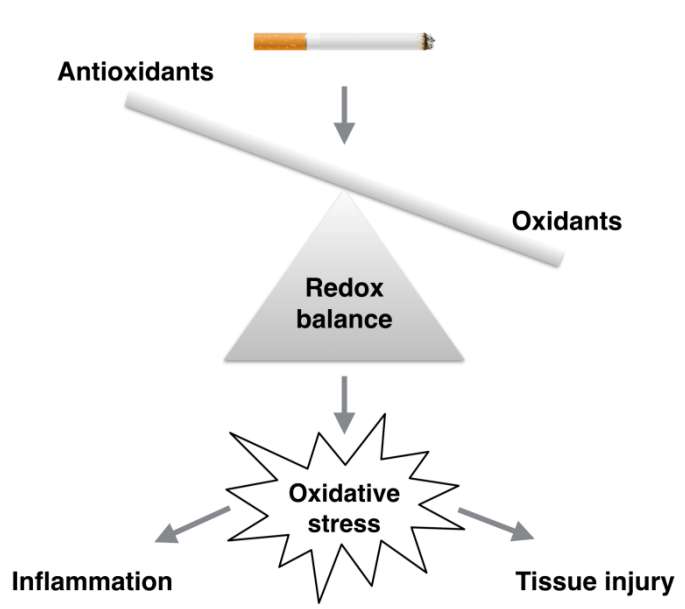

Figure 4. Oxidative stress in emphysema Exposure to cigarette smoke can disrupt the delicate balance between antioxidants and oxidants. As a result oxidative stress leads to inflammation and tissue injury.

Under normal conditions, a pulmonary intra- and extracellular antioxidant defense system protects our lung cells from oxidant damage, by maintaining a balance between oxidants and antioxidants. However, a shift in this delicate balance towards oxidants, resulting from either a depletion of antioxidants or an increase in the level of oxidants, is referred to as oxidative stress (Jin *et al.,* 2015).

Exposure to cigarette smoke has been associated with a disruption of the balance between oxidants and antioxidants. The oxidative stress resulting from the disruption of the balance between oxidants and

antioxidants has been suggested as an important pathogenic mechanism in patients with emphysema (Jin *et al.,* 2014).

In smokers, an increased oxidant burden derives from the fact that cigarette smoke contains various reactive oxygen species (ROS), such as superoxide ($O2^-$) and hydroxyl radicals (•OH), which are present in high concentrations in the gaseous phase of cigarette smoke. These gaseous-phase ROS cause local damage in the lung (Toorn van der *et al.,* 2009). Furthermore, lipid-soluble components in cigarette smoke induce mitochondrial production of ROS in epithelial cells in the lung (Toorn van der *et al.,* 2009). Other factors, such as infections and air pollutants that may exacerbate COPD, also have the potential to increase levels of oxidative stress in the lungs (Rahman, 2005). Moreover, the oxidant burden in the lungs of patients with emphysema is even further enhanced by the release of ROS from macrophages and neutrophils. Macrophages and neutrophils are known to migrate in increased numbers into the lungs upon exposure to cigarette smoke and can generate ROS via the NAPD-oxidase system (Rahman *et al.,* 1996). To crown it all, smoking and exacerbations of COPD result in decreased antioxidant capacity in plasma and in the bronchoalveolar lavage fluid (Rahman *et al.,* 1996).

The contribution of oxidative stress to emphysema is thought to encompass a variety of mechanisms. For example, oxidative stress has been suggested to contribute to lung inflammation via induction of redox-sensitive inflammatory transcription factors such as nuclear factor-κB (NF-κB) (Jin *et al.,* 2014). In addition, oxidative stress has been associated with a strengthening of the protease/antiprotease imbalance and increased alveolar apoptosis by blockage of the VEGF receptor, leading to elevated tissue injury (Figure 4) (Carp and Janoff, 1978; Kasahara *et al*., 2000). For example, oxidants in cigarette smoke can inactivate the antioxidant AAT by oxidation of the methionine residue at its active site (Rahman, 2005). In addition, it has been suggested that the oxidative component of cigarette smoke may stimulate alveolar macrophages to release increased amounts of MMP-9, which is involved in remodeling of the ECM in the alveolar spaces (Rahman, 2005).

1.4 Maintenance of alveolar structure and apoptosis in emphysema

Apoptosis is a tightly regulated form of cell death. Apoptosis is critical for the maintenance of normal tissue homeostasis, and which under normal conditions, is in equilibrium with cell proliferation (Jin *et al*., 2014). In the pathogenesis of emphysema, apoptosis of alveolar epithelial cells is known to play an essential role. In patients with emphysema, increased apoptosis of alveolar epithelial cells is not balanced by an increase in proliferation resulting in loss of alveolar cells (Hodge *et al.,* 2005).

One factor involved in survival of alveolar epithelial cells is vascular endothelial growth factor (VEGF), which is normally abundantly expressed in the adult lung. The receptor for VEGF, mainly type 2 (VEGFR2), is expressed on both epithelial and endothelial cells (Taraseviciene-Stewart and Voelkel, 2008). VEGF is critical for endothelial cell proliferation, lung development and plays a central role in several lung disorders including emphysema. Specifically, it has been suggested that alveolar septal endothelial cells may vitally depend on paracrine and autocrine VEGF survival signals, and are therefore vulnerable to VEGFR2 blockade or VEGF withdrawal (Stevens *et al.,* 2005). Effective transcription of the VEGF

gene is controlled by hypoxia-inducible factor 1α (HIF-1α) in endothelial cells. Synthesis of prostacylin (PGI_2) and nitric oxide (NO) is one of the outcomes of VEGFR2 activation, resulting in increased epithelial cell survival (Figure 3) (Taraseviciene-Stewart and Voelkel, 2008).

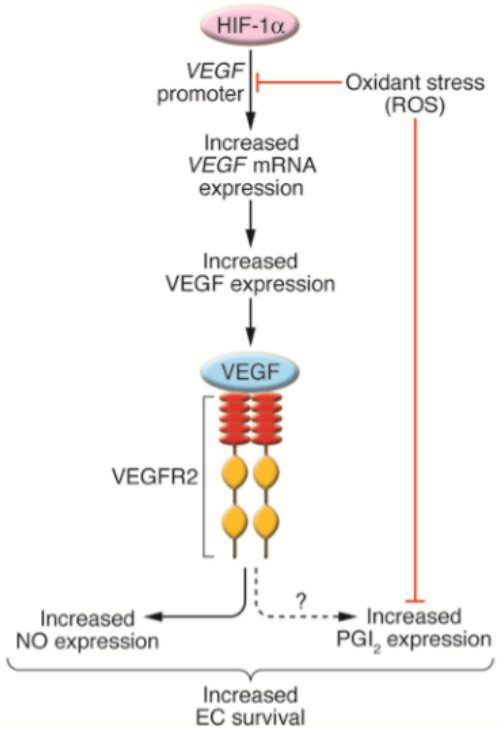

Figure 3. Schematic illustration of the VEGF pathway
The vascular endothelial growth factor (VEGF) pathway is likely to be involved in lung structure maintenance. VEGF gene expression is controlled by hypoxia-inducible factor 1α (HIF-1α). Synthesis of Nitric Oxide (NO) and prostacyclin (PGI_2) is one of the outcomes of VEGF receptor type 2 (VEGFR2) activation, resulting in increased survival of epithelial cells (ECs). Reactive oxygen species (ROS) can damage the promoter region of the VEGF gene and subsequently impair VEGF transcription. Knockout of the VEGF gene or blockade of the VEGFR has been shown to be associated with cell apoptosis and development of emphysema. The link between VEGFR2 activation and $PG1_2$ synthesis still needs to be established.
Adapted from Taraseviciene-Stewart and Voelkel., 2008.

Decreases in VEGF and VEGFR2 at both the mRNA and protein levels have been described in lungs of emphysematous patients and smokers (Kanazawa and Yoshikawa, 2005). In addition, Kasahara and colleagues showed that knockout of the VEGF gene or VEGFR2 blockade caused apoptosis of alveolar cells and subsequently the development of emphysema in rats (Kasahara *et al.,* 2000; Tang *et al.,* 2004). The reduced levels of VEGF and VEGFR2 may lead to a decrease in NO and PGI_2 expression and subsequently diminished survival signals to the alveolar epithelial cells resulting in apoptosis (Taraseviciene-Stewart and Voelkel., 2008). VEGF and VEGFR2 could therefore be in part responsible for the decrease in epithelial cell survival and the inability to maintain alveolar structure in emphysema.

Besides the VEGF-dependent homeostasis of alveolar cells, another mechanism possibly involved in apoptosis of alveolar epithelial cells is an alteration in the expression of apoptotic or anti-apoptotic genes (Jin *et al.,* 2015). As mentioned, oxidative stress caused by cigarette smoke and the inflammatory burden has well been established in emphysematous lungs. Oxidative stress can damage DNA and can lead to the activation of transcription factors such as p53, which is an important transcription factor influencing cell survival. P53 is strongly linked to the DNA damage response through the up-regulation of proapoptotic genes including Bax. More specifically, in response to a stress signal, cytoplasmic p53 rapidly translocates to mitochondria. In mitochondria p53 interacts with multi-domain members of the anti-proapoptotic Bcl-2 family members to either inhibit or activate them. The Bcl-2 family consist of anti-apoptotic members such as Bcl-2 and pro-apoptotic members including Bax. Upon activation Bax insert into the outer mitochondrial membrane and forms dynamic lipid pores that release lethal proteins from the mitochondrial intermembranous space into the cell cytoplasm resulting in apoptosis. P53 participates thus directly in the intrinsic apoptosis pathway by interacting with members of the Bcl-2 family (Vaseva and Moll, 2008). In a study

performed by Imai and colleagues, higher expressions of the pro-apoptotic proteins Bax and Bad were detected in emphysema patients, while this was not the case in healthy controls (Imai *et al.,* 2005).

2. MSC's and regeneration and reparation of destroyed lung tissue caused by emphysema

2.1 General properties of MSCs

MSCs are non-haematopoietic multipotent stromal cells. MSCs, under the influence of appropriate growth factors, can differentiate into multiple cell lines, in particular osteoblasts, chondrocytes, adipocytes and smooth muscle cells. Interestingly, a number of recent reports suggested an additional differentiation capacity of MSCs into a wide range of non-mesodermal and mesodermal adult phenotypes, including cardiomyocytes, hepatocytes, neurons, lung and epithelial cells (Akram *et al*., 2012).

MSCs can be isolated from various sources, but bone marrow derived MSCs (BM-MSCS) are still the most frequently used MSCs in experimental research. Adipose tissue, peripheral blood, the lung, and the myocardium are all documented as potential sources of MSCs, while the placenta, umbilical cord and cord blood have been studied as potential birth-associated sources of MSCs. However, differences within the phenotypes, quality and quantity of MSCs collected at the various sites exist. Although no specific marker for MSCs has yet been identified, there is an abundance of non-specific surface markers for MSCs described (Akram *et al.,* 2012). The International Society for Cellular Therapy has provided guidance on MSC markers: MSCs must express CD73, CD90, CD105 and lack the expression of CD45, CD34, CD14, CD11b, CD19 or MCH class II antigens (Dominici *et al.,* 2006).

MSC based therapy has generated a great interest in clinicians for their anti-inflammatory, immunomodulatory and regenerative capacities. MSCs can secrete multiple anti-inflammatory cytokines. These cytokines modify the microenvironment within damaged tissues. Furthermore, MSCs exert immunomodulatory effects by means of direct cell to cell contact (Jin *et al.,* 2014; Inamdar and Inamdar, 2013). In addition MSCs produce hematopoietic and non-hematopoietic growth factors. A few animals and in vitro studies have shown that MSCs could differentiate into alveolar epithelial cells. Finally, homing to injured tissue, up-regulation of micro-RNA's (miRNA) and protection from proteolytic ECM destruction, are other unique properties of MSCs making them an ideal candidate for the treatment of challenging lung conditions like emphysema (Jin *et al.,* 2014; Inamdar and Inamdar, 2013).

Multiple animal studies have already demonstrated that application of MSCs stimulates wound repair and regeneration with efficient amelioration of a number of clinical conditions, including emphysema (Huh *et al.,* 2011; Zhen *et al.,* 2008). Additionally, MSCs, in general, have established a very good safety profile as validated though clinical studies (Inamdar and Inamdar, 2013; Weiss *et al.,* 2013). However, no beneficial effects on emphysematous characteristics in the lungs of COPD patients after MSC administration were observed in

human clinical trials (Weiss *et al.,* 2013). In this chapter, the precise mechanisms of action of MSC therapy that could contribute to reparation or regeneration of the alveolar epithelial damage caused by emphysema are explored. Besides elucidating the mechanisms of action of MSCs, this chapter discusses possible reasons behind the lack of significant results of MSC therapy in COPD patients, in contrast to promising animal studies.

2.2 Homing and migration of transplanted MSCs

In order to restore alveolar epithelial damage in the lung, MSCs, after administration, must first migrate to the source of injury to subsequently initiate tissue repair. The process by which MSCs migrate to, and engraft in the tissue in which they exert local and functional effects is called homing. Homing involves a cascade of events. After migration, adhesive reactions are initiated between the vascular endothelium at the target tissue and flowing cells. Homing receptors expressed on circulating cells mediate this process, resulting in cell-tethering and rolling contacts on the endothelial surface. This is followed by activation of integrin adhesiveness, triggered by chemokine activation, firm adhesion and subsequently extravasation (Yagi *et al.,* 2010; Sackstein, 2005) (Figure 5). The homing ability of MSCS has already been demonstrated in settings of wound healing and tissue regeneration in various animal models (Yagi *et al.,* 2010).

Integrins are known to play a key role in cell migration, adhesion and chemotaxis. Furthermore, integrins are essential for cell survival and tissue persistence. Specifically, integrin α4/β1, a cell surface heterodimer, and integrin β1, mediate cell-cell and cell-extracellular matrix interactions through adhesion to vascular cell adhesion molecule (VCAM)-1 and to a specific region of the ECM protein fibronectin, called the V-region (Yagi *et al.,* 2010). De Ugarte and colleagues demonstrated that MSCs derived from bone marrow expressed many integrins on their cell surface, including high levels of integrin α4/β1 and β1 (De Ugarte *et al.,* 2003). Furthermore, it has been shown that MSCs interact in a coordinated fashion with endothelial cells by integrin α4/β1-VCAM-1 interaction (Ruster *et al.,* 2006). Fibronectin plays a major role in cell migration, adhesion, growth, and differentiation. Fibronectin can expose its V-region, containing the site for integrin α4/β1 binding which is expressed on MSCs. These fragments of fibronectin enhance integrin α4/β1 mediated cell binding, allowing them to adhere to the surrounding matrix, suggesting that integrin α4/β1-fibronectin interactions plays an important role in transmigration of MSCs into the extracellular matrix of the lung (Figure 5) (Yagi *et al.,* 2010). As mentioned in chapter 1, the protease/antiprotease imbalance and oxidative stress in emphysema, have been shown to be involved in remodeling of the ECM in the lung. Several studies have shown breakdown of the elastin network and decreased interstitial PGs, ECM proteins known to interact and stabilize collagens and fibronectin, in patients with emphysema (Eurlings *et al.,* 2014; Straaten van *et al.,* 1999). Therefore, it could be hypothesized that in patients with emphysema, altered ECM assembly, after administration of MSCs, could lead to reduced adhesion of MSCs to the ECM, less integrin activation, and subsequently less survival of MSCs. This effect could in turn contribute to less significant results of administered MSCs in patient trials, since migration and adhesion of MSCs to the injured lung are crucial factors in alveolar epithelial regeneration in emphysema.

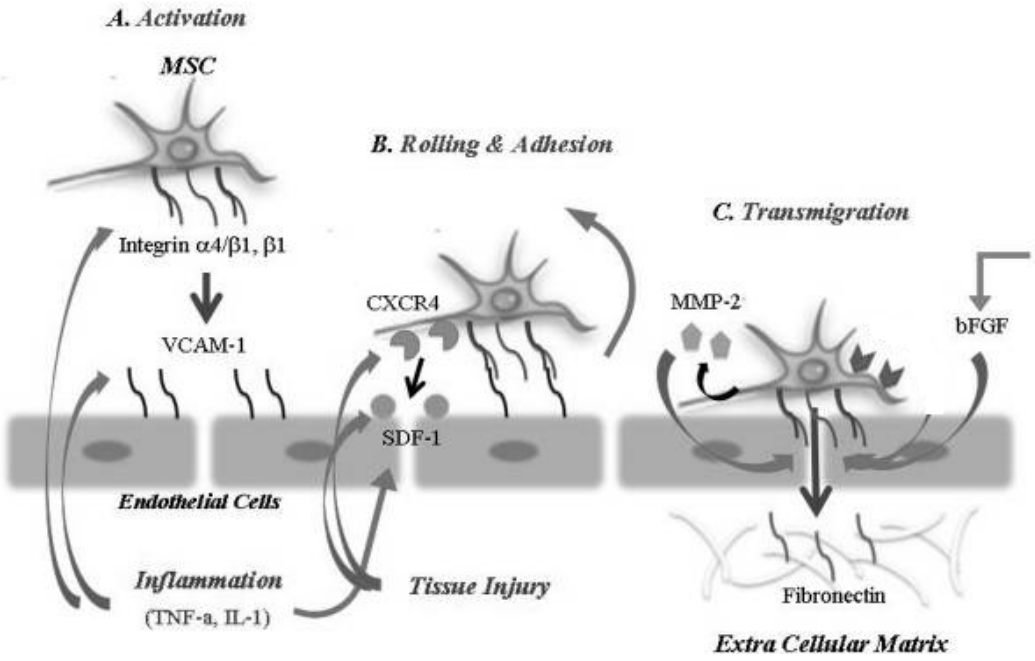

Figure 5. Mechanisms of migration and homing of MSCs
The process of migration and homing of MSCs can be divided in three steps. **A)** Mesenchymal stem cells (MSCs) have been shown to express integrin β1 and/or the integrin α4/β1 complex on their cell surface, stimulated by cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1). These integrins can interact with vascular cell adhesion molecule-1 (VCAM-1), which is expressed on endothelial cells primed by local inflammation. **B)** Besides integrins, MSCs can express chemokine receptor type 4 (CXCR4) which modulates cell-cell contact and rolling with endothelial cells that express and up-regulate stromal-cell-derived factor-1 (SDF-1). **C)** Finally, MSCs can transmigrate into the extracellular matrix by interactions with integrins and fibronectin, which is among others modulated by basic fibroblast growth factor (bFGF) and matrix metalloproteinase-2 (MMP-2). Adapted from Yagi *et al.,* 2010.

Furthermore, Sordi *et al.* reported a chemotactic responsiveness of MSCs to specific chemokines. One important chemokine is stromal-cell-derived factor 1 (SDF-1), which is officially designated as chemokine (C-X-C motif) ligand 2 (CXCL12), a small chemotactic cytokine that activates leukocytes and is induced by proinflammatory stimuli such as TNF-α or interleukin-1 (IL-1). The receptor for this chemokine is called C-X-C chemokine receptor type 4 (CXCR4). MSCs migrated appreciably in response to SDF-1, consistent with their expression of the chemokine receptor CXCR4. Based on these data, they stated that SDF-1/CXCR4 expression is important in MSC adhesion to endothelial cells and migration (Sordi *et al.,* 2005; Yagi *et al.,* 2010). Disturbances in the SDF-1/CXCR4 pathway could therefore result in defective MSC mobilization. Karagiannis and colleagues studied bone-marrow derived MSCs from 15 COPD patients. Measured SDF-1 expression was decreased in bone-marrow derived MSCs from COPD patients compared to healthy controls. They suggested that migration of MSCs from the bone marrow through SDF-1/CXCR4 is defective in COPD patients (Karagiannis *et al.,* 2013). Disturbances in the SDF-1/CXCR4 pathway could therefore might be a challenge in successful migration of administered and resident MSCs to injured lung tissue in COPD patients.

Another factor that has been suggested to modulate MSC migration and adhesion is basic fibroblast growth factor (bFGF), whereas low concentrations of bFGF can lead to an attraction of MSCs (Yagi *et al.,* 2010). Moreover, Steingen *et al.* showed that transendothelial migration of MSCs is at least partially regulated by MMP-2 (Steinegen *et al.,* 2008). Taking everything into consideration, the mechanisms of action responsible for the ability of MSCs to

migrate to the source of injury and initiate tissue repair may involve strong interactions between integrin α4/β1 and VCAM-1 on epithelial cells and the chemotactic responsiveness of MSCs to SDF-1.

2.3 Alveolar differentiative potential of transplanted MSCs

After the administered MSCs have migrated to the source of injury, differentiation of MSCs into the type of epithelial cells present in the lung could help restore alveolar epithelial damage.

The adult lung epithelium is replaced over time. After injury, the lung harbors a remarkable capacity to regenerate and restore its function. The composition of the epithelium in the lung varies along a proximal-distal axis, which is reflected in the diverse physiological functions of the lung. In the most distal region of the lung, approximately 90% of the alveolar epithelium is composed of a flattened alveolar type 1 (AT1) cells (Voclkaert and de Langhe., 2014). These AT1 cells are in close proximity to the capillary endothelium, which allows for rapid and efficient gas exchange, and cuboidal alveolar type 2 (AT2) cells that express surfactant. These epithelial regions of the lung are maintained and repaired by distinct stem cell populations. Lineage tracing, identification of all progeny of a single cell, during normal homeostasis has identified three main stem cell populations responsible for maintaining the lung epithelium: club cells, basal cells and AT2 cells (Volckaert and de Langhe., 2014).

Club cells are the predominant stem cell population responsible for maintaining the bronchiolar epithelium. As a population, club cells are replaced over time by new club cells derived from basal cells. The alveolar epithelium is primarily maintained by AT2 cells, which can self-renew and can give rise to the flattened AT1 cells (Volckaert and de Langhe, 2014). Furthermore, additional distal progenitor cell populations have been shown to contribute to the regeneration of alveolar epithelium, including an integrin (Itg) α6/$\beta4^{+}$ (Itgα6$\beta4^{+}$) alveolar epithelial stem cell population, which has the potential to give rise both AT2 and club cells in vitro and in vivo (Chapman *et al.,* 2011; McQualter *et al.,* 2010). Furthermore, bronchioalveolar stem cells (BASCs), another population of stem cells located at bronchioalveolar duct junctions, can self-renew and give rise to both chronchiolar and alveolar cell lineages in vitro and in vivo. To what extent these additional cell populations contribute to alveolar repair after injury is not clear (Kim *et al.,* 2005; Lee *et al.,* 2014).

Lung stem cells must give rise to the appropriate number of differentiated progeny in order to achieve homeostasis and to restore the functional organ after injury such as alveolar break down seen in emphysema (Voclkaert and de Langhe, 2014). The behavior of the epithelial progenitors is controlled by the interplay between intrinsic transcriptional programs and extrinsic signals. These extrinsic signals are provided by the niche; the local tissue environment that hosts and influences the behaviors or characteristics of stem cells and comprises both ECM and other cell types. For example, fibroblast growth factor 10 (Fgf10) is expressed in several stem cell niches in the lung and influences stem cell maintenance and activation after injury (Volckaert and de Langhe, 2014). In addition, delivery of Fgf10 in the lungs of rats has been shown to increase the amount of lung resident mesenchymal stem cells in treated lungs (Tong *et al.,* 2016).

The beneficial role of transplanted MSCs in emphysema has been attributed in part to the differentiation of MSCs into alveolar epithelial cells. However, the exact type of cell is an area of controversy. Both differentiation of MSCs into AT1 and/ or AT2 cells has been shown in rat models of cigarette smoke- and lipopolysaccharide-induced emphysema, and in bleomycin-induced lung injury (Zhao *et al.,* 2014; Rojas *et al*., 2005; Jin *et al.,* 2014). This ability of MSCs to engraft in lung tissue and differentiate into alveolar cells suggests that exogenously administered MSCS may contribute to the repair of the alveolar epithelium following injury. However, little is known about the detailed mechanisms underlying the epithelial differentiation potential of MSCs in vivo.

Sun *et al.* investigated the possible regulation mechanisms of MSC differentiation in treatment for acute lung injury. Acute lung injury is a clinical syndrome, characterized by acute hypoxemic respiratory failure and lung tissue edema, finally leading to lung fibrogenesis. Sun and colleagues suggested that the differentiation process of MSCs may be regulated by various cytokines and special signal pathways at the injury sites in the lung. For example, they demonstrated that canonical Wnt/ β-catenin signaling is involved in regulating the process of epithelial differentiation of MSCs. Wnt/β-catenin signaling is a crucial regulator in tissue repair, wound closure, fibrosis and tissue remodeling. Activated Wnt signaling inhibited the epithelial differentiation process of MSCs in a co-culture system. However, inhibition of Wnt signaling caused by Wnt antagonist Dickkopf-I (DKKI) promoted MSCs to differentiate into alveolar epithelial cells including type 2 alveolar epithelial cells. These findings suggest a strong link between Wnt/β-catenin signaling and the epithelial differentiation of MSCs towards lung epithelial cells (Sun *et al.,* 2013; Wang *et al.,* 2009).

The differentiation of MSCs into specific cells at the injury sites has been considered a very important process in the effect of MSCs therapy. Unfortunately, differentiation of MSCs in vivo and engraftment rates are still very low (Ingenito *et al.,* 2012). This suggests that the mechanisms by which MSCs protect the lung might not only be via their ability to engraft and differentiate into alveolar epithelial cells, but also by other mechanisms. MSCs can also have a reparative effect through paracrine signaling, by releasing biologically active molecules that affect survival, proliferation and differentiation of the surrounding cells. Analysis of MSCs conditioned medium indicate that MSCS are able to secrete many known mediators of tissue repair including growth factors, cytokines and chemokines, specifically, hepatocyte growth factor (HGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and keratinocyte growth factor (KGF). These humoral factors secreted by MSCS may play an important role in approving tissue damage caused by emphysema (Katsha *et al.,* 2011; Maxson *et al.,* 2012). Various animal models of emphysema or emphysema related lung injuries revealed that paracrine mechanisms, in contrast to differentiation of MSCs, are essential in amelioration of lung tissue injury. Katsha *et al.* showed that MSCs could ameliorate elastase-induced emphysema in mice. Furthermore, they suggested that release of paracrine factors derived from MSCs was the main mechanism responsible for the observed protection of lung tissues from elastase injury (Katsha *et al.,* 2011). Other animal model-based studies demonstrated that MSC-paracrine factors attenuated pulmonary fibrosis through modulation of inflammation and suppression of fibrogensis (Akram *et al.,* 2013).

In the lung, development of epithelial cells depends on precise coordination of signals, such as Fgf, Sonic Hedgehog (Shh), retinoic acid, Notch, and TGF-β. Disruption of these signals can result in dramatic changes in differentiation of the lung epithelium. Recent studies, including a genome-wide association analysis, suggest that some molecular regulators described to be involved in developmental processes in the lung may be altered in patients with COPD (Shi *et al.,* 2009). For example, retinoic acid has been shown to have stage-specific effects on lung development, and could down regulate maturation of lung epithelial cells. In addition, altered TGF-β signaling has been implicated in the pathogensis of emphysema. Disruption of the TGF-β signaling results in abnormalities in the respiratory tract and the immune system. Specifically, blockade of TGF-β signaling in embryonic lung MSCs results in retarded lung branching, whereas overexpression of TGF-β could arrest lung growth and epithelial cell differentiation. Thus, appropriate TGF-β signaling activity is essential for normal lung development. It could be hypothesized that changes in the microenvironment in patients with COPD and emphysema could disturb the differentiation process of MSCs leading to relatively low rates of engraftment and differentiation of MSCs towards alveolar epithelial cells, since the fate of stem cells in vivo is mainly regulated by the microenvironment (Shi *et al.,* 2009)

To conclude, MSCs can migrate to sites of injury repairing damaged tissue, and facilitating tissue regeneration. Both differentiation of MSCs into alveolar epithelial cells and paracrine signaling by MSCs influencing proliferation and differentiation of surrounding cells, have been implicated as mechanisms by which MSCs can possibly improve tissue damage.

2.4 Inhibition of inflammation by transplantation of MSCs

One of the mechanisms postulated for MSC protection against emphysema is suppression of the chronic inflammatory response by modulating the release of soluble (anti)-inflammatory molecules and activation of cellular anti-inflammatory pathways (Jin *et al.,* 2014). Several studies have shown that MSCs actively inhibit the function of several immune cells through secreted cytokines, growth factors and enzymatic action (Yagi *et al.,* 2010). For example, administration of MSCs in a rat model of cigarette smoke induced emphysema has been shown to improve emphysematous pathology in these animals, partly via down-regulation of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6 and MCP-1 (Guan *et al.,* 2013). Moreover, infusions of allogeneic MSCs suppressed levels of circulating C-reactive protein, an annular protein found in blood plasma whose levels rise in response to inflammation, in a clinical trial with patients suffering from COPD (Weiss *et al.,* 2013). Furthermore, MSCs can alter the cytokine secretion profile of dentritic cells (DCs), naïve and effector T cells, and natural killer (NK) cells to induce a more anti-inflammatory phenotype. Specifically, MSCs caused mature DCs type 1 to decrease secretion of TNF-α and mature DC type 2 to increase interleukin-10 (IL-10), an anti-inflammatory cytokine. MSCs can cause T-cells to decrease IFN-y expression and increase the proportion of regulatory T suppressor cells. (Yagi *et al.,* 2010). These results indicate that MSC administration can suppress inflammatory processes via paracrine mechanisms.

Besides secretion of soluble anti-inflammatory mediators, MSCs are capable of modulating the immune system through interactions with a wide range of immune cells. Macrophages are the predominant immune effector cells and act as mediators of the inflammatory

response. Gu *et al.* proposed that MSCs are able to reverse inflammatory processes and restore impaired lung function in emphysema through their interaction with macrophages (Gu *et al.,* 2015). MSC administration alleviated airway inflammation and emphysema through the down-regulation of cyclooxygenase-2 (COX-2) and COX-2 mediated prostaglandin E2 (PGE2) production, though the effect on alveolar macrophages. COX-2 is an enzyme linked to inflammatory responses. PGE2 is a lipid mediator derived from metabolism of arachidonic acid by COX, and is an important mediator in inflammation. Co-culture experiments showed that MSCs down-regulated COX-2/PGE2 in macrophages through inhibition of the activation-associated phosphorylation of p38, mitogen-activated protein kinase (MAPK) and ERK (Gu *et al.,* 2015).

The described immune modulating properties of MSCs are rather complex. As mentioned, immune modulation by MSCs is not only attributed to secretion of soluble factors, but is also dependent on MSC-to immune cell contact. In addition, a study by Waterman and colleagues established a connection between the stimulation of specific Toll-like receptors (TLRs) and the immune modulating responses of human MSCs. TLRs are able to recognize danger signals. Activation of TLRs leads to profound cellular and systemic responses that mobilize innate and adaptive host immune cells. The TLRs consist of a relatively large family of evolutionary conserved receptors: TLR1-TLR9 (Waterman *et al.,* 2010). Waterman *et al.* observed distinct effects after stimulation of a specific type TLR, namely TLR3, compared with activation of TLR4, another type of TLR. TLR3 stimulation of MSCs supports the immunosuppressive effects of MSCs, while TLR4 activation of MSCs provides a pro-inflammatory signature (Waterman *et al.,* 2010). These results suggest that MSCs can be induced to develop into two diverse but homogenously acting phenotypes; exposure with TLR4 polarizes MSCs towards a pro-inflammatory MSC1 phenotype important for early injury responses, whereas TLR3 exposure polarizes MSCs toward an immunosuppressive MSC2 phenotype essential to later-anti-inflammatory responses that help resolve tissue injury (Waterman *et al.,* 2010). Emphysema is associated with an enhanced chronic inflammatory response in the lungs. Although MSCs have multiple anti-inflammatory characteristics, it could be hypothesized that in patients with emphysema, lung resident and administered MSCs may polarize towards a more pro-inflammatory MSC1 phenotype, and therefore contribute to the establishment of the inflammatory response and tissue injury. This could in part explain the lack of significant results of MSC therapy in patients with emphysema.

2.5 Inhibition of protease release by MSC transplantation

In emphysema, a protease/antiprotease imbalance contributes to alveolar wall destruction and airspace enlargement via degradation of ECM proteins and promoting apoptosis of structural cells in the alveolar walls (Jin *et al.,* 2014). Pulmonary administration of MSCs has been shown to reverse the induction of the proteases MMP-9 and MMP-12 in the lungs of rats with cigarette smoke-induced emphysema, both at the mRNA and protein levels (Figure 6). The mechanistic basis of this effect is not completely understood, however, it has been attributed in part to the inhibition by MSCs of a positive feedback loop, involving the release of proteases by inflammatory and structural cells activated by cigarette smoke (Guan *et al.,* 2013).

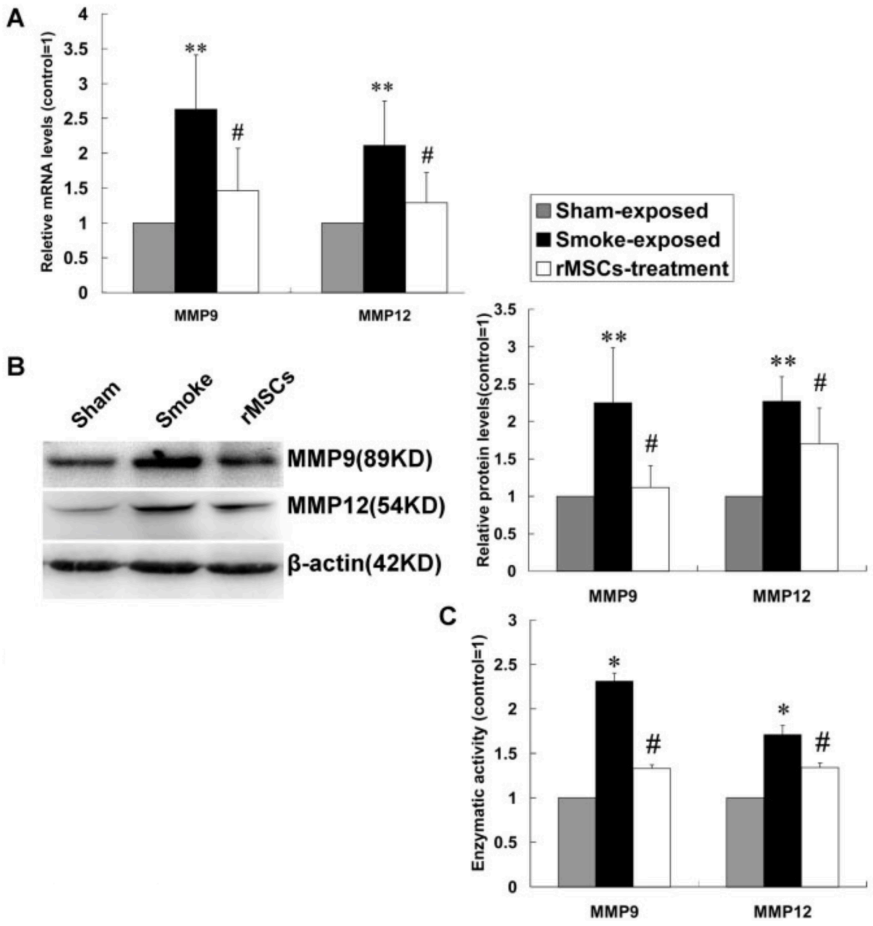

Figure 6. Rat MSCs down-regulated the levels of MMP9 and MMP12 in lung tissue
Guan and colleagues analyzed the **A)** mRNA, and **B)** protein levels of matrix metalloproteinase-9 (MMP9) and matrix metalloproteinase-12 (MMP-12) by Real-time PCR and Western blot respectively in rats after MSCs administration. **C)** Enzyme activity of MMP9 and MMP12 was measured by gelatin zymograph. Data is expressed as mean ±SD. *P< 0.05, **P<0.01 versus sham exposed rats, #P<0.05 versus cigarette exposed rats. Adapted and edited from Guan *et al.,* 2013.

2.6 Inhibition of oxidative stress by MSC transplantation

The contribution of oxidative stress to the development of emphysema is thought to encompass a variety of functions. For example, oxidative stress in emphysema has been suggested to enhance lung inflammation via induction of redox-sensitive inflammatory transcription factors such as NF-kB. Modulation of the redox environment by MSCs is an area of emerging interest (Jin *et al.,* 2014). Increased survival in rats with lipopolysaccharide-induced lung injury after transplantation with bone marrow derived MSCs has been shown to be accompanied by decreased levels of oxidative stress (Li *et al.,* 2012). In addition, transplantation of bone-marrow derived MSCs is known to decrease oxidative stress in the brain of a rat model of spontaneous stroke. These encouraging results suggest that MSCs may also decrease oxidative stress in animal models of cigarette smoke-induced emphysema. However, the effects of MSCs on oxidative stress in emphysema are not yet fully understood (Calió *et al.,* 2014).

In addtion, Cho *et al*. showed that MSC-mediated resolution of liver injury may occurs through a specific antioxidative process. After being injected with carbon tetrachloride (CCL_4), mice were injected with bone marrow derived MSCs. CCL_4 treatment generates free radicals that rigger a cascade of events, resulting in fibrosis in the liver. The treatment with CCL_4 up-regulated the level of reactive oxygen species (ROS) in liver cells, this effect was attenuated by co-culturing with MSCs. Furthermore, MSCs increased superoxide dismutase

(SOD) activity. SOD catalysis the conversion of superoxide to H_2O_2, the latter is converted into water and oxygen by catalase and peroxidase. Therefore, SOD is a major antioxidant defense that protects tissues within the body from oxidative stress. Since SOD secreted by MSCs decreased levels of ROS in injured liver cells and improved hepatic endothelial dysfunction, one could speculate a similar mechanism could be involved in reducing oxidative stress by MSCs in alveolar epithelial cells (Cho *et al.,* 2012).

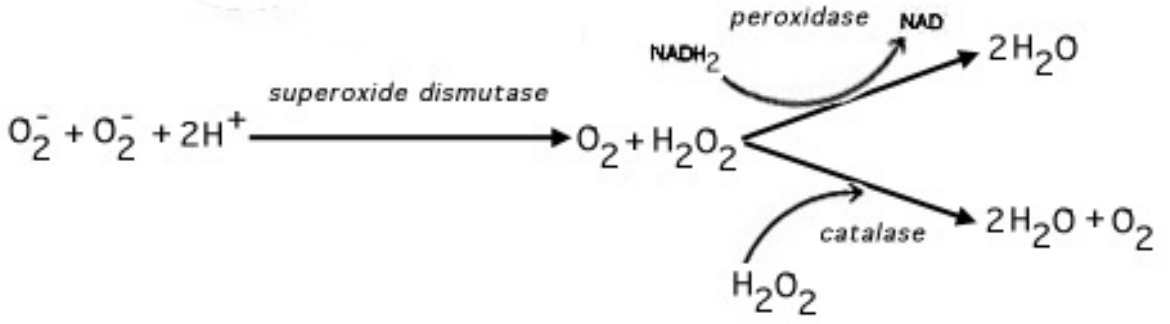

Figure 9. The action of superoxide dismutate, catalase and peroxidase in oxidative stress
Superoxide dismutase (SOD), catalase and peroxidase are enzymes that can detoxify oxygen radicals that are inevitably generated by living systems in the presence of O_2. Mesenchymal stem cells (MSCs) can increase SOD activity (Cho *et al.,* 2012). This mechanism may contribute to the amelioration of oxidative stress, which is one of the pathological processes leading to development of emphysema.
Adapted from Todar., 2012.

Recently, it has been shown that MSCs have the extraordinary capacity of executing mitochrondrial transfer. Oxidative stress can results in damaged mitochondria, which subsequently leads to even higher levels of oxidative stress. It has been shown in a mouse model of induced acute lung injury that the therapeutic effect of MSCs was associated with mitochondrial transfer to alveolar epithelial cells and thereby allowed the mouse to recover from lung injury. In these mice, bone marrow derived MSCs formed connexin 43(Cx43)-containing gap junctional channels (GJCs) with the alveolar epithelium, releasing mitochondria-containing microvesicles that the epithelium subsequently engulfed. This mitochondrial transfer process increased levels of alveolar ATP and protected the mice against acute lung injury by restituting alveolar bioenergetics and improving lung function (Islam *et al.,* 2012). A possible role of mitochondrial transfer in amelioration of lung damage in emphysema needs to be further investigated.

2.7 Inhibition of alveolar cell apoptosis by MSC transplantation

Apoptosis of alveolar epithelial cells is known to play a pivotal role in the pathogenesis of emphysema. As mentioned in chapter 1, blocking the VEGF signaling pathway leads to apoptosis of the alveolar cell; and decreases in VEGF and VEGF receptor 2 (VEGFR2) at both the mRNA and protein levels have been described in emphysematous patients and smokers (Jin *et al.,* 2014; Kanazawa and Yoshikawa, 2005). Interestingly, MSCs may beneficial inhibit alveolar cell apoptosis since they have been described to stimulate VEGF secretion and VEGFR2 induction. Therefore, measured amelioration by MSC transplantation of alveolar cell apoptosis in the lungs of papain- or cigarette smoke-induced rat models of emphysema has been postulated to involve reversal of the effects of cigarette smoke

exposure on the VEGF signaling pathway (Guan *et al., 2013;* Zhen *et al*., 2010). Figure 6 shows part of the results of research done by Guan and colleagues, showing that mRNA and protein levels of VEGF in lungs were significantly lower in cigarette smoke exposed rats compared with sham exposed rats, while these levels were higher in MSCs-treated rats (Figure 7 A-B). One hypothesis is that MSCs transplantation in rats can promote VEGF release from alveolar epithelial cells by regulating the lung local microenvironment, together with VEGF release from the MSCS, which may account for the elevated VEGF in lungs and amelioration of alveolar cell apoptosis (Guan *et al.,* 2013).

MSCs may also suppress alveolar cell apoptosis and ameliorate emphysema by an alternative mechanism. This mechanism has been suggested to involve alterations in the expression of apoptotic or anti-apoptotic genes in these cells. For example, it has been reported that the apoptotic gene *Bax* and the anti-apoptotic gene *Bcl-2* are repressed and induced respectively, after pulmonary administration of MSCs in a papain-induced rat model of emphysema (Zhen *et al.,* 2008).

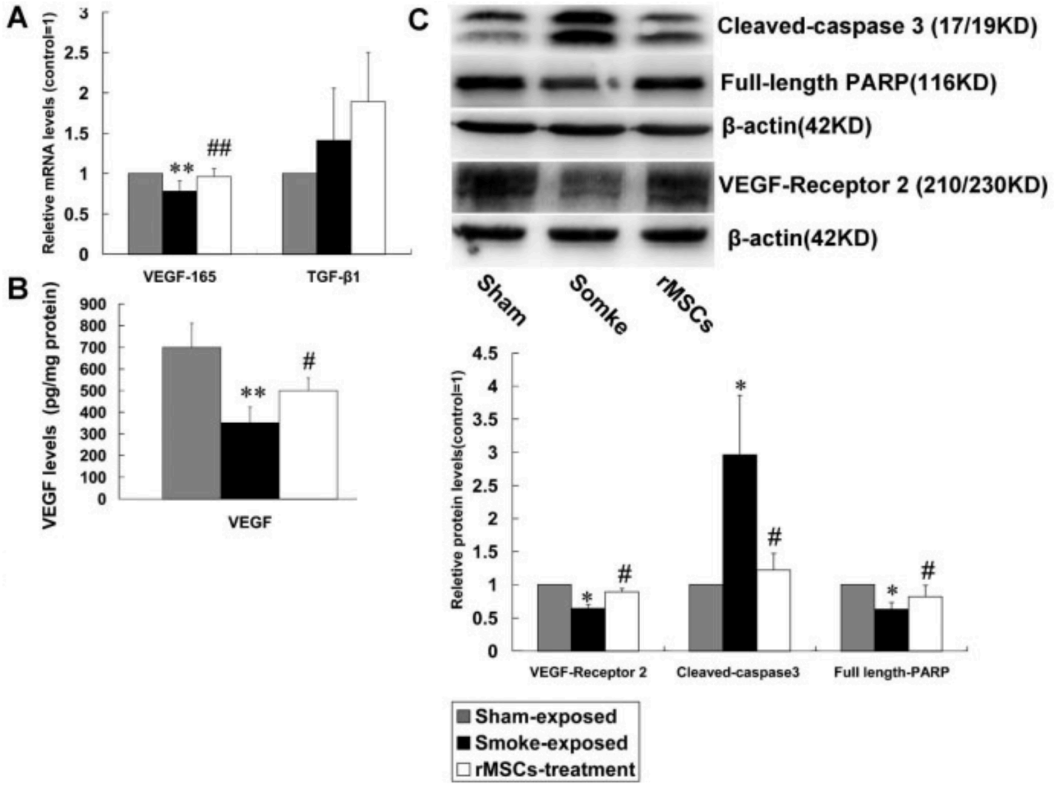

Figure 7. MSCs up-regulate levels of VEGF and VEGF receptor 2
Guan and colleagues investigated levels of vascular endothelial growth factor -164 (VEGF164) and Transforming growth factor (TGF)-β1 in lungs of rats exposed to cigarette smoke and treated with mesenchymal stem cells (MSCs). **A)** real time PCR to measure mRNA levels and **B)** ELISA to measure protein levels were performed. **C)** Apoptosis related proteins, cleaved-caspase3 en Poly (ADP-ribose) polymerase (PARP) in lungs of rats exposed to cigarette smoke and treated with MSCs were assessed by western blot. Data are expressed as mean ±SD. *P< 0.05, **P<0.01 versus sham exposed rats, #P<0.05 versus cigarette exposed rats.
Adapted and edited from Guan *et al.,* 2013.

A third mechanism for MSC mediated amelioration of alveolar apoptosis is the suppression of alveolar levels of cleaved caspase 3, which is a key player in the apoptotic programme in epithelial cells (Kim *et al,* 2012). Caspases are a family of cysteine proteases that are activated during apoptosis. Caspase 3 is the ultimate apoptotic proenzyme in most types of cells. Activation of caspase 3 requires proteolytic processing of its inactive symogen into activated fragments resulting in cleaved caspase 3. Cleaved caspase 3 is primarily responsible for the cleavage of full length Poly (ADP-ribose) polymerase (PARP), which plays a central role in the execution of the apoptotic program in epithelial cells (Figure 8). Therefore, activation of caspase 3 suggests cell apoptosis. Guan et al showed that MSCs

administration inhibited lung cell apoptosis and therefore protected the epithelial in the lung, supported by reduced cleaved-caspase 3 and increased full-length PARP levels (Figure 7C) (Guan *et al.,* 2013).

Taking everything in consideration, MSCs can suppress alveolar cell apoptosis via multiple mechanisms including stimulation of VEGF secretion and VEGFR2 induction, repression of the apoptotic gene *Bax* and stimulation of the anti-apoptotic gene *Bcl-2*, and reduction of cleaved-caspase 3 levels.

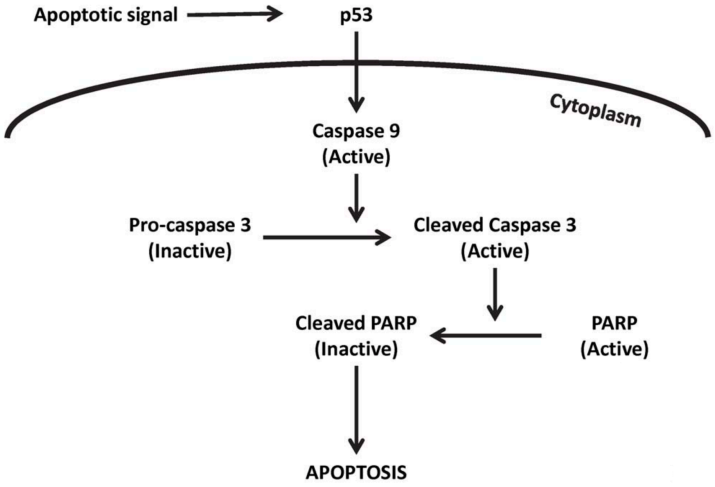

Figure 8. Pathway of caspase-3 mediated apoptosis After an apoptotic signal via p53, activated caspase 9 can cleave caspase-3 resulting in active fragments of cleaved caspase 3. Cleaved caspase 3 is primarily responsible for the cleavage of full length poly (ADP-ribose) polymerase (PARP), which plays a central role in the execution of the apoptotic program in epithelial cells. Therefore, activation of caspase 3 suggests apoptosis of epithelial cells. Adapted and edited from Malhotra *et al.,* 2013.

2.8 Other protective mechanisms of MSCs

In addition to the protective mechanisms of MSCs mentioned above, recent studies have recognized another novel mechanism that could contribute to the protective and regenerative capacities of MSC therapy. For example, Lee and colleagues performed a proteomic analysis of MSC-conditioned medium which revealed the presence of a number of proteins including CD63, CD81, moesin, lactadherin (MFGE8), heat-shock protein 90 (hsp90), and hsp70. These proteins have been reported to be associated with secreted vesicles known as exosomes (Lee *et al.,* 2012). Exosomes are small heterogeneous microvesicles stored within multivesicular bodies (MVB) and released upon fusion with the plasma membrane. Exosomes have been recognized as important mediators of intercellular communication, especially in the immune system. In addition, exosomes can act as a vector for the transfer of genetic information such as mRNA and micro-RNAs to recipient cells. Micro-RNAs are critical regulators of gene expression and hence many cellular functions in health and disease. In cells in the airway, microRNA expression profiles can be regulated by multiple factors, including growth factors, inflammatory agents, mechanical forces and hypoxia. Furthermore, micro RNAs have been demonstrated to play a critical role in many inflammatory diseases and asthma because of their anti-inflammatory effects (Lee *et al.,* 2012). Lee et al demonstrated that the protective functions of MSCs in lung injury are partly mediated by these secreted microvesicles. Administration of MSC secreted exosomes (MEX) led to an up-regulation of the miRNA-17 superfamily of micro-RNA clusters and miRNA-204 in a murine model of pulmonary hypertension. They hypothesized that MEX might be one of the paracrine anti-inflammatory mediators of MSC action in the lung (Lee *et al.,* 2012). However, a possible role of MEX in amelioration of lung damage in emphysema needs to be further investigated.

Discussion

COPD is a progressive lung disease with high rates of mortality and morbidity. The term COPD includes both emphysema and chronic obstructive bronchitis. This review focused on emphysema, which is defined by the enlargement of airspaces as a result of alveolar breakdown in the adult lung. The pathogenesis of emphysema is a complex process and is unable to be attributed to a single mechanism. Multiple pathological processes occur simultaneously and are often interrelated. In addition, many aspects of the pathobiology of human emphysema remain unclear (Taraseviciene-Stewart and Voelkel, 2008). In this review, recent literature was used to provide an overview of our current knowledge of the molecular pathogenesis of emphysema.

Chronic inflammation can be considered as one of the key aspects in the development of emphysema. Cigarette smoke activates immune cells including macrophages, neutrophils and T-cells, which subsequently release pro-inflammatory substances that mediate alveolar wall destruction. Furthermore, in emphysema, the delicate balance between proteases and antiproteases can shift towards a dominance of proteases, resulting in degradation of ECM-proteins and eventually proteolytic lung destruction. In patients with emphysema, an increase in apoptosis of alveolar epithelial cells is not balanced by an increase in proliferation, resulting in alveolar breakdown, which is partly mediated by the VEGF pathway. Finally, oxidative stress has been suggested as a pathogenic mechanism in patients with emphysema, which, among others, contributes to lung inflammation (Taraseviciene-stewart and Voelkel, 2008; Inamdar *et al.,* 2013; Jin *et al.,* 2014).

As of today, no treatment has been found to repair or reverse the damage done to the lungs by emphysema. MSCs, however, because of their anti-inflammatory and protective abilities, are a promising therapeutic alternative for emphysema, although many questions regarding the mechanisms of action of MSC therapy in lung reparation and regeneration remain unanswered. After exploring the pathogenesis of emphysema, this review aimed to investigate the underlying mechanisms of action of MSCs that could contribute to regeneration or reparation of alveolar epithelial damage caused by emphysema.

First, MSCs have been shown to migrate to the source of injury and initiate tissue repair. The homing ability of MSCs is made feasible by strong interactions between integrin α4/β1 on the MSCs and VCAM-1 on epithelial cells and a chemotactic responsiveness of MSCs to SDF-1. Furthermore, MSCs have been shown in several animal models to be able to differentiate in both AT1 and AT2 cells, which suggest that administered MSCs may contribute to repair of the alveolar epithelium following injury. Recently, Wnt/β-catenin signaling has been shown to be involved in regulating the process of epithelial differentiation of MSCs. Paracrine signaling by MSCs further influences proliferation and differentiation of the surrounding cells and contributes to repair of tissue damage in the lung. Modifying the host immune response by the release of soluble anti-inflammatory molecules and direct contact with macrophages via the COX-2/PGE2 pathway, is another valuable quality making MSCs a promising therapeutic tool for emphysema. Finally, inhibition of protease release, reduction of oxidative stress mediated by levels of SOD, and prevention of apoptosis of alveolar epithelial cells by

stimulation of VEGF secretion and alteration of the apoptotic-anti-apoptotic gene ratio by MSCs have been shown to contribute to prevention of emphysema in various mice and rat models. An overview of the mechanisms of action of MSCs in emphysema is shown in figure 10.

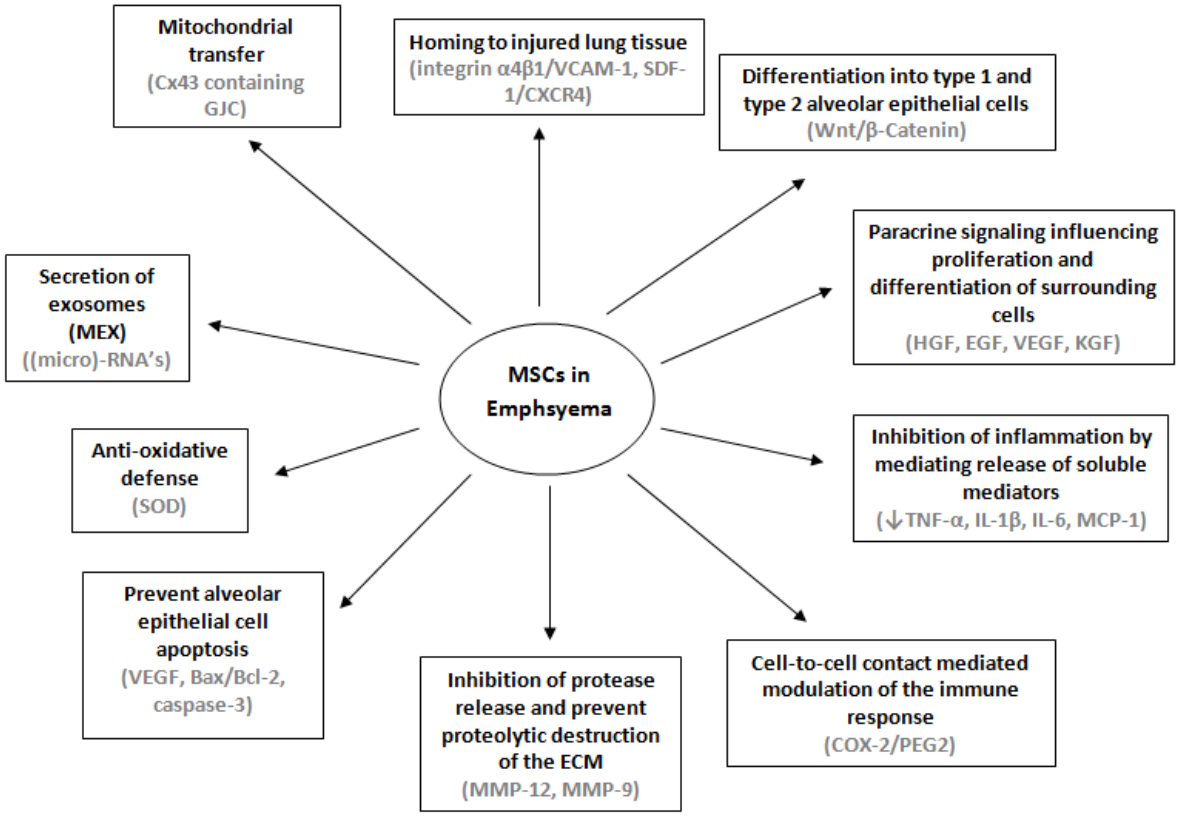

Figure 10. MSC therapy in emphysema
MSCs are potential candidates for treatment of emphysema. Possible protective mechanisms of action for MSCs, and involved signaling pathways and molecules, as understood from multiple (animal) experiments are shown. MSCs are known to impart anti-inflammatory and immunomodulatory effects. Furthermore, homing to injured tissue, prevention of alveolar cell apoptosis, inhibition of protease release, differentiation into alveolar cells and paracrine signaling by MSCs have been shown. Furthermore, other mechanisms of action of MSCs such as mitochondrial transfer and up-regulation of microRNAs by secretion of exosomes have recently been recognized. Abbreviations: MSC: mesenchymal stem cell, VCAM-1: vascular cell adhesion molecule-1, SDF-1: stromal cell derived factor-1, CXCR4: C-X-C chemokine receptor type 4, HGF: hepatocyte growth factor, EGF: epidermal growth factor, VEGF: vascular endothelial growth factor, KGF: keratinocyte growth factor, TNF-α: tumor necrosis factor-α, IL-1β: interleukin-1β, IL-6: interleukin-6, MCP-1: monocyte chemotactic protein-1, COX-2: cyclo-oxygenase-2, PEG2: prostaglandin E2, ECM: extracellular matrix, MMP-12: matrix metalloproteinase-12, MMP-9: matrix metalloproteinase-9, SOD: superoxide dismutase, RNA: ribonucleic acid, Cx43: connexin 43, GJC: gap junctional channel.

Although multiple pre-clinical animal models of emphysema show encouraging results concerning the alleviation of emphysema by MSC therapy, in human clinical trials no beneficial results of MSC therapy in COPD have been observed yet. Besides unraveling the mechanisms of action of MSCs, this review aimed to explore possible reasons behind the lack of significant results of MSC therapy in COPD patients, in contrast to promising animal studies. For example, the breakdown of the elastin network and interstitial PGs in patients with emphysema, as a result of protease/antiprotease imbalance and oxidative stress, could lead to reduced adhesion of MSCs to the ECM, less integrin activation and possibly less survival of MSCs, after administration (Eurlings *et al.,* 2014; Straaten van *et al.,* 1999). Furthermore, disturbances in the SDF-1/CXC4 pathway, measured in bone-marrow derived MSCs from COPD patients, could lead to defective MSC mobilization (Karagiannis *et al.,*

2013). Since migration and adhesion of MSCs to the injured lung are crucial factors in alveolar epithelial regeneration in emphysema, this could contribute to less significant results of MSC therapy.

The fact that ECM breakdown in emphysema might prevent MSCs from migrating towards injured tissue in the lung, suggests that the measured beneficial effect of MSCs in improving alveolar tissue damage in emphysema-induced mice models might be the result of paracrine functions of MSCs in stead of differentiation of MSCs at the injured alveolar epithelium. MSCs are capable of secretion of various anti-inflammatory mediators and growth-factors. Several animal studies whereby administered MSCs improved emphysematous characteristics in the lung, attributed these beneficial effects to paracrine mechanisms of MSCs. In addition, several studies mentioned low engraftment rates of MSCs and differentiation of MSCS to be rare in animal and human cells (Fritzell *et al.,* 2009; Liebler *et al.,* 2008). Lung development is a complex process and depends on very precise coordination of signals. In COPD changes in the differentiation prolife of the airway epithelium takes place. It could be hypothesized that changes in the microenvironment in patients with COPD and emphysema could disturb the differentiation process of MSCs leading to relatively low rates of engraftment and differentiation of MSCs towards alveolar epithelial cells, since the fate of stem cells in vivo is mainly regulated by the microenvironment (Shi *et al.,* 2009).

Interspecies differences should also be considered when translating information from murine or rat models to humans. For example, there are important interspecies differences in the distribution and abundance of specific cell types in the airways. These differences may influence the response of the lung to particular types of injury. Moreover, some mouse models may be unable to reproduce all the characteristics of the disease phenotype of emphysema seen in humans (Shi *et al.,* 2009).

BM-MSCs are still the most frequently used MSCs in experimental research. However, MSCs can be isolated from various sources and differences between phenotype, quality and quantity of MSCs collected at these various sites exist. It could be possible that MSCs isolated from different sources result in different outcomes when administrated as a therapy for emphysema (Akram *et al.,* 2012). Riccardi and colleagues isolated, expanded and characterized MSCs from normal adult human lungs, lung resident human MSCs (L-MSCs), and compared these cells with human BM-MSCs (Ricciardi *et al.,* 2012). They found no differences in terms of immunophenotype, stemness gen profile, mesodermal differentiation potential and modulation of immune cells such as T, B and NK cells. However, L-MSCs did show higher epithelial cell polarization, although they questioned the real capability of acquiring epithelial functions by MSCs. This specific characteristics of lung-MSCs may be useful in reparation of alveolar epithelial damage in emphysema (Ricciardi *et al.,* 2012). Hoffman and colleagues found lung retention efficiency and evasion of phagocytosis to be higher for L-MSCs than BM-MSCS on 4 days and 32 days after transplantation of MSCs in mice. They mentioned paucity of receptors on BM-MSCs for endothelial ligands as a proposed mechanism involved in low engraftment of BM-MSCS in lung tissue. In this study, L-MSCs consistently expressed higher levels of several surface proteins, including ICAM-1, PDGFRα, and Itgα2. Furthermore, these proteins were shown to modulate essential

engraftment-related functions; adherence, migration and invasion, in L-MSCs. They concluded that L-MSCs exhibit phenotypic and functional characteristic that are distinct from BM-MSCs (Hoffman *et al.,* 2011). Ratajczak *et al.* believed that the family of BM-MSCs consists of commited tissue-specific stem cells for various organs. This theory believes that only the lung-specific/commited BM-MSCs can be driven to differentiation into type 2 alveolar epithelial cells (Ratajczak *et al.,* 2014). Besides the source, determination of the optimal route of administration (intravenously vs intratracheal) and dose are important aspects that require more study.

The molecules and pathways involved in regulating the differentiation of MSCs in vivo are thus complex, and there is need for further research on the exact regulatory mechanisms involved in differentiation of MSCs and approaches that increase survival and engraftment of MSCs in host organs (Sun *et al.,* 2014). In addition, we could shift our attention from using BM-MSCs towards L-MSCs in experimental research because L-MSCs tend to showed higher epithelial cell polarization and lung retention efficiency. Moreover, the evaluation of MSCs as safe and effective therapeutic modality in the treatment of COPD including emphysema is still in its infancy. There has only been one fully completed trail evaluating the safety and efficacy of non-modified bone-marrow derived MSs in the treatment of COPD. The recently completed study, sponsored by 'Mesoblast International Sàrl', was evaluating the efficacy and safety of the use of MSCs in patients with moderate to severe COPD. A total of 62 patients received 4 monthly infusions of 100 x 106 allogeneic MSCs or a placebo. The patients were followed up for a time period of 2 years after the first infusion, at the end of which safety, pulmonary function, systemic inflammation, quality of life, and a 6-min walk test (6MWT) were evaluated. During the course of this study, no adverse events were observed. However, no significant changes in pulmonary function were detected during this study either (Weiss *et al.* 2013). A larger trail and a more effective dosage and treatment schedule may be necessary to evaluate efficacy more accurately.

To conclude, the unique properties of MSCs in homing, differentiation, immunomodulation, restoring protease/antiprotease balance and inhibition of alveolar epithelial cell apoptosis and oxidative stress make them an ideal candidate for the treatment of challenging lung conditions like emphysema. However, further research is necessary to unravel all the unsolved mysteries of the mechanisms of action of MSCs in emphysema both in vivo and in vitro, in order to further develop and establish MSCs as a novel therapeutic tool to ameliorate emphysema in COPD patients.

Acknowledgements

I would like to thank prof. dr. Irene Heijink for supervising this thesis, for the very helpful meetings, and for taking the time to offer constructive feedback on my progress.

References

Akram, K.M., Samad, S., Spiteri, M.A, & Forsyth, N.R. (2012). Mesenchymal stem cell therapy and lung disease. *Advances in biochemical engineering/biotechnology,* 130: 105-129.

Akram, K.M., Samad, S., Spiteri, M.A., & Forsyth, N.R. (2013). Mesenchymal stem cells promote alveolar epithelial cell wound repair in vitro through distinct migratory and paracrine mechanisms. *Respiratory Research,* 14: 9.

Calió, M.L., Marinho, D.S., Ko, G.M., Riberio, R.R., Carbonel, A.F., Ovama, L.M., Ormanji, M., Guirao, T.P., Calió, P.L., Reis, L.A., Simoes, M.J., Lisboa-Nascimento, T., Ferreira, A.T., & Bertoncini, C.R. Transplantation of bone marrow mesenchymal stem cells decreases oxidative stress, apoptosis, and hippocampal damage in brain of a spontaneous stroke model. *Free Radical Biology & Medicine,* 70:141-154.

Carp, H., & Janoff, A. (1978). Possible mechanisms of emphysema in smokers. In vitro suppression of serum elastase-inhibitory capacity by fresh cigarette smoke and its prevention by antioxidants. *The American review of respiratory disease,* 118:617-621.

Chapman, H.A., Li, X., Alexander, J.P., Brumwell, A., Lorizio, W., Tan, K., Sonnenberg, A., Wei, Y., & Vu, T.H. (2011). Integrin alpha6beta4 identifies an adult distal lung epithelial population with regenerative potential in mice. *Journal of clinical investigation,* 121: 2855-2862.

Cho, K.A., Woo, S.Y., Seoh, J.Y., Han, H.S., & Ryu, K.H. (2012). Mesenchymal stem cells restore CCL_4-induced liver injury by an antioxidative process. *Cell Biology International,* 36:1267-1274.

Churg, A., Wang, R.D., Xie, C., & Wright, J.L. (2003). Alpha-1-antitrypsin ameliorates cigarette smoke-induced emphysema in the mouse. *American Journal of Respiratory and Critical Care Medicine,* 168: 199-207.

Danielson, K.G., Baribault, H., Holmes, D.F., Graham, K.E., & Lozzo, R.V. (1997). Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *The Journal of Cell Biology,* 136: 729:43.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., & Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy,* 8(4): 315-317.

Eurlings, I.M.J., Dentener, M.A., Cleutjens, J.P.M., Peutz, C.J., Rohde, G.G.U., Wouters, E.F.M., Reynaert, N.L (2014). Similar matrix alterations in alveolar and small airway walls of COPD patients. *Biomed Central Pulmonary Medicine*, 14:90.

Fritzell, J.A., Mao, Q., Gundavarapu, S., Pasquariello, T., Aliotta, J.M., Ayala, A., Padbury, J.F., & Paepe de, M.E. (2009). Fate and effects of adult bone marrow cells in lungs of normoxic and hyperoxic newborn mice. *American Journal of Respiratory Cell and Molecular Biology,* 40: 575-587.

Guan, X.J., Song, L., Han, F., Cui, Z., Chen, X., Guo, X., & Xu, W. (2013). Mesenchymal stem cells protect cigarette smoke-damaged lung and pulmonary function partly via VEGF-VEGF receptors. *Journal of Cellular Biochemistry,* 114: 323-335.

Hautemaki, R.D., Kobayashi, D.K., Senior, R.M., & Shapiro, S.D. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science,* 277: 2002-2004.

He, A., Jian, Y., Chun, G., Sun, Y., Li, J., & Wang, J. (2009). The antiapoptotic effect of mesenchymal stem cell transplantation on ischemic myocardium is enhanced by anoxi preconditioning. *Canadian Journal of Cardiology,* 25(6): 353-358.

Hodge, S., Hodge, G., Holmes, M., & Reynolds, P.N. (2005). Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. *European Respiratory Society*, 25(3): 447-454.

Hoffman, A.M., Paxson, J.A., Mazan, M.R., Davis, A.M., Tyagi, S., Murthy, S., & Ingenito, E.P. (2011). Lung-derived Mesenchymal stromal cell post-transplantation survival, persistence, paracrine expression, and repair of elastase-injured lung. *Stem cells and development*, 20(10): 1779-1792.

Huh, J.W., Kim, S.Y., Lee, J.H., Lee, J.S., Van Ta, Q., Kim, M., Oh, Y.M., Lee, Y.S., & Lee, S.D. (2011). Bone marrow cells repair cigarette smoke-induced emphysema in rats. *American Journal of Physiology. Lung cellular and molecular physiology,* 301(3): L255-66.

Imai, K., Mercer, B.A., Schulman, L.L., Sonett, J.R., & D'Armiento, J.M. (2005). Correlation of lung surface area to apoptosis and proliferation in human emphysema. *The European Respiratory Journal,* 25(2): 250-80.

Inamdar, A.C., & Inamdar, A.A. (2013). Mesenchymal stem cell therapy in lung disorders: pathogensis of lung diseases and mechanism of action of mesenchymal stem cell. *Experimental Lung Research,* 39: 315-327.

Ingenito, E.P., Tsai, L., Murthy, S., Tyagi, S., Mazan, M., Hoffman, A. (2012). Autologous lung-derived msenchymal stem cell transplantation in experimental emphysema. *Cell transplantation,* 21: 175-189.

Irshad, M., & Chaudhuri, P.S. (2002). Oxidant-antioxidant system: role and significance in human body. *Indian Journal of Experimental Biology,* 40(11): 1233-9.

Islam, M.N., Das, S.R., Emin, M.T., Wei, M., Sun, L., Westphalen, K., Rowlands, D.J., Quadri, S.K., Bhattacharya, S., & Bhattacharya, J. (2012). Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nature Medicine,* 18: 759-765.

Jin, Z., Pan, X., Zou, K., Bi, H., Wang, L., Yu, L., & Wang, Q. (2014). Biological effects and mechanisms of action of mesenchymal stem cell therapy in chronic obstructive pulmonary disease. *Journal of International Medical Research,* 43(3): 303-310.

Jones, R.L., Noble, P.B., Elliot, J.G., & James, A.L. (2016). Airway remodelling in COPD: it's not asthma! *Respirology.* 21(8): 1347-1356.

Kanazawa, H., & Yoshikawa, J. (2005). Elevated oxidative stress and reciprocal reduction of vascular endothelial growth factor levels with severity of COPD. *Chest*, 128: 3191-3197.

Kanazawa, H., & Yoshikawa, J. (2005). Elevated oxidative stress and reciprocal reduction of vascular endothelial growth factor levels with severity of COPD. *Chest*, 128: 3191-3197.

Karagiannis, K., Antoniou, K., Moraitaki, D., Psaraki, A., Soufla, G., Kalpadaki, C., Siafakas, N., & Tzanakis, N. (2013). Impaired migration of bone marrow mesenchymal stem cells in COPD. *European Respiratory Journal,* 42: 245.

Kasahara, Y., Tuder, R.M., Taraseviciene-Stewart, L., Le Cras, T.D., Abman, S., Hirth, P.K., Waltenberger, J., & Voelkel, N.F. (2000). Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *Journal of Clinical Investigation*, 106(11): 1311-1319.

Katscha, A.M., Ohkouchi, S., Xin, H., Kanehira, M., Sun, R., Nukiwa, T., & Saijo, Y. (2011). Paracrine factors of multipotent stromal cells ameliorate lung injury in an elastase-induced emphysema model. *Molecular Therapy,* 19: 196-203.

Kim, C.F., Jackson, E.L., Woolfenden, A.E., Lawrence, S., Babar, I., Vogel, S., Crowly, D., Bronson, R.T., & Jacks, T. (2005). Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell*, 121: 823-835.

Kim, S.Y., Lee, J.H., Kim, H.J., Park, M.K., Huh, J.W., Ro, J.Y., Oh, Y.M., Lee, S.D., & Lee, Y.S. (2012). Mesenchymal stem cell-conditioned media recovers lung fibroblasts from cigarette smoke induced damage. *Lung Cellular and Molecular Physiology,* 302:L891-L908.

Laennec, R.T. (1819). De auscultation mediate; ou, Traité du diagnostic del maladies des poumon et du Coeur. Brosson et Chaude. Paris, France.

Laennec, R.T. (1834). A treatise on the diseases of the chest. Longman. London, United Kingdom.

Lee, C., Mitsialis, S.A., Aslam, M., Viali, S.H., Vergadi, E., Konstantinou, G., Sdrimas, K., Fernandez-Gonzalez, A., & Kourembanas, S. (2012). Exsomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia induced pulmonary hypertension. *Circulation*, 126(22): 2610-2611.

Lee, J.H., Bhang, D.H., Beede, A., Huang, T.L., Stripp, B.R., Bloch, K.D., Wagers, A.J., Tseng, Y.H., Ryeom, S., & Kim, C.F. (2014). Lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-thrombospondin-1 axis. *Cell,* 156: 440-455.

Liebler, J.M., Lutzko, C., Banfalvi, A., Senadheera, D., Aghamohammadi, N., Crandall, E.D., & Borok, Z. (2008). Retention of human bone marrow-derived cells in murine lungs following bleomycin-induced lung injury. *American Journal of Physiology. Lung Cellular and Molecular Phsyiology,* 295: L285-L292.

Li, J., Li, D., Liu, X., Tang, S., & Wei, F. Human umbilical cord mesenchymal stem cells reduce systemic inflammation and attenuate LPS-induced acute lung injury in rats. *Journal of Inflammation,* 9:33.

Malhotra, U., Zaidi, A.H., Kosovec, J.E., Kasi, P.M., Komatsu, Y., Rotoloni, C.L., Davison, J.M., Irvin, C.R., Hoppo, T., Nason, K.S., Kelly, L.A., Gibson, M.K., & Jobe, B.A. (2013).

Prognostic value and targeted inhibition of survinin expression in esophageal adenocarcinoma and cancer-adjacent squamous epithelium. *PLoS ONE* 8(11): e78343.

Maxson, S., Lopez, E.A., Yoo, D., Danilkovitch-Miagkova, A., & LeRoux, M.A. (2012). Concise review: role of mesenchymal stem cells in wound repair. *Stem cells translational medicine,* 1(2): 142-149.

McQualter, J.L., Yuen, K., Williams, B., & Bertoncello, I. (2010). Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. *Proceedings of the National Academy of Sciences,* 107: 1414-1419.

Merrilees, M.J., Ching, P.S.T., Beaumont, B., Hinek, A., Wight, T.N., Black, P.N. (2008). Changes in elastin, elastin binding protein and versican in alveoli in chronic obstructive pulmonary disease. *Biomed Central Respiratory Research,* 9:41.

Morrison, D., Rahman, L., Lannan, S., & Macnee, W. (1999). Epithelial permeability, inflammation and oxidant stress in the air spaces of smokers. *American Journal of Respiratory and Critical Care Medicine,* 159: 473-479.

Pons, A.R., Sauleda, J., Noguera, A., Pons, J., Barceló, B., Fuster, & A., Agustí, A.G. (2005). Decreased macrophage release of TGF-beta and TIMP-1 in chronic obstructive pulmonary disease. *European Respiratory Journal,* 26: 60-66.

Rahman, I. (2005). Oxidative stress in pathogensis of chronic obstructive pulmonary disease. Cellular and molecular mechanisms. *Cell biochemistry and biophysics,* 43: 167-188.

Rahman, I., Morrison, D., Donaldson, K., & MacNee, W. (1996). Systemic oxidative stress in asthma, COPD, and smokers. *American Journal of Respiratory and Critical Care Medicine,* 154: 1055-1060.

Ricciardi, M., Malpeli, G., Bifari, F., Bassi, G., Pacelli, L., Kamdje, A.H.N., Chilosi, M., & Krampera, M. (2012). Regulatory properties of Mesenchymal Stromal cells derived from human lung and bone marrow. *PLoSONE,* 7(5): e35639.

Rojas, M., Xu, J., Woods, C.R., Mora, A.L., Spears, W., Roman, J., & Brigham, K.L. (2005). Bone marrow-derived cells as progenitors of lung alveolar epithelium. *American Journal of Respiratory Cell and Molecular Biology,* 33: 145-152.

Ruster, B., Gottig, S., Ludwig, R.J., Bristian, R., Muller, S., Seifried, E., Gille, J., & Henschler, R. (2006). Mesenchymal stem cells display coordinated rolling and ashesion behavior on endothelial cells. *Blood*, 108(12): 353-363.

Sackstein, R. (2005). The lymphocyte homing receptors: gatekeepers of the multistep paradigm. *Current Opinion in Hematology,* 12(6): 444-450.

Saetta, M., Turato, G., Maestrelli, P., Mapp, C.E., & Fabbri, L.M. (2001). Cellular and structural bases of chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine*, 163: 1304-1309.

Sharafkhaneh, A., Hanania, N.A., Kim, V. (2008). Pathogenesis of emphysema. From the bench to the bedside. *Proceedings of the American Thoracic Society*, 5: 475-477.

Snider, G.L., Kleinerman, J., Thurlbeck, W.M., & Bengali, Z.H. (1985). The definition of emphysema. *The American review of respiratory disease,* 132: 182-185.

Sordi, V., Malosio, M.L., Marchesi, F., Mercalli, A., Melzi, R., Giordano, T., Belmonte, N., Ferrari, G., Leone, B.E., Bertuzzi, F., Zerbini, G., Allavena, P., BOnifacio, E., & Piemonti, L. (2005). Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood,* 106(2): 419-427.

Steingen, C., Brenig, F., Baumgartner, L., Schmidt, J., Schmidt, A., & Bloch, W. (2008). Characerization of key mechanisms in transmigration and invasion of Mesenchymal stem cells. *Journal of Molecular and Cellular Cardiology,* 44(6): 1072-1084.

Stevens, T., Kasper, M., Cool, C., & Voelkel, N. (2005). Pulmonary circulatin and pulmonary hypertension. In *Endothelial cells in health and disease.* W.C. Aird. Editor. Taylor & Francis Group. Boca Raton, Florida, USA. 417-438.

Straaten van, J.F.M., Coers, W., Noordhoek, J.A., Huitema, S., Flipsen, J.T.M., Kauffman, H.F., Timens, W., Postma, D.S. (1999). Proteoglycan changes in the extracellular matrix of lung tissue from patients with pulmonary emphysema. *Modern Pathology,* 12(7): 697-705.

Sun, Z., Gong, X., Zhu, H., Wang, C., Xu, X., Cui, D., Qian, W., & Han, X. (2013). Inhibition of Wnt/β-Catenin signaling promotes engraftment of mesenchymal stem cells to repair lung injury. *Journal of Cellular Physiology,* 229:213-224.

Tang, K., Rossiter, H.B., Wagner, P.D., & Breen, E.C. (2004). Lung-targeted VEGF inactivation leads to an emphysema phenotype in mice. *Journal of applied physiology,* 97: 1559-1566.

Taraseviciene-Stewart, L., & Voelkel, N.F. (2008). Molecular pathogenesis of emphysema. *The journal of clinical investigation,* 118: 394-402.

Todar, K. (2012). Nutrition and growth of bacteria. Retrieved from: http://textbookofbacteriology.net/nutgro_4.html

Tong, L., Zhou, J., Rong, L., Seeley, E.J., Pan, J., Zhu, X., Liu, J., Wang, Q., Tang, X., Qu, J., Bai, C., & Song, Y. (2016). Fibroblast growth factor-10 (FGF-10) mobilizes lung-resident mesenchymal stem celsl and protects against acute lung injury. *Scientific reports,* 12: 6-21642.

Toorn van der, M., Rezaya, D., Kauffman, H.F., Bakker, S.J.L., Gans, R.O.B., Koëter, G.H., Choi, A.M.K., Oosterhout van, A.J.M., & Slebos, D.J. (2009). Lipid-soluble components in cigarette smoke induce mitochondrial production of reactive oxygen species in lung epithelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology,* 297(1): L109-L114.

Ugarte, D.A., Alfonso, Z., Zuk, P.A., Elbarbary, A., Zhu, M., Ashjian, P., Benhaim, P., Hedrick, M.H., & Fraser, J.K. (2003). Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunology Letters,* 89(2-3): 267-70.

Vaseva, A.V., & Moll., U.M. (2008). The mitochondrial p53 pathway. *Biochimica et Biophysica Acta,* 1787(5): 414.

Vestbo, J., Hurd, S.S., Agustí, A.G., Jones, P.W., Vogelmeier, C., Anzueto, A., Barnes, P.J., Fabbri, L.M., Martinez, F.J., Nishimura, M., & Stockley, R.A. (2013). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease, GOLD executive summary. *American Journal of Respiratory and Critical Care Medicine,* 187: 347-365.

Volckaert, T., & De Langhe, S. (2014). Lung epithelial cells and their niches: Fgf10 takes center stage. *Fibrogenesis and tissue repair,* 7:8.

Wang, Y.J., Sun, Z.R., Qui, X.F., Li, Y., Qjn, J.Z., & Han, X.D. (2009). Roles of Wnt/beta-catenin signaling in epithelial differentiation of mesenchymal stem cells. *Biochemical and Biophysical Research Communications,* 390: 1309-1314.

Waterman, R.S., Tomchuck, S.L., Henkle, S.L., & Betancourt, A.M. (2010). A new Mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosppresive MSC2 phenotype. *PLoS ONE,* 5(4): e10088.

Wecht, S., & Rojas, M. (2016). Mesenchymal stem cells in the treatment of chronic lung disease. *Respirology: offical journal of the Asian Pacific Society of Respirology,* 21(8): 1366-1375.

Weiss, D.J., Casaburi, R., Flannery, R., LeRoux-Williams, M., & Tashkin, D.P. (2013). A placebo-controlled, randomized trial of mesenchymal stem cells in COPD. *Chest*, 143(6): 1590-1598.

Yagi, H., Soto-Gutierrez, A., Parekkadan, B., Kitagawa, Y., Tompkins, Kobayashi, N., & Yarmush, M.L. (2010). Mesenchymal stem cells: mechanisms of immunomodulation and homing. *Cell transplant,* 19(6): 667-679.

Zhao, Y., Xu, A., Xu, Q., Zhao, W., Li, D., Fang, X., & Ren, Y. (2014). Bone marrow mesenchymal stem cell transplantation for treatment of emphysemic rats. *International Journal of Clinical and Experimental Medicine,* 7: 968-972.

Zhen, G., Liu, H., Gu, N., Zhang, H., Xu, Y., & Zhang, Z. (2008). Mesenchymal stem cells transplantation protects against rat pulmonary emphysema. *Frontiers in Bioscience,* 13: 3415-3422.

Zhen, G., Xue, Z., Zhao, J., Gu, N., Tang, Z., Xu, Y., & Zhang, Z. (2010). Mesenchymal stem cell transplantation increases expression of vascular endothelial growth factor in papain-induced emphysematous lungs and inhibits apoptosis of lung cells. *Cytotherapy*, 12: 605-614.

Zheng, T., Kang, M.J., Crothers, K., Zhu, Z., Liu, W., Lee, C.G., Rabach, L.A., Chapman, H.A., Homer, R.J., Aldous, D., De Sanctis, G.T., Underwood, S., Graupe, M., Flavell, R.A., Schmidt, J.A., & Elias, J.A. (2005). Role of cathepsin S-dependent epithelial cell apoptosis in IFN-gamma-induced alveolar remodeling and pulmonary emphysema. *Journal of Immunology*, 174(12): 8106-15.