Stem Cell-Based Therapy for Lung Disease

Janette K. Burgess
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Editors

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Preface

Abnormal lung tissue repair and regeneration has recently emerged as a potential driving force underlying the pathogenesis and progression of many lung diseases, and research in the field of lung tissue regeneration has rapidly developed during the past years. Worldwide, there is a momentum to understand the mechanisms underlying the potential of stem and progenitor cells as drivers of repair and as a possible source of reparative factors in the lung. Their application in cell-based therapeutic strategies is an evolving subject of investigation.

Nevertheless, the field of stem cells and their mechanisms in lung pathologies is complex, and the challenge of realizing the clinical potential of stem cell-based regenerative medicine in the lung is considerable. Translating findings from preclinical settings to clinical applications remains a significant hurdle for the field. In addition, it has recently been recognized that the extracellular matrix provides crucial bio-instructive cues for cells within the microenvironment, both resident and incoming trafficking endogenous and exogenously introduced potential therapeutic cells, and this should be taken into account for when considering cell-based therapies.

This book provides a comprehensive overview of the current state of the art of the field of stem cell-based therapies in lung pathologies for a broad audience including medical residents, fellows, general practitioners, as well as master students, postgraduate students, and postdoctoral research fellows. The overview includes a description of stem cell types and mechanisms involved in lung (re)generation as well as their effects in preclinical models and clinical studies on various lung diseases. The book concludes with a description of novel delivery strategies.

Part I describes abnormalities in lung tissue repair and deficiencies in stem/progenitor cells from lung disease patients and subsequently provides an up-to-the-minute overview of our current understanding of the stem/progenitor cell populations resident in the lung. Furthermore, the role of stromal support in the maintenance and differentiation of progenitor cells is described. A comparison is made in the potential of mesenchymal stem/stromal cells obtained from different origin (body location) sources for use in therapeutic strategies for lung disorders. Also, the use of the stem/stromal cell secretome for therapeutic approaches in lung diseases is summarized. Part II provides a summation of preclinical evidence and clinical applications in various chronic lung diseases, including chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, bronchopulmonary dysplasia (BPD), pulmonary arterial

hypertension (PAH), acute respiratory distress syndrome (ARDS), and cystic fibrosis (CF). Being a monogenic disorder, the potential of using CRISPR-Cas9 gene editing in induced pluripotent stem cells (iPSCs) as an approach for CF is also discussed. In Part III, the use of spraying as a novel delivery route for stem cells in the lungs is discussed. Finally, the state of the art for the potential application of stem cells as therapeutic agents in pulmonary medicine is summarized.

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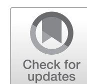

1 Chronic Lung Pathologies That Require Repair and Regeneration

Roderick de Hilster, Minghui Li, Wim Timens, Machteld Hylkema, and Janette K. Burgess

Abbreviations

AEC	Alveolar epithelial cell
BPD	Bronchopulmonary dysplasia
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CL-	Chloride
COPD	Chronic obstructive pulmonary disease
DAD	Diffuse alveolar damage
ECM	Extracellular matrix
FDA	Federal drug agency
HCO3-	Bicarbonate
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
MSC	Mesenchymal stem cell
PAH	Pulmonary arterial hypertension
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
UIP	Usual interstitial pneumonia
WHO	World Health Organisation

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1.1 Introduction

Lung diseases are, when cumulatively added, the main cause of mortality worldwide [1, 2], and the full impact of these diseases is yet to be realised. The incidence and accuracy of diagnosis of chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis, continue to increase worldwide. Lung diseases affect both the individual and the surrounding society extensively, with impact of these diseases on the family, work place and health economics, including increasing costs for health care while a majority continues to be incurable. For most chronic lung diseases little progress has been made, in recent years, in the development of therapeutic strategies for managing these burdensome pathologies. There is an urgent need to increase our understanding of the mechanisms underlying these diseases and for innovative approaches that will lead to the clinical breakthroughs that are currently lacking.

The lung is built of airways and lung parenchyma and blood vessels. Specialised cells that reside within, and in relation with, defined regions of the extracellular matrix (ECM) make

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up the main building blocks of these components. As one of the few organs that are directly exposed to environmental insults, the lung has an effective endogenous repair system that enables homeostasis to be maintained within the tissue. Whilst the exact mechanisms underlying most chronic lung diseases are not well understood, in many instances it is recognised that an aberration of the inherent repair process probably contributes to the pathophysiology that results in the diseased outcome [3–9]. The lung has a slow yet constant cell turnover that nevertheless cannot always cope with the loss of tissue and cells during (severe) injury or chronic disease [5]. During ageing, the respiratory system undergoes structural remodelling affecting both elements of the ECM and the cells, which leads to loss of elasticity and enlargement of alveolar spaces, with eventual airway narrowing because of loss of elastic recoil [10]. The result is a lung more susceptible to both acute and chronic insults, which becomes dysfunctional and with a lower breathing capacity that debilitates the patient [11].

Endogenous progenitor cells (stem cells) have been recognised in many organs, including the lungs [12–15]. Understanding the role of stem cells in maintaining a population of cells that are able to facilitate the endogenous repair processes that maintain tissue homeostasis is currently an area of intense research interest. Emerging knowledge of how these repair processes are disrupted in chronic lung diseases and the potential to capitalise upon the regenerative capacity of these cell populations as the much-anticipated advance for clinical management for these devastating diseases is raising the hopes of the field worldwide.

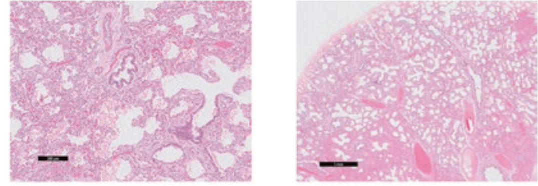
1.2 Chronic Lung Disease Pathologies That May Benefit from Regeneration Approaches

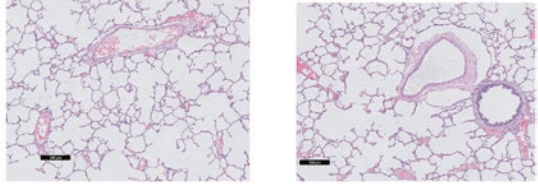
1.2.1 Chronic Obstructive Pulmonary Disease (COPD)

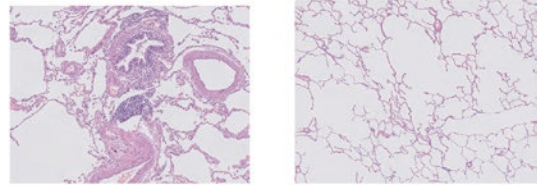
Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation that is not fully reversible and results mainly from the interplay between genetic susceptibility and environmental stimuli [16]. COPD is currently the third leading cause of death in the world [17], attributed to exposure to smoke by cigarette smoking and/or indoor cooking or by other harmful particles [19]. Alpha l-anti-trypsin deficiency is a genetic cause responsible for a minority of COPD incidence [18, 19]. The incidence of COPD is more often seen at a higher age, in particular because of the slow development of the disease before the clinical deterioration becomes apparent; the disease diagnosis therefore increases with age, peaking for patients between age 65 and 74, although patients who develop disease at a younger age usually have more severe disease. More than three million people died of COPD in 2012 accounting for 6% of all deaths worldwide. Globally, the COPD burden is projected to increase in coming decades because of continued exposure to COPD risk factors, as mentioned above, and ageing of the population seriously impacting the health costs associated with managing these patients.

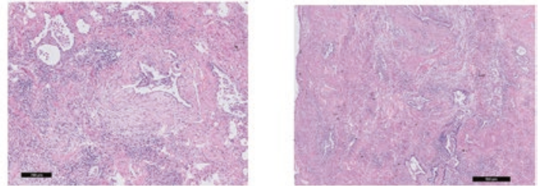
1.2.1.1 Pathology of COPD

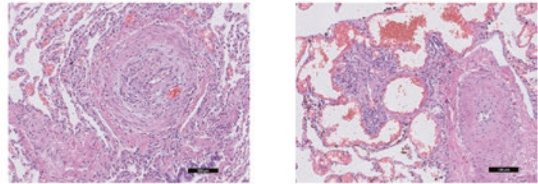
The pathological changes in COPD are observed in the central airways, small airways, alveolar space and vasculature [20–22] (see Fig. 1.1). The central airway alterations include goblet cell hyperplasia, which are associated with the enlargement of mucus-producing glands and squamous cell metaplasia [23]. These changes are related to enhanced mucus production and cough in chronic bronchitis. The small airways, usually defined as airways with less than 2 mm internal diameter without cartilage, are considered the major site of increased airflow resistance in most patients with COPD [24]. Small airway wall thickening is observed, with increased smooth muscle mass and infiltration of inflammatory cells, and some alterations of epithelial cell differentiation leading to a variable shift of club- and ciliated cells to more goblet cells. This small airway wall thickening together with the loss of peri-bronchial elastic recoil is considered as the predominant cause of airflow limitation [10, 21]. Infiltration of

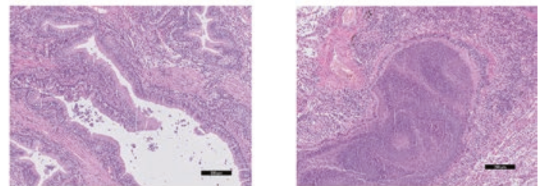












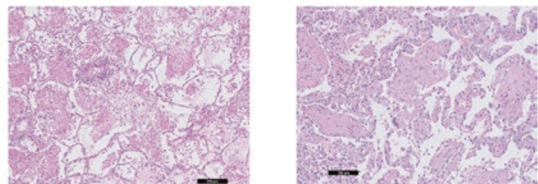


Fig. 1.1 Representative photomicrographs of human lung tissue sections stained with hematoxylin and eosin illustrating disease pathological features from a 10-week-old infant who died from bronchopulmonary dysplasia, and adults with chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, pulmonary arterial hypertension, cystic fibrosis and acute respiratory distress syndrome compared to normal adult lung

the small airway walls mainly by macrophages and (CD8) T lymphocytes can contribute to the severity of airway limitation [24]. The presence of macrophages and T lymphocytes, particularly $CD8^+$ T cells, may in addition contribute to changes of the alveolar walls in COPD [25–27]. The chronic presence of these inflammatory cells leads to damage of the alveolar walls, and eventually, because of the lack of tissue repair in COPD [28, 29], could lead to emphysema. Alteration of the pulmonary vasculature is now also considered as a vital component of COPD. This is caused by smooth muscle hypertrophy, which is associated with increased deposition of elastin and collagen [23, 30, 31] accompanied by apoptosis of the endothelial cells in the arterioles [32], induced by the hypoxic conditions in the COPD lung.

1.2.1.2 Treatment of COPD

There is currently no cure available for COPD, but treatment can control the symptoms, reduce the risk of complications and exacerbations and help slow the progression of the condition. Smoking cessation is the most effective intervention in any treatment plan for COPD, stopping smoking could decrease the risk of death by 18% [33]. Several kinds of medications are used to treat the symptoms and complications of COPD [34]. Bronchodilators, with long-acting and short-acting forms, can relax the muscles of the airways to help relieve coughing and shortness of breath and make breathing easier [35]. Theophylline can help improve breathing and prevent exacerbations, although with strong side effects [36]. Anti-inflammatory medications, inhaled glucocorticosteroids are commonly combined with long-acting bronchodilators to reduce inflammation in the airways and reduce mucus production [37]. Antibiotics have been successfully used for treatment and prevention of acute exacerbations of COPD [38]. Oxygen therapy and pulmonary rehabilitation programs also provide additional therapies for people with moderate or severe COPD. Surgery is an option for COPD patients with severe emphysema who are not helped sufficiently by medications alone, including lung transplantation.

1.2.2 Lung Fibrosis

Pulmonary fibrosis describes a group of interstitial lung diseases (ILDs), mainly characterized by progressive extracellular matrix (ECM) remodelling, increased ECM deposition and irreversible scarring [39]. The quite random accumulation of excess fibrotic ECM leads to mostly irregular stiffening of the lung, with irregular compliance resulting in inadequate ventilation and diffusion with reduced oxygen transfer, breathing difficulties and eventually respiratory failure [40]. It is estimated that ILDs account for approximately 20% of the spectrum of lung diseases encountered in the practice of pulmonary medicine with varying degrees of pulmonary fibrosis and respiratory dysfunction [39]. Pulmonary fibrosis includes diseases such as scleroderma, radiation and chemotherapy-induced fibrosis and the most common form is idiopathic pulmonary fibrosis (IPF). Although it is challenging to provide evaluations as to how many people are affected by IPF, it is estimated IPF affects approximately three million people worldwide. Approximately 50,000 new cases are diagnosed each year and the death toll due to IPF totals about 40,000 patients in the US each year. How many people are affected by IPF in Europe is not completely known but current estimates suggest that between 37,000 and 40,000 people will be diagnosed each year [41]. More importantly, it is anticipated that the number of individuals diagnosed with IPF will continue to increase, and by 2025 about 132,000 patients are expected to suffer this intractable and debilitating disease. The prognosis for patients with IPF is worse than for most cancers including breast cancer, prostate cancer, some forms of leukaemia and lymphoma [42–45].

IPF is an irreversible and life-threatening lung degenerative disease characterized by the presence of lung scarring, immune infiltrates, and inflammation, which typically leads to respiratory failure. The clinical course of the disease is characterized by a progressive decline in exercise capacity, difficulty breathing, recurrent infections and severe impairment in lung function, which makes the patients dependent on long-term oxygen treatment [46, 47].

A myriad of agents such as allergens, chemicals, radiation and environmental particles are risk factors implicated in the pathogenesis of pulmonary fibrotic diseases [40, 46, 48]. The most consistent risk factor for pulmonary fibrotic diseases is cigarette smoking [46, 49]. Injury caused by these triggers leads to wound-healing responses which are generally divided in three phases: injury, inflammation and repair [40]. The presence of a persistent irritant or repeated injury could cause a dysregulation at one or more of these phases.

1.2.2.1 Pathology of IPF

Idiopathic pulmonary fibrosis (IPF) is one of the most well-researched forms of ILDs and is the most commonly diagnosed [46]. IPF is a chronic, progressive fibrotic ILD which is characterized by myo(fibroblast) proliferation, interstitial inflammation and fibrosis within the alveolar wall (see Fig. 1.1), the cause of which is unknown and portrays the histological picture of usual interstitial pneumonia (UIP) [50, 51]. UIP usually presents as severe fibrosis with peripheral alveolar septal thickening and distortion of tissue architecture with a honeycomb structure made up of subpleural cystic airspaces with irregular fibrotic walls and irreversible dilatation of bronchi and bronchioles (bronchiectasis) [49].

Classically IPF was thought to be driven by a chronic inflammatory process; however, although inflammation likely still contributes to the pathogenesis, increasing evidence indicates that an uncontrolled healing response can gradually evolve into a pathologic fibrotic response when important regulatory mechanisms are disrupted and persistent inflammation follows. The persistent inflammation can result in a local milieu of pro-fibrotic cytokines and growth factors such as IL-13 and TGF-β1 [52–54]. The pro-fibrotic environment causes fibroblasts to transform into myofibroblasts which are the main producers of ECM which stay active in the presence of TGF-β, resulting in scarring and destruction of the lung architecture [53]. Increasing research results indicate that the fibrotic response is also driven by abnormally activated alveolar epithelial cells (AECs) [43]. AECs produce mediators that induce the formation of fibroblast and myofibroblast foci through the proliferation of resident mesenchymal cells, attraction of circulating fibrocytes and stimulation of epithelial to mesenchymal transition. The mechanisms that link IPF with ageing and aberrant epithelial activation are unknown; recent research results suggest that the abnormal recapitulation of developmental pathways and epigenetic changes may have a role in driving these changes [44].

1.2.2.2 Treatment of IPF

Two antifibrotic drugs have been approved by the FDA to combat pulmonary fibrosis, namely Nintedanib and Pirfenidone [43, 55]. Nintedanib is a small molecule inhibitor of the receptor tyrosine kinases of the PDGF receptor, FGF receptor and vascular endothelial growth factor receptor, which are believed to play important roles in the pathogenesis of IPF [51, 56]. Pirfenidone has multiple, different, not fully understood, mechanisms of action; however, it appears to have antifibrotic properties via regulation of pro-fibrotic growth factors such as TGF-β and tumour necrosis factor-α (TNF-α) [51, 57, 58]. Both drugs slow the progression or reduce the risk of acute exacerbations of IPF but do not stop or reverse pathophysiology, meaning the search for a cure continues and lung transplantation remains the only treatment for IPF that improves quality of life and survival [55, 59, 60].

1.2.3 Bronchopulmonary Dysplasia (BPD)

Annually, globally 15 million babies are born premature (defined as before 37 weeks of gestation) and approximately 2.4 million babies are born before 32 weeks of postmenstrual age [61]. Bronchopulmonary dysplasia (BPD) is the most common chronic lung disease in preterm infants, affecting ~10–50% of all infants born before 32 weeks, and has long been defined by the need for supplemental oxygen and/or mechanical ventilation 28 days after birth ("old BPD") [62]. However, the advances in obstetric and neonatal care over the last half century have resulted

in changes in pathophysiology and clinical presentation of BPD, and a new type of BPD has developed [63, 64]. More preterm infants born in the early stages of lung development presently suffer from BPD, with a frequency inversely correlated with gestational age. BPD develops as a result of lung injury caused by maternal pre-eclampsia, chorioamnionitis, postnatal ventilation, hyperoxia and/or inflammation, leading to arrested alveolar and microvascular development (fewer and larger alveoli), airway hyperreactivity and pulmonary hypertension [62, 65]. This indicates that the pathogenesis of BPD is multifactorial, which means that it is difficult to predict and also prevent short- and long-term consequences of the disease.

1.2.3.1 Pathology of BPD

Whereas the histopathologic lesions in children with "old BPD" are mostly characterized by fibrosis and inflammation, pathology of the "new BPD" is rather characterized by tissue simplification and arrest of alveolarization (fewer and larger alveoli) [63]. An example of lung tissue sections from a 10 weeks old infant with "new" BPD is shown in Fig. 1.1.

1.2.3.2 Treatment of BPD

Over the years, treatment strategies for BPD improved and gentle ventilation strategies and effective non-invasive ventilation devices were implemented to reduce prolonged mechanical ventilation and oxygen exposure. Besides these approaches, other more targeted therapies including low-dose hydrocortisone, non-invasive surfactant instillation, retinoic acid and anti-inflammatory strategies have been trialled, all which have been limited by serious side effects [64]. In addition, with increased survival of infants, interest in stem cell-related therapies has emerged as mesenchymal stem cells (MSCs) play a key role in alveolarization and lung tissue repair [13]. Clinical trials of MSCs in chronic lung diseases have demonstrated short-term safety and tolerability; however, studies have also shown populations of MSCs with adverse pro-inflammatory and myofibroblastic characteristics [66].

In hyperoxic rodent models of BPD, as shown by a systemic review of the literature, MSC treatment resulted in a significant improvement in lung injury, with the primary outcome of lung alveolarization and secondary outcomes, including inflammation, pulmonary hypertension, lung fibrosis, apoptosis and lung angiogenesis [67]. Likewise, MSC-derived conditioned media conferred therapeutic benefit for alveolarization, pulmonary artery remodelling, and angiogenesis. Cell-based therapies may represent the next breakthrough therapy for the treatment of BPD; however, there remain barriers to implementation as well as gaps in knowledge of the role of endogenous MSCs in the pathogenesis of BPD.

1.2.4 Pulmonary Arterial Hypertension (PAH)

Pulmonary hypertension describes conditions in which high blood pressure affects the arteries in the lungs and the right side of the heart. It is a chronic, progressive condition that can lead to right heart failure which can be lethal if not treated appropriately. As the presentation of this disease is similar to many other lung diseases (shortness of breath (dyspnea)—initially while exercising then building up while at rest, fatigue, dizziness or fainting (syncope), chest pressure or pain), this can lead to a delay in accurate timely diagnoses. Pulmonary hypertension is classified into five groups, based on World Health Organisation (WHO) classifications (groups) defined by the pathophysiology of the disease [68–70]. The most common form is WHO group 1, which refers to patients who have pulmonary arterial hypertension (PAH).

PAH is a rare disease, affecting about 15 people per million worldwide. The cause of PAH is generally unknown, it is often referred to as idiopathic PAH, although genetic predisposition is recognised in familiar PAH. There are a number of pre-disposing conditions, including systemic sclerosis (particularly in the presence of ILD), human immunodeficiency virus and methamphetamine exposure, that are recognised to increase the prevalence of PAH [71–73].

1.2.4.1 Pathology of PAH

In PAH in early/mild lesions the small arteries in the lungs (<500 μm diameter) become obstructed, which leads to an increase in blood pressure due to the increased resistance. The obstruction is driven by remodelling of the artery walls including hypertrophy in the medial layer, proliferation and fibrotic (ECM deposition) changes in the intima and thickening of the advential layer accompanied by inflammatory infiltrates [69, 74]. In more severe disease, the so-called complex or plexiform lesions develop with a glomeruloid vascular process and a dilated venous part added to the obstructed arteriole [69] (see Fig. 1.1). Vasoconstriction, associated with overproduction of endothelin-1 (vasoconstrictor) or underproduction of nitric oxide or prostacyclin (or both) (vasodilators), is often a disease characteristic. Mutations in the bone morphogenic protein receptor type II gene are the most common cause of familiar PAH [75] and are also thought to play a role in sporadic cases though it is less clear exactly how.

1.2.4.2 Treatment of PAH

Patients with untreated PAH have a median life expectancy of 2.8 years [76]. However, recent advances in therapeutic approaches for the management of PAH have advanced the survival times significantly [68, 77, 78]. Over the last two decades, advances in understanding and pursuit of the pathophysiology of PAH have enabled the development of pharmaceutical agents that target three critical pathways; prostacyclin analogues replace the deficiency of endogenous prostacyclins, endothelin receptor antagonists counteract the overproduction of endothelin and phosphodiesterase-5 inhibitors act to account for the reduced activity of the nitric oxide pathway [79, 80]. Worldwide accepted treatment algorithms have significantly improved the survival outcomes for many PAH patients [68]; however, it remains a fatal disease.

1.2.5 Cystic Fibrosis (CF)

Cystic fibrosis (CF) is a progressive life-threatening genetic disease involving multiple organs, including the lungs, pancreas, sweat glands, biliary tract, salivary glands and the vas deferens. The lung pathology is the major cause of disease morbidity and generally is the cause of death in these patients. CF is an autosomal recessive disease affecting approximately 75,000 worldwide. It is most common in Caucasians of northern European descent, and least common in Asian-Americans [81]. The life expectancy of an individual with CF has increased steadily since the recognition of the disease in the 1950s; with advances in symptomatic treatments the estimated life expectancy for individuals with CF, born in 2016, is greater than 45 years (Cystic fibrosis registry 2016).

1.2.5.1 Pathology of CF

CF is the most common lethal autosomal recessive disease in Caucasians, resulting from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). More than 1700 mutations have been recognised in the CFTR gene that lead to CF [82]. These mutations are divided into six classes depending on how the change affects the protein synthesis, trafficking, function or stability of the CFTR [83]. The CFTR, expressed apically in epithelial cell membranes, is a transmembrane channel that is important for regulating cellular salt and fluid homeostasis. It is a chloride (Cl^-) and bicarbonate (HCO_3^-) ion channel. Mutations in this channel result in disrupted flow of Cl^- and HCO_3^- through epithelial cells of multiple organs, resulting in aberrant functioning of those organs.

In the lung, disruption of the CFTR impacts the hydration of mucus at the epithelial cell surface (the lack of Cl^- transport) [84] and allows tethering of mucus, rather than clearance as a result of the lack of the key alkalisation effector (HCO_3^-) [85, 86]. These mutations result in the accumulation of a thick, sticky mucus in the airways driving chronic inflammation and enabling recurrent respiratory infections usually initiating soon after birth. Animal studies reflect the pathological changes in the airways, also suggesting the trachea is narrowed. The neutrophil influx, in response to the ongoing airway inflammation, leads to further inflammation as a result of

the release of neutrophil elastase and other pro-inflammatory cytokines [87, 88]. This vicious cycle drives remodelling of the airway wall tissues, resulting in air trapping and bronchiectasis (see Fig. 1.1).

1.2.5.2 Treatment of CF

The main treatment approaches for CF aim to modulate symptoms but are not able to cure the disease [89]. Preventing and reducing the severity of respiratory infections is a major focus, usually with antibiotics. Airway inflammation is addressed with steroids and non-steroidal anti-inflammatory drugs, while β agonists are used in combination with hypertonic saline to reduce the viscosity of the mucus and relax the airways. More recently there have been exciting developments of therapeutics aimed at "correcting" and "potentiating" CFTR. Vertex Pharmaceuticals have obtained FDA approval for the use of VX-770 (CFTR corrector, ivacaftor, trade name Kalydeco) [90, 91] and VX-809 (CFTR potentiator, lumacaftor) [92] for clinical use in CF patients with specific mutations [93]. Combination therapies that aim to improve the functionality and stability of the CFTR at the epithelia cell surface are now being developed [94]. These approaches have made significant advances and improvements in the quality of life for many patients with CF, but they are not effective for all mutations of CFTR. These approaches help to reduce the ongoing inflammation, but it is not clear if they are able to address the structural remodelling in the lung tissues.

1.2.6 Acute Respiratory Distress Syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) is a relatively common life-threatening syndrome that affects 200,000 adults annually in the US and is the cause of almost 75,000 deaths each year. Worldwide more the three million people are affected, accounting for 10% of admissions to intensive care units annually. It is a major clinical problem that is the end result of a number of aetiologies of lung injury. ARDS is an acute cause of rapidly progressing respiratory failure which is often associated with multiple organ failure. Many events have been suggested as risk factors for the development of ARDS. Respiratory infections are the most common causes, particularly bacterial pneumonia, although there is increasing recognition of the impact of respiratory viruses, pathogenic fungi and parasites (especially in immunocompromised patients). There is an emerging awareness of genetic susceptibilities in ARDS patients [95].

1.2.6.1 Pathology of ARDS

The pathophysiology of ARDS is recognised to occur in three overlapping phases [96, 97] with a characteristic and pathognomonic histopathology termed diffuse alveolar damage (DAD). The initial injury to the lung causes a disruption of the alveolar endothelium and epithelial cell barrier accompanied by oedema in the airspaces [98]. In the case of an infectious origin, neutrophils and macrophages are the major cell types that infiltrate the airspaces, but with many aetiologies inflammation is inconspicuous. In a somewhat later phase, there is also leakage of thrombin and protein amongst others leading to hyaline membranes. There are also vascular changes leading to the formation of microthrombi and changes in vasomotor tone. The last phase is a repair phase in which the alveolar epithelium is regenerated through proliferation of the type II cells and differentiation to type I cells. This enables restoration of the permeability of the basal membranes and allows fluid clearance from the airspaces. The vascular changes also reverse during this stage. The final stage can be complete resolution but very often is characterized by impressive thickening of alveolar septa by fibroblast proliferation without obvious collagen fibrosis with subsequent severe lung function problems and often a poor prognosis. In some cases a fibrotic phase can follow where collagen and other ECM proteins are deposited resulting in a partially stable fibrotic lung. The factors regulating the fibrotic/fibroblastic phase in DAD/ARDS are not known but mechanical ventilation, in particular

with high pressure and high oxygen concentration is thought to be an important pathogenetic component [99].

1.2.6.2 Treatment of ARDS

Current therapeutic management of ARDS consists of the use of supportive therapeutic approaches that are beneficial for all critical care patients as there are no effective ARDS-specific therapies to date. Lung-protective mechanical ventilation is the major intervention for ARDS patients. For a subset of patients there is evidence that fluid management is also beneficial [52]. While the mortality of ARDS has been declining modestly in recent years [100], likely due to improvements in supportive therapies, the overall mortality rates remain unacceptably high and the long-term impact for survivors is also considerable [101].

1.3 Conclusion

It is clear that there is an urgent need, for many chronic lung diseases, to develop better therapeutic approaches. Given the lack of curative treatments and the often-progressive pathology of the lung diseases described in this chapter, particular interest exists in mechanisms aimed at helping with the repair of the lung structure and function. There is emerging excitement about the potential for stem/progenitor cell-based therapeutic approaches, but there is much still to be done to understand the full potential of these approaches for lung patients [102].

Preclinical studies suggest that cell therapy using mesenchymal stromal cells represents a potential new treatment strategy for lung diseases [102–104]. In these models, MSCs displayed the potential to regenerate and restore the architecture of lung tissue, reflected by their ability to repair airway epithelial and endothelial cells. Although it is not clear what the exact mechanism behind this is, it possibly involves the secretion of various growth factors and cytokines. However, as yet, no cell-based therapy has been shown to be both safe and effective for any lung disease in patients so the field waits to see the developments in the next few years.

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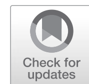
Part I

Stem/Stromal Cells Populations in the Lung



2 Stem/Progenitor Cell Populations Resident in the Lung and the Role of Stromal Support in Their Maintenance and Differentiation

Irene H. Heijink and Nick H. T. ten Hacken

2.1 Introduction

Lungs are vital organs for respiration, being enabled by their complex three-dimensional organization [1]. Airway tubes bifurcate into millions of highly vascularized alveolar sacs, the alveoli, which are responsible for gas exchange. The gas exchange surface of the lungs makes up one of the largest surface areas of the human body. The alveoli receive air from the conducting airways, starting in the trachea, bifurcating into the bronchi and bronchioles, and ending in the terminal bronchioles, which divide into the alveolar ducts from which the alveoli arise. The proximal parts of the airways, the trachea and bronchi, are supported by cartilage, while the distal bronchioles are not.

The different cell types of which the human lungs are composed of are the epithelium, endothelium, pleural mesothelium, airway and vascular smooth muscle, pericytes, fibroblasts, neurons, and immune cells including alveolar macrophages [2]. These cell types can often be classified further based on their position along the respiratory tree. Lung epithelial cells can be subdivided into airway tracheal and bronchial (airway) types and alveolar types. Gas exchange is facilitated by the close interaction between capillaries and alveolar type 1 (AT1) pneumocytes, flat-shaped epithelial cells that accommodate the transfer of oxygen into the blood stream. The AT1 cells line the alveolar surface together with AT2 cells. Cuboidal-shaped AT2 cells serve as progenitor cells for AT1 cells, contributing to the regeneration of alveolar tissue upon injury by re-epithelialization. The trachea and bronchi are lined with columnar pseudostratified epithelial cells, including basal, secretory, ciliated, and neuroendocrine cells as well as submucosal glands [3]. Throughout the airways, the tracheobronchial epithelial layer is separated from the underlying mesenchyme, blood and lymphatic vessels, nerves, and cartilage by the basement membrane [2]. The transitional region between

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terminal bronchioles and alveoli is referred to as the bronchioalveolar duct junction [4].

In addition to gas exchange, the lungs have a key role in host defense, forming a selective barrier against inhaled pathogens and toxicants. Both the airway and alveolar epithelium exert critical functions in this host defense. The airway epithelium forms a physical barrier by formation of tight junctions. Additionally, it provides a chemical barrier, producing antioxidants, antiproteases, and mucus, in which particles can be trapped and subsequently removed by ciliary beating. Moreover, the airway epithelium is part of the innate immune defense, expressing pattern recognition receptors that recognize molecular patterns on pathogenic microbes, parasites, fungi, and allergens in order to initiate proinflammatory cascades. In the alveoli, epithelial cells produce surfactants, which bind to glycomolecules on microbes to induce opsonization and clearing of pathogens.

The lung is a complex organ developing from a bud of the foregut during embryogenesis and containing a large variety of cell lineages of endodermal, mesoderm, and ectodermal origin [5, 6]. Many of the epithelial cells in the adult lung arise from a few progenitor cells in the embryonic foregut endoderm [7]. In the adult stage, the lung is a conditionally renewing organ. During homeostasis, the turnover of epithelial cells in the lung is relatively low compared to other continuously renewing organs, such as the gut and bone marrow. Airway epithelial cell turnover is approximately 1% per day, with complete replacement of the proximal airway epithelium taking more than 4 months [4]. Nevertheless, the lung has a remarkable ability to regenerate and replace epithelial cells upon injury by microbial infections, inhaled toxicants, and other harmful substances [6]. In an example case-report of the remarkable ability of the lung to regenerate, a woman with a right-sided pneumonectomy showed an increase in vital capacity over the course of a 15-year follow-up. The authors of the case reported a 64% increase in alveoli, as determined using serial CT scans and magnetic resonance imaging [8]. Neoalveolarization and lung regeneration require various types of stem cells as well as stromal support, although involvement of stem cells was not directly shown in this case report.

Stem cells are defined as uncommitted cells that during adulthood are predominantly present in the bone marrow. They are characterized by the ability to differentiate into other cell types and to sustain themselves (self-renewal). Stem cells can be more or less specialized in their lineage and are classified based on ectodermal, mesodermal, or endodermal germ layer origin [9]. This ranges from totipotent, being able to differentiate into all cell types in the embryonic stage, to pluripotent, multipotent, and oligopotent, being restricted to differentiate into only one or two cell types. Cells that are able to differentiate into cell types responsible for the function of the tissue are referred to as progenitor cells. These progenitors include basal, secretory, and club cells in the proximal airway and AT2 cells in the distal lungs [7], as identified by “lineage-tracing” experiments, where the offspring of repairing cells, i.e., the cells that are able to enter the cell cycle upon injury, is tracked.

The aim of this chapter is to provide an overview of the progenitors in adult lung tissue and the regulation of their maintenance and differentiation by the microenvironment during lung developmental as well as repair processes, when developmental pathways are often reactivated. As most work has been done in mouse studies, the current knowledge from animal studies will be summarized and translated to what is known from human lungs. In order to understand the regenerative processes in the lung, we will first provide insight into the complex three-dimensional organization and composition of the lung, its function, and the processes involved in lung development.

2.2 Overview of the Human Respiratory System

2.2.1 Anatomy and Cellular Composition of the Lung

The respiratory system is divided in two parts, the upper and lower respiratory tracts, which are separated by the pharynx. The upper part comprises the nose, paranasal sinuses, and the nasopharynx. The lower respiratory tract starts at the larynx

and then continues into the thorax as the trachea, before dividing into numerous orders of smaller airways, the bronchi and bronchioles, and ending in the terminal bronchioles, to reach the alveoli (Fig. 2.1). The airway divides about 23 times, terminating in approximately 30,000 pulmonary acini, each containing more than 10,000 alveoli. In the normal adult, the lungs weigh approximately 1 kg, have a total volume of 6 L, and a height of 24 cm in the range of normal breathing. The right lung is divided by a major and minor fissure into tree lobes: the upper, middle, and lower lobe; the left lung has one major fissure dividing it into an upper and lower lobe. Figure 2.2 demonstrates the 5 lobes and 20 segments of the lung. The airways are composed of several layers of tissue, going from the inside to the outside: respiratory epithelium, lamina propria, smooth muscle layer, submucosal connective tissue, cartilage, and adventitia (Fig. 2.3), including various different cell types: the epithelium, airway and vascular smooth muscle, pericytes, fibroblasts, endothelium, neurons, and immune cells such as alveolar macrophages. Going from proximally to distally, several changes can be observed [11, 12]: the epithelial layer becomes thinner and undergoes progressive transition from a tall, pseudostratified columnar, ciliated form to a simpler, cuboidal, non-ciliated form. Goblet cells are numerous proximal, but decrease in number and are absent in the terminal bronchioles, which is restricted to the other secretory cell type, the club cells. Also, the serous and mucous glands become progressively less numerous in the narrower airways and are not present in the bronchioles and terminal bronchioles. The smooth muscle layer transits from bands or a spiral network to a continuous layer. As the airway size decreases more

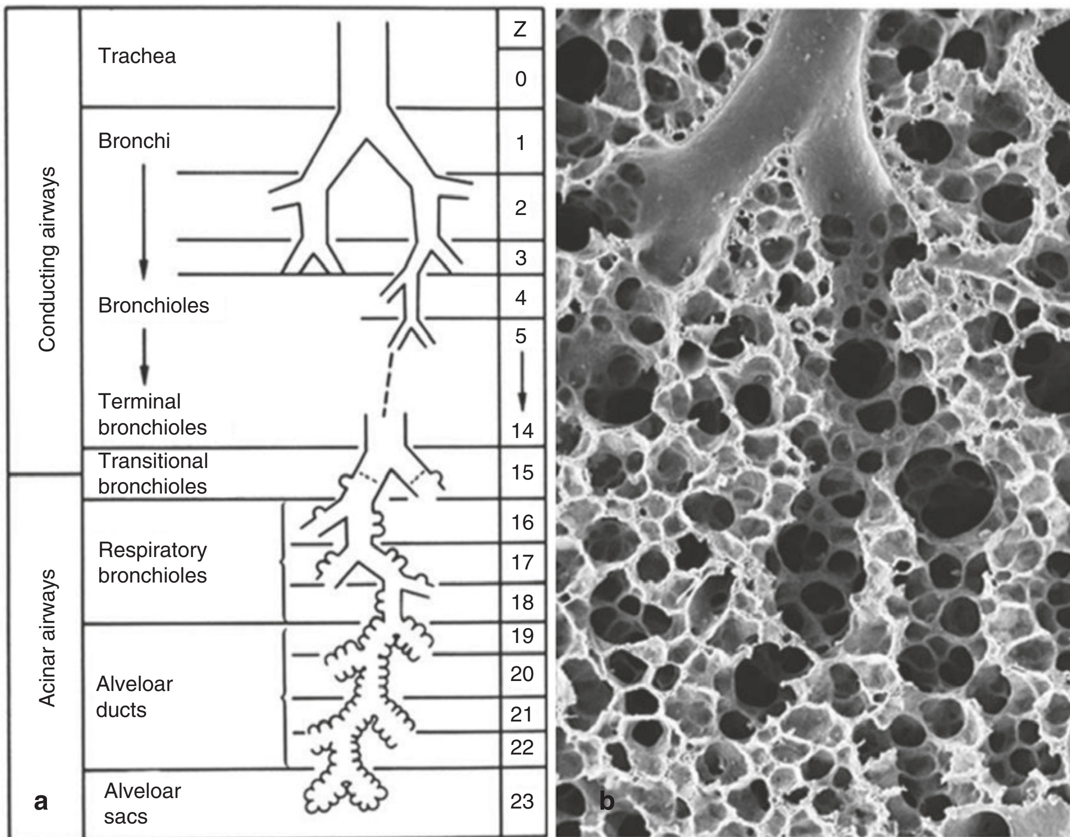

Fig. 2.1 (**a**) Hierarchy of airways, with surface complexity occurring in the last eight generations by formation of alveoli on the acinar ducts (**b**) [10]. (Reprinted with permission of the American Thoracic Society. Copyright © 2019 American Thoracic Society. Weibel, E.R. 2013. It takes more than cells to make a good lung. AJRCCM; vol 187, pp. 342–346)

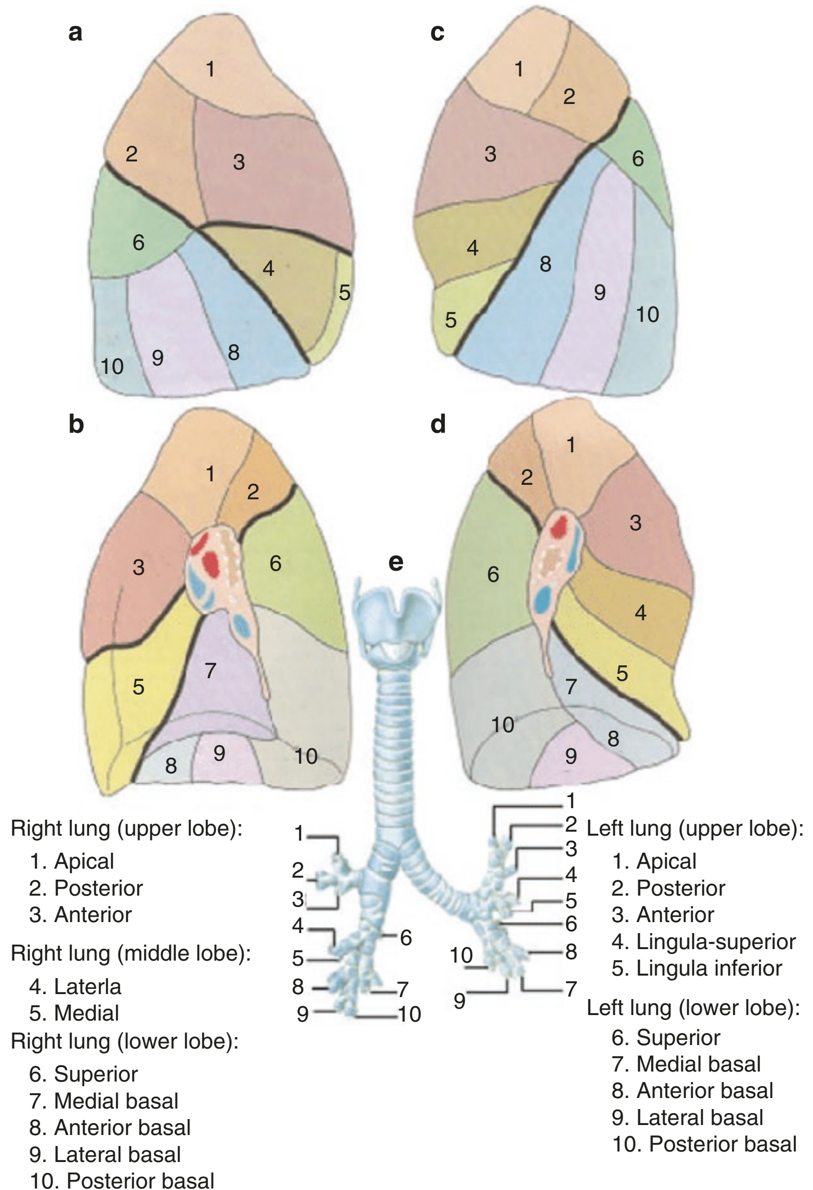

Fig. 2.2 Airways, lobes, and segments of the lung. (**a**) right lung (lateral view), (**b**) right lung (medial view), (**c**) left lung (lateral view), (**d**) left lung (medial view), (**e**) segmental or tertiary bronchi. Each tertiary bronchus is numbered with respect to its position in the matching numbered bronchopulmonary segment. The medial basal segment of the lung is mostly not present. The lingula on the left side is the counterpart of the right middle lobe. Figure Design and fabrication of pulmonary embolism phantom for planar and SPECT V/Q imaging quality assurance—Scientific Figure on ResearchGate. (Available from: https://www.researchgate.net/figure/Bronchopulmonary-segments-a-Right-lung-lateral-view-b-Right-lung-medial-view-c_fig1_45276172)

distally, the contribution of smooth muscle to the airway wall thickness increases and is maximal at the level of terminal bronchioles. Proximally, the cartilage prevents the trachea and bronchi from collapsing during ventilation, diminishing more distally. As mentioned in the introduction of this chapter, the presence of local stem cells and progenitors is responsible for the regeneration and/or repair of the airway epithelium [7, 13]. For example, in the large airways the basal cells differentiate into and replenish the more superficial and differentiated ciliated and goblet cells in the mucosa, as described in the following sections.

The airways end in the alveolar compartment, or pulmonary parenchyma. This region includes the alveolar walls and spaces at the level of the alveolar sacs, ducts, and respiratory bronchioles [11]. At this level, gas-exchange takes place, the most important function of the lung. An extensive capillary network courses through and comes into close contact with alveolar gas. Figure 2.4 shows the alveolar wall barrier, where AT1 pneumocytes

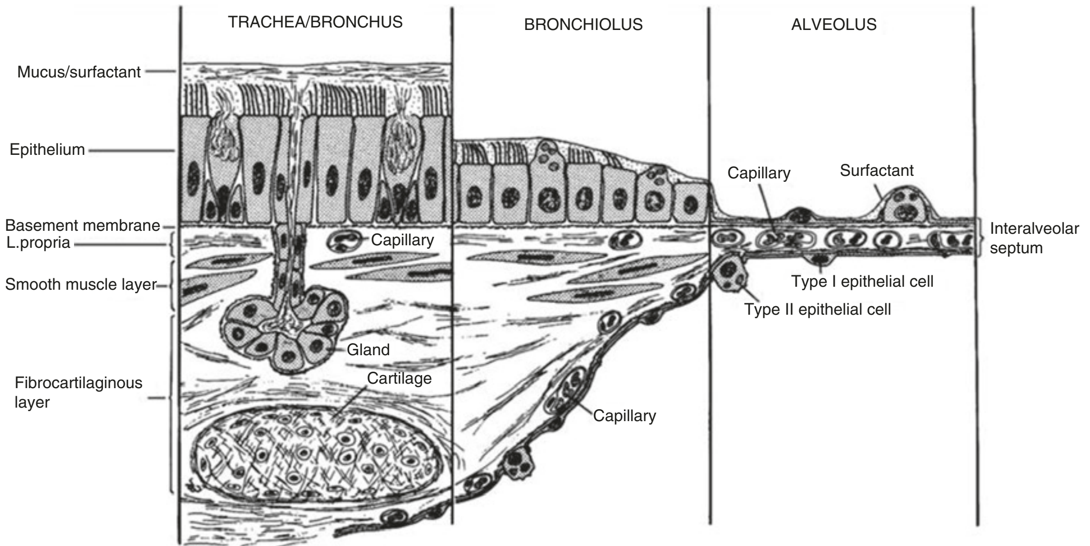

Fig. 2.3 Airway wall composition schematic diagram of the composition of the airway wall starting proximally (trachea) and ending distally (alveolus). (From: Michael A Grippi, Jack A Elias, Jay A Fishman, Robert M Kotloff, Allan I Pack, Robert M Senior, Mark D Siegel: Fishman's Pulmonary Diseases and Disorders, fifth edition. www.accessmedicine.com. Copyright 2015 McGraw-Hill Education)

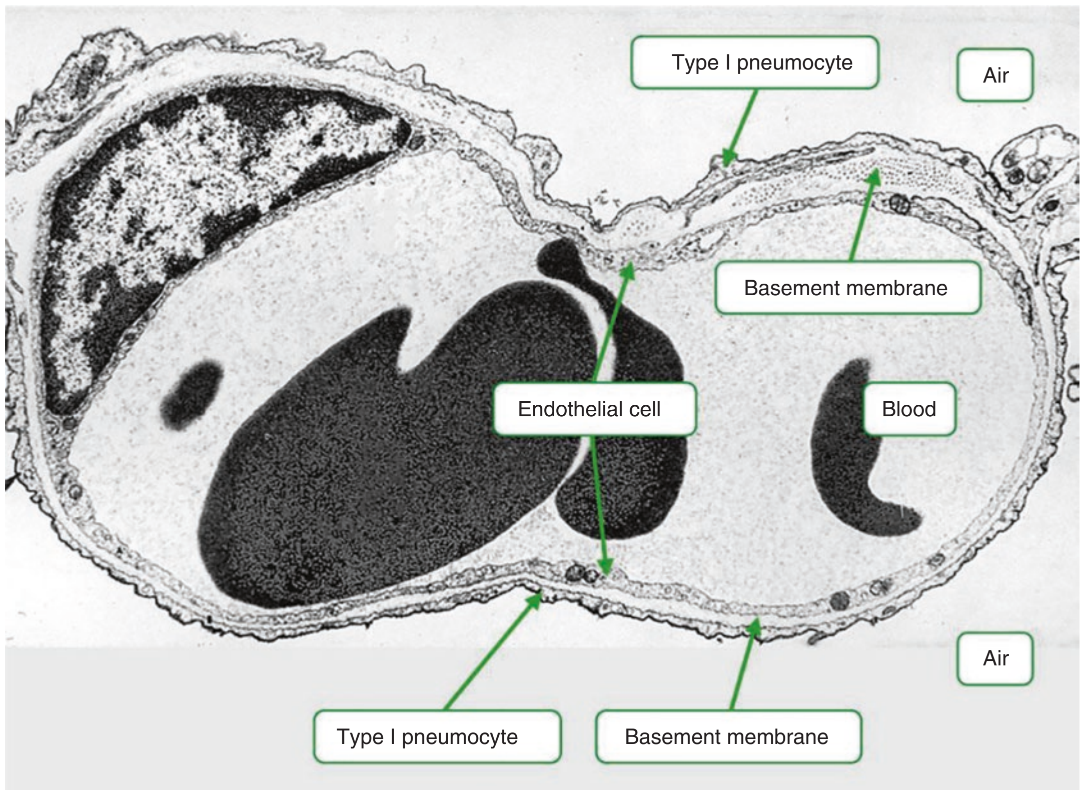

Fig. 2.4 Structures that compose the air–blood barrier. (Available from: http://histology.med.yale.edu/respiratory_system/respiratory_system_reading.php)

are relatively flat and AT2 pneumocytes are cuboidal. AT1 cells have long, ultrathin extensions that line more than 95% of the alveolar surface, their primary function being facilitating gas exchange. In addition, AT1 cells form a physical barrier. AT2 cells, in their turn, produce surfactant that contains a high amount of lipids and contributes to the stability of the alveoli, maintaining structural integrity and reducing surface tension in the alveoli. Additionally, AT2 cells are involved in regenerative processes; they may proliferate and display hyperplasia upon injury, and act as progenitors of AT1 cells.

2.2.2 Physiology and Pathophysiology

To maintain normal gas exchange to the tissues, an adequate volume of air must pass through the lungs for provision of oxygen (O_2) and removal of carbon dioxide (CO_2) [11]. At rest, a normal, healthy person breathes approximately 500 mL of air per breath at a frequency of 12 to 16 times per minute, resulting in a ventilation (VE) of 6–8 L/min, a CO_2 flow (VCO_2) of 0.2 L/min outwards, and an O_2 flow (VO_2) of 0.25 L/min inwards. The counterpart of the ventilation is the pulmonary circulation, which depends on the cardiac output and has a blood flow of 5 L/min at rest. For adequate gas exchange it is important that local ventilation meets local perfusion in a 1:1 (V/P) ratio. In case of locally poor perfusion, part of the ventilation is not used, which is called “wasted” or “dead space” ventilation. This wasted ventilation reduces the efficiency of local CO_2 excretion and has to be compensated by other parts of the lung that have normal V/P ratios. In case of locally poor ventilation (low V/P ratio) there is “wasted perfusion” or shunting that contributes to lower oxygenation of the blood. Importantly, within one lung many areas with different V/P ratios may exist together.

The most important pathophysiological mechanisms that lead to hypoxemia are:

- Low oxygen tension or oxygen fraction of ambient air (e.g., living at high altitude).
- Hypoventilation with hypercapnia (e.g., due to respiratory depressing agents).
- Low ventilation/perfusion ratio’s (e.g., due to asthmatic bronchospasm).
- Shunting (zero ventilation due to, e.g., complete lung collapse).
- Reduced diffusion across the alveolo-capillary membrane (due to a thickened membrane, e.g., in a number of interstitial lung diseases).
- In pulmonary medicine, a number of lung function tests may help to detect and monitor disease. Diseases can even be categorized according to the test that is needed to detect them:
- Obstructive lung diseases, e.g., asthma, chronic obstructive pulmonary disease (COPD), comprising both chronic bronchitis and emphysema, asthma COPD overlap syndrome (ACOS), cystic fibrosis, bronchopulmonary dysplasia (BDP).
- Restrictive lung diseases, e.g., interstitial lung diseases, neuromuscular diseases.
- Vascular lung diseases, e.g., lung embolism, primary pulmonary hypertension.

In obstructive lung diseases the patency of the airway lumen is chronically or intermittently reduced by (a combination of) luminal mucus (chronic bronchitis), thickened airway wall (chronic bronchitis, asthma), smooth muscle contraction (asthma, chronic bronchitis), or reduced elastic recoil forces (emphysema). Emphysema is characterized not only by airway obstruction but also by loss of alveolar tissue as well as the corresponding lung capillaries; in that perspective it is also an example of a vascular disease. The gold test to demonstrate airway obstruction is by performing a forced expiration maneuver in a spirometer, showing a reduced forced expiratory volume in 1 s (FEV1).

In restrictive lung diseases the lung can be stiff, i.e., noncompliant to be enlarged during breathing, and the capacity to contain air is reduced. In addition, the capacity to contain air may also be reduced by nonpulmonary causes, including a rigid thoracic cage or a neuromuscular disease. In restrictive parenchymal lung diseases, the patency of the airway lumen is frequently increased, leading to exceptionally high FEV1/FVC ratios. The gold test to demonstrate restriction is by performing a bodybox

to assess whether total lung capacity (TLC) is reduced. Additionally, the alveolo-capillary membrane is frequently thickened in combination with a reduced alveolo-capillary surface area, leading to a reduced diffusion capacity for O_2. This limitation is assessed by the so-called CO-diffusion test.

In pure vascular lung diseases, the lung mechanics may be completely normal, showing no signs of obstruction (airway disease) or restriction (parenchymal disease). A lung function test to detect vascular disease is the CO-diffusion test, because for the uptake of CO (and O_2), perfusion of the lung is needed. Another frequently used test is echocardiography, which indirectly measures pulmonary hypertension due to pulmonary vascular disease.

In order to understand how disturbed lung mechanics in lung disease can be restored and how lung tissue damage can be repaired, it is crucial to understand the repair mechanisms of the lung and the lung developmental processes that are reactivated during lung repair.

2.2.3 Lung Development

The lung is a complex organ developing from a bud of the foregut during the fifth week of gestation, containing a high variety of cell lineages of endodermal, mesodermal, and ectodermal origin [5, 6]. Cell layers originating from these three germ layers come together in a parallel and serially linked network of tubes, strictly dictated by the genetic blueprint of the present cells [9]. During this complex developmental process, well-balanced signaling of several growth factors is essential, including Wingless/Intergase-1 (WNT) ligands, fibroblast growth factors (FGFs), keratinocyte growth factor (KGF), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), retinoic acid (RA), transforming growth factor (TGF)-β, sonic hedgehog ligands, and Notch ligands [14]. The development of the lung can be arbitrarily divided into five stages (Table 2.1), where these overlapping signaling cascades play a role. The primary lung buds arise from the ventral wall of the anterior foregut (endoderm). Afterwards, the proximal conducting airways start to form by extensive branching, followed by distal septation to generate the gas exchange units (alveoli) of the mature lung [15]. These processes are accompanied by coordinated differentiation of the airway and distal lung epithelium, leading to a regionally specific pattern of cell types. Formation of a functional lung also requires the simultaneous development of both the pulmonary vascular system and bronchial vascular system. Each of these steps in lung development relies on inductive cues and mutual interactions between the respiratory epithelium and the surrounding mesenchyme [15]. For respiratory lineage specification, the surrounding ventral mesenchyme secretes critical factors including FGFs, WNT ligands, BMPs, RA, and TGF-β [15]. For branching morphogenesis, a cardinal mesenchymal signal that drives epithe-

Table 2.1 Stages of lung development

Stage	Time	Growth characteristics	Important genes/growth factors
Embryonic	4–7 weeks	• Beginning organogenesis • Development of major airways	Nkx2–1, BMPs, FGFs, WNT ligands, retinoic acid, VEGF
Pseudoglandular	5–17 weeks	• Bronchial tree formation • Beginning of parenchyma differentiation	FGFs, WNT ligands, PDGF, BMP
Canalicular	16–26 weeks	• Conducting airways • Epithelial differentiation	WNT ligands, FGFs, TGF-β
Saccular	24 weeks term	• Air space expansion • Beginning alveolarization	VEGF, PDGF
Alveolar	First 18 months	• Remodeling with continued septation and alveolarization • Maturation of capillary bed	PDGF, VEGF

Modified from Chap. 1 in Clinical and Respiratory Medicine 4th edition. *BMP* bone morphogenetic protein, *FGFs* fibroblast growth factor, *TGF-β* transforming growth factor-β, *VEGF* vascular endothelial growth factor

lial branching is FGF10 [15]. Differentiation of the conductive airway epithelium also critically depends on signals from the neighboring mesenchyme, although the inductive cues are not fully clear [15]. Next to its role as a source of signals, the mesenchyme itself receives cellular contributions from the cardiac mesoderm before differentiating into multiple lineages [15]. The processes of lung vascular development are poorly understood, but endothelial cells from vascular precursors have been suggested to play a role in distal lung vasculogenesis, where mesenchymal factors like Sox17 contribute to the formation of a normal microvascular network [15]. In the phase of alveolar maturation, thin walled septa are formed covered with AT1 and AT2 pneumocytes. The mesenchyme contains key cells that drive this transition, where signaling pathways including TGF-β, PDGF, and WNT signaling play an orchestrating role. In the later embryonic phases, other factors that influence lung development are fetal respiration, pulmonary and intrauterine fluid, hormonal and paracrine factors, nutritional factors and postnatal disease.

2.3 Overview of Progenitor Cell Populations of the Lungs Characterized in Animal Studies

2.3.1 Progenitors in the Airways

The same signaling cascades that are active during lung development also play a crucial role during various repair processes in adulthood [14]. Many of the currently known epithelial progenitors in the adult lung have been identified in mouse models, and it is of importance to take into consideration that there are differences between human and mouse epithelium in the lungs. These differences will be highlighted later in this chapter, where we will discuss the translation of knowledge of progenitor cell populations in the lung to human.

In the murine lungs, the basement membrane separates the tracheobronchial epithelial layer from the underlying mesenchyme, blood and lymphatic vessels, nerves, and cartilage throughout the conducting airways [2]. The trachea and bronchi are lined by columnar pseudostratified epithelial cells. In the trachea and proximal bronchi, these include basal, secretory, ciliated, and neuroendocrine cells as well as submucosal glands, while the distal part of the airway is lined with columnar epithelial cells, including ciliated cells, goblet cells, club cells, and neuroendocrine cells [3]. Of note, the murine respiratory tree differs considerably from human, as discussed later in this chapter, and the murine distal intralobar airways and bronchioles do not contain basal cells.

Throughout the murine airways, basal epithelial cells represent the progenitor/stem cells of the bronchiolar epithelium that are able to self-renew and differentiate into secretory and ciliated cells, both during homeostasis and during repair responses upon injury. Whereas ciliated and goblet cells are involved in different effector functions of the airway epithelium, including mucociliary clearance, the basal and non-ciliated secretory club cells display stem/progenitor cell properties and are thought to be crucial for effective wound repair. These basal progenitor cells are characterized by the expression of nerve growth factor receptor (NGFR)/CD271, transformation-related protein p63 (trp63), and cytokeratins (CK)5 and CK14 [16, 17]. Basal murine tracheal epithelial cells positive for these markers have been shown capable of self-renewal and give rise to ciliated and goblet cells in three-dimensional culture systems [18]. In vivo lineage-tracing experiments of *Ck14*-expressing basal cells in the mouse trachea have been performed, following the fate of these epithelial cells upon epithelial injury. These studies have shown that basal cells are able to self-renew and generate club and ciliated cells [19]. In turn, basal cells from submucosal gland ducts that express the marker Trop2 (trophoblast cell surface antigen 2) and integrin (Itg) α6 have been shown able to give rise to CK5 and CK14 positive cells basal in vitro [20]. Recent lineage tracing studies have shown that submucosal gland duct myoepithelial cells can give rise to several epithelial cell types and regenerate the tracheal epithelium in mouse models following severe

airway injury using naphthalene [21]. Another in vivo lineage-tracing study demonstrated that club cells, characterized by secretoglobin, family 1A member 1 (*Scgb1a1*) expression, themselves also constitute a source of airway progenitor cells, generating basal cells after epithelial injury [22]. A small subset of these club cells, referred to as variant club cells, has been shown resistant to treatment with the toxic compound naphthalene, a type of injury that depletes the majority of club cells [7]. Characteristic of stem cells, variant club cells can thus repopulate the damaged airway tissue. Variant club cells reside in two niches, adjacent to neuroepithelial bodies and at bronchoalveolar duct junctions [7]. The bronchoalveolar duct junction has been shown to harbor a specific population of club cells that is responsible for the repair of terminal bronchioles and the maintenance of epithelial diversity upon naphthalene-induced damage [23]. Furthermore, a specific subset of club cells that starts to proliferate, self-renew, and give rise to differentiated bronchiolar cell types after lung injury has been identified and referred to as bronchioalveolar stem cells (BASCs). These cells have been reported as progenitors of both distal airways and the alveolar bed. In vivo studies demonstrated that their numbers increase after naphthalene-induced injury [24]. Postnatally, BASCs are characterized by the co-expression of *Scgbl1a1* and *Sftpc*, the gene encoding surfactant protein C (SPC) [22]. While showing that these cells are involved in the maintenance and repair of airway epithelium, Rawlins et al. were not able to confirm that these BASCs maintain and repair alveolar epithelium [22]. Rather, they proposed that the trachea, bronchioles, and alveoli are maintained by distinct populations of epithelial progenitor cells [22]. Nevertheless, the role of BASCs cells in tissue regeneration has been supported by in vitro studies showing that these cells are able to self-renew and differentiate into both bronchiolar (club cells) and alveolar colonies [25]. Others documented that BASCs are positive for the stem cell marker Sca-1, epithelial marker EpCAM (CD326) as well as for CD49f, laminin receptor Itg α6β4, CK5, and p63, while being negative for SPC as well as hematopoietic (CD34, CD45) and endothelial cell markers (CD31) [26]. In addition to BASCs, basal cells are positive for the stem cell markers Sca-1 and aldehyde dehydrogenase (ALDH)1 [27] and a rare cell population of neuroendocrine cells [28] have been characterized as region-specific stem/progenitor cells, contributing to airway epithelial maintenance and repair upon injury. Whereas Sca-1/ALDH1 positive cells were found in the trachea, neuroendocrine cells form clusters in the lower mouse airways, known as neuroendocrine bodies (NEBs), located at the same spatial domain as club cells. As mentioned above, together with the bronchoalveolar duct junction, NEBs have also been proposed as niche of variant club cells that function as bronchiolar progenitor cell [19, 26, 28]. Furthermore, neuroendocrine cells have been found that share their calcitonin gene-related peptide-positive (CGRP positive) neuroendocrine cell lineage origin with alveolar cells in the distal lung, both being able to renew and generate club and ciliated cells in the murine lung following acute injury induced by naphthalene [29].

Together, various subsets of basal epithelial cells within the proximal and distal airways have been identified as potential stem and progenitor cells, playing a role in epithelial regeneration upon damage, as summarized in Fig. 2.5. The specific progenitor cell subset that is recruited and activated upon injury is likely context-dependent, e.g., on the type of injury, the severity, and the specific location [6]. Indeed, results from different mouse models of lung injury have confirmed that different local epithelial stem and progenitor cell subsets are recruited to regenerate specific airway epithelial cell lineages during homeostasis and upon tissue damage, as reviewed by Bertoncello and McQualter [6].

2.3.2 Alveolar Progenitors

In the alveoli, epithelial cells are in close contact with capillaries, whereby oxygen can be transferred from the inhaled air to the circulation. Additionally, they are surrounded by stroma, supporting the maintenance of epithelial homeostasis as well as their regenerative responses. The

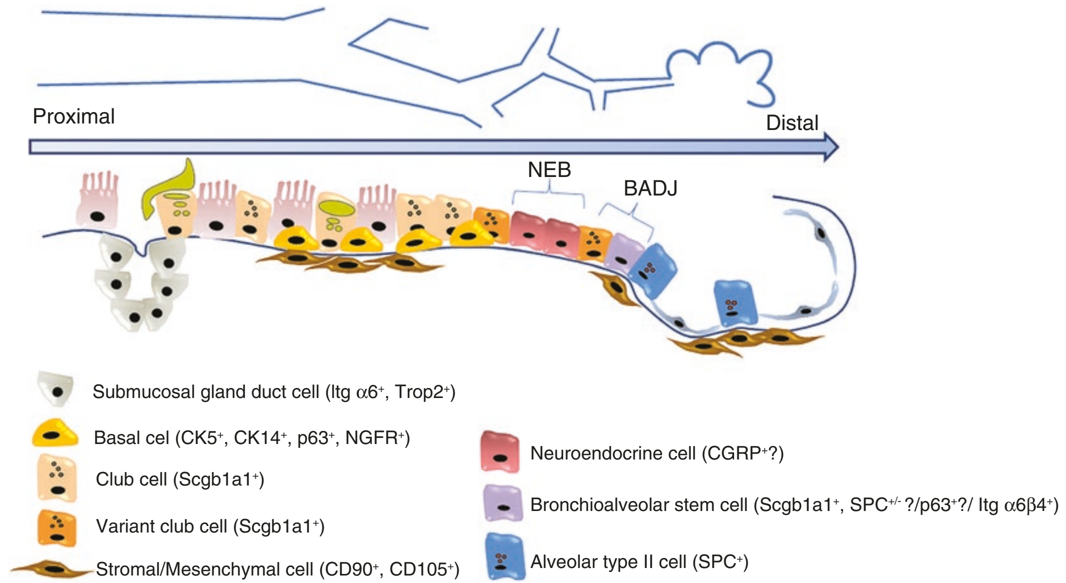

Fig. 2.5 Lung epithelial stem and progenitor cell candidates. Schematic of proposed lung epithelial candidate stem or progenitor cells and their niches in the proximal conducting airways and distal alveoli. Cells whose localization or existence in the human lung is not yet clear or accepted are indicated with question marks. *BADJ* bronchoalveolar duct junction, *NEB* neuroepithelial body. Marker abbreviations used for each cell subtype include the following: *CGRP* calcitonin gene-related peptide, *CK* cytokeratin, *Itg* integrin, *Scgb1a1* secretoglobin, family 1A member 1, *SPC* surfactant protein C

stromal niche consists of extracellular matrix, soluble factors, and mesenchymal cells [3, 26], including fibroblasts, which can be mainly subdivided into myofibroblasts and lipofibroblasts [2].

The flat-shaped AT1 cells, specialized in exchange of oxygen and characterized by the expression of podoplanin (pdpn/T1α), aquaporin 5 (AQP5), etc., make up for more than 95% of the gas exchange area [2]. AT2 cells contain lamellar bodies that produce and recycle surfactants, which are involved in host defense and responsible for the maintenance of structural integrity and reduction of surface tension in the alveoli. In addition, AT2 are capable of self-renewal and of differentiation into ATI cells [30], acting as major alveolar progenitor cells in the alveoli [21]. In contrast, AT1 cells have limited proliferative capacity upon lung damage [31], although AT1 cells expressing the AT1 marker Hopx have been shown to display plasticity in the adult lung [32]. Lineage-labeled *Hopx* positive cells were found to proliferate and generate AT2 cells following pneumonectomy [32].

As mentioned earlier, BASCs cells have been identified as progenitors of both AT2 and AT1 cells [24]. Furthermore, lineage-tracing studies have provided evidence that alveolar epithelial cells can derive from Scgb1a1 positive club cells following bleomycin-induced lung injury [33] and from p63/CK5/CK14 positive basal airway cells following influenza virus infection [34]. In addition, a resident niche of epithelial progenitor cells expressing Itg α6β4 has been identified to have regenerative potential within the alveolar epithelium [35]. This population, that hardly expressed sftpc, displayed self-renewal capacity and differentiated toward mature alveolar cell types ex vivo [35]. In the bleomycin model of lung injury, the same authors used epithelial fate-mapping experiments to trace the alveolar epithelial lineage, showing that the majority of AT2 cells in fibrotic areas were progenitor cells negative for the AT2 marker sftpc. Thus, it was concluded that a stable alveolar epithelial progenitor population, potentially the α6β4 integrin (itg) positive cells, may be responsible for the

maintenance and differentiation into AT2 cells after parenchymal injury in the adult lung [35].

Additionally, a subset of alveolar cells comprising approximately 20% of adult AT2 cells has been documented to represent an alveolar epithelial progenitor lineage [36]. This subset expressed the gene *Axin2*, a direct target of the developmental WNT signaling pathway. Activation of Axin2 has been shown to be crucial for differentiation into both AT2 and AT1 cells. In the murine lung, *Axin2* expressing WNT-responsive epithelial cells, acting as major facultative progenitor cells in the distal lung, were reported to be restricted to the alveolar region and to co-express *Sftpc*, while only few of these cells also express AT1 markers such as Hopx [36]. These AT2 progenitor cells are thought to be stable during homeostasis, but were shown to rapidly start expanding and regenerating the alveolar epithelium in response to lung injury by influenza virus [36]. This was supported by in vitro studies showing their potential to grow in organoid models, for which progenitor cells require the ability to differentiate and self-organize into three-dimensional structures [36]. In this model, AT2 progenitors specifically expressed the conserved cell surface marker Transmembrane 4 L six family member 1 (TM4SF1), a protein previously identified on epithelial tumor cells, and may thus constitute an evolutionarily conserved alveolar progenitor. Similar studies were performed by Nabhan et al. who also lineage-labeled *Axin2* positive AT2 cells in mice [37]. Here, only 1% of the AT2 cells was found to express Axin2 during homeostasis. The authors proposed that the required WNT signal is provided by a single PDGF receptor A (PDGFRA) expressing fibroblast near each of the AT2 stem cells, in order to maintain the stemness and control the fate of the daughter cell. Once daughter cells leave this WNT niche, they differentiate into AT1 cells. The authors showed that upon severe injury by diphtheria toxin, autocrine WNT signaling is activated in the bulk of AT2 cells, recruiting them as progenitor cells. Despite the differences observed in these two studies, both confirm an important role for WNT signaling in regulating the regenerative properties of AT2 cells [37].

Collectively, to date AT2 cells constitute the most important source of progenitors in the alveoli. Support from the mesenchyme through specific developmental signaling pathways such as WNT signaling appears to be crucial for alveolar repair. Other progenitor populations, including epithelial cells expressing Itg α6β4, may have regenerative potential in the lung parenchyma as well. Alveolar progenitors are summarized in Fig. 2.5.

2.4 Overview of Mesenchymal Stem Cell Populations and Function in the Lung Characterized in Animal Studies

2.4.1 Mesenchymal Populations in Lung Development and Repair

Niches for resident mesenchymal stem cells that replenish stromal cell populations, including lipofibroblasts, myofibroblasts, and smooth muscle cells, have been identified in many adult tissues and more recently in the lung. Mesenchymal progenitors that have clonogenic potential and can differentiate into multiple mesenchymal lineages in vitro are often referred to as mesenchymal stem cells, or nowadays, mesenchymal stromal cells (MSCs) [38]. Their in vivo counterparts can be referred to as tissue-resident mesenchymal progenitor cells, to distinguish them from the more differentiated smooth muscle [38]. During embryonic development, cells that express FGF10 in the mesenchyme have been documented to be progenitors of mesenchymal cell lineages [39]. During adult lung homeostasis, a subpopulation of *Fgf10*-expressing cells was shown to be present in mesenchymal niches, representing a pool of resident MSCs that were positive for the stem cell marker Sca-1 [40, 41]. Resident mesenchymal progenitor cells of the mouse lung were also identified as a side population of cells negative for the hematopoietic marker CD45, positive for Sca-1 as well as various mesenchymal markers and being capable of differentiation into multiple mesenchymal lineages [42].

Mesenchymal cells constitute a niche of airway epithelial stem cells and increasing numbers of studies in mice have shown that communication between the epithelium and underlying stromal cells is crucial for alveologenesis, normal homeostatic maintenance, and repair responses upon lung injury [43–45]. Therefore, we will also discuss the role of mesenchymal cells in lung epithelial homeostasis, maintenance of epithelial barrier and epithelial regeneration. In the alveolar beds, epithelial cells are mainly surrounded by fibroblasts of the lipofibroblast and myofibroblast type [2]. Although the exact function of the different types of fibroblasts is still unclear [2], myofibroblasts are contractile, generate elastin, and regulate airflow in the alveolar ducts, while lipofibroblasts store lipids [46] and transfer triglycerides to adjacent AT2 cells for surfactant production, protect the lung against oxidative stress [47], and are a major source of lung-specific developmental growth factor FGF10. The different mesenchymal cell types that have been identified in murine studies to coordinate the proliferation and differentiation of alveolar progenitors are PDGFRA positive and secrete a variety of paracrine growth factors [30]. Various growth factors have been implicated in mesenchymal-epithelial crosstalk during developmental and repair processes, including FGFs, such as FGF10 and/or FGF18, WNT ligands, TGF-β, BMPs, and hepatocyte growth factor (HGF) [14, 48–50]. Notably, FGF10 has been described as a cardinal factor for directing differentiation in the developing lung [51]. During alveologenesis, FGF10 acts both in an autocrine and a paracrine fashion to drive differentiation of early lung mesenchymal progenitors into multiple lineages, including lipofibroblasts and smooth muscle cells [39]. Lineage-tracing of *Fgf10*-positive cells in a mouse model has demonstrated that these cells contribute to the formation of parabronchial and vascular smooth muscle and at later developmental stages of alveologenesis also to lipofibroblasts, but not myofibroblasts [41]. A mesenchymal subpopulation of leucine-rich repeat-containing G protein-coupled receptor (lgr)6 positive smooth muscle cells surrounding airway epithelia has been shown to promote airway differentiation of epithelial progenitors via WNT-FGF10 cooperation. Genetic depletion of *Lgr6* positive cells impaired airway epithelial repair upon diphtheria toxin injury in vivo [45].

Mesenchymal progenitor cells, e.g., fibroblasts and MSCs, have proven difficult to distinguish on the basis of their secretory profile or surface molecule expression in vitro, yet mainly MSCs have been implicated as key elements in the regeneration of multiple organs [52]. MSCs have been proposed to constitute a key element of epithelial progenitor niches along the respiratory tree [26]. Moreover, MSCs are the most widely described stem cell type in regenerative medicine, having promising effects in cell-based regenerative strategies in mouse models of lung disease [9], due to their paracrine function in combination with immunosuppressive effects. Because of these implications, we will focus on MSCs in this chapter.

2.4.2 Mesenchymal Stromal/Stem Cells and Lung Repair

MSCs are multipotent stem cells with self-renewal ability, originating from the mesoderm and residing in various tissues, including bone marrow, adipose tissue, muscle, and lung [52]. Although different types of tissue-resident MSCs have been described, it is not clear yet whether these cells are specific for regeneration of only the tissue from which they originate, or whether their heterogeneity allows them to differentiate into various types of cells [9]. Differences between lung tissue MSCs and those from other locations will be extensively described in Chap. 3. The exact location of lung-resident MSCs within their own lung tissue niche in vivo is not fully clear, because of the lack of specific markers allowing their precise recognition [9]. According to the international criteria to identify MSCs (International Society for Cellular Therapy MSC criteria) [53], lung-resident MSCs express various nonspecific, non-hematopoietic mesenchymal surface markers, including CD29 (Itg β1), CD44, CD73, CD90 (Thy-1), CD105, and MHC class I antigens, and have the ability to self-renew

and differentiate towards adipocytes, chondrocytes, osteoblasts, and smooth muscle cells, of which only the latter type is of relevance in the lung. Whether MSCs are able to differentiate into alveolar epithelial cells is still under debate. Although various studies have provided evidence for the expression of alveolar markers when MSCs of different tissue origins were subjected to alveolar differentiation protocols [54–56], it remains controversial whether MSCs are able to differentiate into functional alveolar epithelial cells in vivo. However, as mentioned above, the actual contribution to tissue renewal by replacement of differentiated cell types is not regarded as the most important function of MSCs, which is the support of site-specific epithelial as well endothelial regenerative responses through paracrine effects. Furthermore, in vitro studies have shown that specifically MSCs from lung origin are able to organize into alveolar-like networks, producing elastin to repair and regenerate elastic fibers and producing growth factors that promote neo-alveolarization and vascularization [57]. Thereby, they provide a structural ECM network that may enables alveolar regeneration.

Indeed, in addition to the secretion of growth factors, mesenchymal cells in the lung deposit ECM molecules, such as elastins, collagens, fibronectin, laminins, tenascins, and proteoglycans that not only provide tissue structure, strength, and elasticity, but also regulate cell activities through integrin and growth factor binding and signaling [9]. Mouse models have demonstrated that elastin is critical for the formation of alveoli, and decreased elastin deposition results in defective alveolarization [58]. Moreover, MSCs are able to secrete factors with local anti-inflammatory capacity (e.g., IL-10, PGE2, IL1RA, IDO, microRNAs) [9], and may thus act to reduce damage in various inflammatory lung diseases.

Numerous in vitro and in vivo studies in mouse and larger animal models have provided evidence for MSCs (from different origins) to support lung regenerative responses, as overviewed in Chaps. 3–8 and 10–12 of this book. Indeed, the described MSC-mediated effects in lung repair have been attributed to paracrine effects. For example, lung-derived MSCs (LMSCs) were shown to increase the deposition of extracellular matrix and normalize lung tissue architecture in an ovine model of elastase-induced lung damage, where autologous LMSCs were used [59]. MSCs from bone marrow were shown to directly affect alveolar epithelial and endothelial cells, reducing apoptosis [60, 61]. In addition to promoting epithelial repair in the alveoli, MSCs have been implicated in airway epithelial repair. A significant increase in BASCs was observed in a mouse model of neonatal hyperoxia-induced damage upon systemic treatment with MSCs or MSC-derived conditioned medium [33]. In vitro, MSCs increased the growth efficiency of BASCs, indicating a direct effect of MSCs on BASCs. MSCs and MSC-derived factors thus play a role in the repair of alveolar lung injury and in the restoration of distal airway epithelia as well [33]. These mouse studies have been supported in vitro by studies using human MSCs, showing that these are able to induce both alveolar and small airway epithelial migratory responses by secretion of various paracrine factors [62] and to promote the regenerative potential of human airway epithelial cell lines [63]. In the following sections we will further translate the findings on stem/progenitor populations identified in the mouse lungs and the supportive role of MSCs to human.

2.5 Translation of Knowledge of Progenitor Cell Populations in the Lung to Human

Before translating the different stem/progenitor populations identified in the mouse to human, it is important to highlight the differences in the organization of the epithelial layer. Wansleeben and colleagues [1] have provided a comprehensive review where they report that, while the basic organization of pseudostratified mucociliary epithelium and underlying mesenchyme is similar in the mouse and human trachea, the distal intralobar airways and bronchioles in mice have a more simple columnar epithelium. In the human lung, the trachea, proximal bronchi, and bronchioles are larger, with a diameter of >3 mm.

In the mouse lung, with fewer bifurcations of the bronchi, the diameter of the trachea and proximal main stem bronchi is 1.5 mm and cartilage rings are restricted to these areas. In the human lung, cartilage plates are found much deeper. Similarly, submucosal glands are confined to the most proximal region of the trachea in the mouse lung, but are present deeper into the human lung. The columnar pseudostratified epithelium in the proximal airways in the human lung is taller than in mice, consisting of multiple cell layers. More goblet cells are present, while club cells are restricted to the smallest airways (~1 mm diameter) in the human lung. Here, basal cells are present in the pseudostratified epithelial lining all the way down to the terminal bronchioles, whereas no basal cells are present in the more distal areas of the mouse lung, consisting mainly of club and ciliated cells. The phenotype of the epithelium of the smallest respiratory bronchioles of the human lung is still largely unknown [1].

The first evidence for the presence of cells with stem/progenitor potential in the adult human lung has come from a study where c-Kit (CD117) positive stem cells isolated from human distal lung tissue were expanded in vitro and injected into damaged mouse lungs [8], where they were shown to integrate structurally and to have regenerative functions. However, this observation has not yet been replicated by others [9] and this article has recently been retracted. Until now, only a few studies have attempted to isolate and characterize adult epithelial stem cells in the human lung. Mostly, basal epithelial cells from the proximal airways have been isolated, with progenitor capacity as demonstrated by the ability to differentiate into mucociliary epithelium during air–liquid interface culture and to generate spheroid/organoid structures [6]. These basal cells were able to form complex three-dimensional structures when grown in Matrigel. This indicates that basal epithelial cells from human airways have a similar putative progenitor role as observed in murine models. Accordingly, as reviewed by Nikolic et al. [2], in both human and mouse adult airways, basal cells are the progenitor cells that self-renew and differentiate into secretory and ciliated cells during homeostasis and repair processes [17, 19, 64–66]. Similar to mice, it is thought that human ciliated epithelial cells in the airways are terminally differentiated and not able to self-renew after injury [67–69]. Furthermore, similar to their murine counterparts, human basal epithelial cells have been reported to express NGFR, Itg α6, CK14, p63 as well as ALDH, and to be able to generate organoids where secretory and ciliated cells are present. The use of the combination of markers NGFR, Itg α6 and CD49f has also confirmed the isolation of human basal epithelial cells with stem cell potency [17, 70]. Furthermore, CD166 positive cells from human proximal submucosal gland ducts that co-expressed ALDH were shown capable of forming spheres expressing CK5 and CK14, with some mucus- and serous-secreting cells [71]. In the human lungs, secretory cells are predominantly of the mucous subtype [72], and it is doubted whether these mucus-secreting cells retain the ability to function as stem/progenitor cells. Also, it is unclear if a specific human club cell subset exists that qualifies as progenitor cell [65]. Of note, a basal side population of cells has been identified in the human lungs that expresses breast cancer resistance protein (BCRP1), CK5, and p63, and has been proposed as a major airway epithelial stem cell involved in repair of the conducting airways [73]. These BCRP1/CK5 p63 positive cells were highly clonogenic, with the ability to give rise to a multilayered differentiated epithelium in air–liquid interface culture and also proposed to be capable of generating club cells [73, 74].

As for the existence of neuroendrocrine progenitor cells, morphometric studies in humans have shown that these cells make up less than 1% of the population and are not often found in clusters or only in small clusters [75]. Of note, the number of NEB clusters has been reported to be highest during development and in young adults [75, 76] and decreases with age. BASCs and other rare populations of stem/progenitor cells in the distal airways have not yet been identified in humans [2].

As for the alveolar progenitors in the human lung, it has been confirmed that epithelial cells derived from human lung parenchyma are able to organize in three-dimensional organoid structures in Matrigel. Human organoids made up of

AT1 and AT2 cells were shown to be responsive to WNTs and WNT inhibitors in the same way as their murine counterparts [5]. Similar to the Itg α6β4 positive alveolar epithelial progenitor identified in mouse studies, a resident niche of epithelial progenitor cells expressing Itg α6β4 along with epithelial marker E-cadherin has been identified in human lungs [35, 77], being capable of self-renewal and differentiation into basal and club cells in Matrigel, although differentiation into AT2 cells was not observed [35]. Furthermore, Oeztuerk-Winder et al. have cloned putative E-cadherin/Itg α6 positive human lung epithelial stem cells, which additionally expressed Lgr6. In vitro, these cells could be serially passaged and were able to generate differentiated human bronchioalveolar epithelial tissue using a model of transplantation of a single cell under the kidney capsule of recipient immune-deprived (CD-1 nude) mice. The Axin positive AT2 stem cell population that has been identified in studies of Nabhan et al. and Zacharias et al. [36, 37] has also been suggested to exist in the human alveoli [36], acting as functional alveolar progenitors in organoid cultures.

Together, similar progenitor populations as identified in animal models may exist in the human lung. However, we need to bear in mind the differences in composition of the different cell layers in the human and animal lung, and realize that much more research needs to be conducted to confirm the existence and function of specific progenitor subsets in the human lung. Similar to animal models, the recruitment and activation of a specific progenitor cell subset in the human lungs may be context-dependent, thus varying with the heterogeneity of a specific lung disease.

2.6 Characterization of Mesenchymal Stem/Stromal Cells and Their Function in the Human Adult Lung

There is substantial evidence for the presence of tissue resident mesenchymal progenitor cells in the adult human lung [38]. Mesenchymal lineage cells isolated from adult human lungs by plastic adherence express the mesenchymal markers CD73, CD90, and CD105 and have the capacity to differentiate into adipocytes, chondrocytes, and osteocytes [78]. While the presence of lipofibroblasts in the human lungs is controversial [2, 79], supportive evidence exists that MSCs reside within the lung tissue in healthy humans [52, 80]. MSCs were first found to be present in fetal lung tissue [81] and a subset of adult human bronchial fibroblasts was reported to display similar MSC properties [82], as further outlined in Chap. 3 of this book. Westergren-Thorsson and coworkers have shown that MSCs can be isolated from bronchial biopsies in the central airway of lung transplanted patients [83, 84]. In situ staining revealed that these CD90/CD105 cells are located perivascularly [85]. As long as 16 years after lung transplantation, MSCs were shown to be exclusively donor lung-derived [84]. These data indicate not only that MSCs are tissue-resident but also that a tissue-specific niche of MSCs exists.

In line with the supportive effects on lung repair that have been observed in animal studies, human bone marrow-derived MSCs (BM-MSCs) have been shown to express various growth factors, including FGF2, PDGFA, and VEGF, and to support epithelial repair responses [63]. In another study, human BM-MSCs have been shown to induce alveolar and small airway epithelial migratory responses by secreting fibronectin, lumican, periostin, and insulin-like growth factor-binding protein 7 (IGFBP7), thus being able to promote repair processes [62]. Further, human-derived BM-MSCs have been shown capable of reducing alveolar damage through HGF cytoprotective effects in an elastase mouse model of emphysema [86], while treatment with adipose-derived human MSCs stem cell in mice attenuated lung and systemic injury induced by cigarette smoking [87]. However, in the first clinical trials in humans using autologous BM-MSCs, little beneficial efficacy has been observed. In 2013, a prospective, double-blind, placebo-controlled trial was published, which included 62 severe emphysema patients [88]. While the intravenous administration of BM-MSCs was safe and no side effects were reported during the 2 years of follow-up, no

improvements in lung function, quality of life or exacerbation incidence were observed. A post-hoc analysis revealed a mild systemic anti-inflammatory effect in patients with higher than 4 mg/L titer of the inflammatory marker c-reactive protein (CRP), showing a significant reduction of CRP levels in the first month of treatment. Although the beneficial effects were limited, they support the anti-inflammatory actions of MSCs observed in various animal models. Additional studies have demonstrated that intravenously administered BM-MSCs and placenta-derived MSCs are well tolerated in emphysema and IPF patients respectively [89, 90]. Again, no promising clinical effects were observed, although Stolk et al. report a threefold increase of the platelet and endothelial marker CD31 within the alveolar septa three weeks after infusion. This could have implications for perfusion of the lungs. Improved lung perfusion upon elastase-induced injury has been observed in a large animal (ovine) model of emphysema, where LMSCs were administered endobronchially [59]. However, substantial evidence for this notion is still lacking.

More fundamental knowledge on the function of human MSCs is clearly needed and further studies on the source, dosage, and administration are warranted. For instance, instead of intravenous administration, endobronchially delivered LMSCs were used in the ovine model and demonstrated to have beneficial effects, normalizing lung tissue architecture and increasing ECM deposition upon elastase-induced injury [59]. Furthermore, the use of autologous MSCs in the treatment of age-related lung diseases like COPD may be disadvantageous, as ageing and cellular senescence may lead to exhaustion of the stemness of MSCs [91]. BM-MSCs from aged humans have been shown to express markers of cellular senescence and exhaustion [92]. It can also be anticipated that the origin of MSCs is critical, and that MSCs are specific for regeneration of only the tissue from which they originate. In this respect, LMSCs may be better equipped for lung repair than for instance BM-MSCs. This notion is supported by the animal studies described earlier in this chapter. While both BM-MSCs and LMSCs had protective effects on lung damage in elastase-induced emphysema [57], LMSCs were more beneficial than BM-MSCs, displaying prolonged lung retention which is likely due to the expression of specific adhesion molecules. In a study that compared human LMSCs and BM-MSCs in vitro, LMSCs were observed to express higher levels of the stemness-related marker nestin, which may have consequences for their ability to differentiate into different cell types [93]. In addition, Westergren-Thorsson et al. have shown that lung-resident MSC possess lung-specific properties [85]. They are smaller, possess a higher colony-forming capacity, have a different cytokine profile compared to BM-MSC and express different levels of the lung-specific genes *FOXF1* and *HOXB5* compared to BM-MSCs. Importantly, we have recently observed that LMSCs specifically express crucial growth factors involved in lung repair, including FGF10 and HGF, which are expressed to a much lower extent in BM-MSCs and adipose-derived cells [94], while BM-MSCs were more specialized in gene expression of ECM molecules. The differences in gene expression, secretome, and proteome profiles between LMSCs and BM-MSCs will be discussed extensively in the next chapter.

In addition to their paracrine profile, part of the beneficial effects of MSCs has been attributed to the transfer of healthy mitochondria. MSCs have been reported to transfer mitochondria to alveolar epithelia by the formation of gap junctional channels [95]. This transfer was shown to reduce cigarette smoke-induced damage in a rat model [95]. Here, delivery of BM-MSCs attenuated cigarette smoke-induced airspace enlargement, although the precise mechanisms involved in the rescue of epithelial cell damage upon mitochondrial transfer remain to be elucidated. Later, Sinclair et al. isolated two populations of human lung MSCs from either digested parenchymal lung tissue of healthy individuals or from lung transplant recipients' bronchoalveolar lavage fluid [52]. Similar to the previous studies on the marrow counterparts, human lung-derived MSCs were able to transfer mitochondria to human bronchial epithelial cells in vitro [96].

Finally, Mendez, and coworkers have shown some promising evidence that human MSCs isolated from bone marrow and adipose tissue are able to transdifferentiate into lung epithelial cells when seeded on decellularized lung scaffolds, expressing SPC and/or contributing to club like cell populations [56]. This enhances the possibility of using these cells for lung cell therapies and tissue engineering, although these findings need to be confirmed in vivo.

Together, findings in human MSCs so far confirm the beneficial properties of these cells from animal studies, especially with respect to their paracrine function.

2.7 Concluding Remarks

In this chapter we provided an overview on progenitors identified in adult lung tissue and how their maintenance and differentiation is regulated by mesenchymal cells during lung developmental and repair processes. Work performed in animal studies has led to important insight into the role of progenitor cells and stromal support in these processes. Part of the progenitor populations and repair mechanisms identified in animal models may also exist in the human lung, although there are also important differences in the composition of the different cell layers in the human and animal lung, which need to be taken into account when translating findings from animal studies to human. Furthermore, the recruitment and activation of specific progenitor cell subsets may strongly depend on the context, thus varying with the heterogeneity of the disease. As for regenerative approaches, cell-based strategies based on the supportive role of mesenchymal cells, particularly MSCs, are promising. Nevertheless, clinical trials in humans so far have demonstrated that much more knowledge is needed before these cells can be used effectively in the treatment of human lung disease. The following chapters of this book provide an overview of the promises and setbacks of the use of MSCs in regenerative strategies for lung disease.

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3 Comparison of the Regenerative Potential for Lung Tissue of Mesenchymal Stromal Cells from Different Sources/Locations Within the Body

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3.1 Introduction

Mesenchymal stromal cells (MSCs) were originally discovered in the bone marrow, where they constitute a small percentage (0.01–0.001%) of the bone marrow cells [1]. Today however, MSCs have been isolated from a variety of human tissues, such as umbilical cord [2, 3], adipose tissue [4, 5], and lung tissue [6–9], but also from other species such as mouse [10–12], horse [13], and sheep [14]. The number of publications regarding MSC from different cell sources is continuously increasing, and some researchers believe that all organs have their own pool [15]. In contrast, Bianco et al. claimed that MSCs are not mesenchymal cells and should therefore be called skeletal stem cells. Based on this hypothesis, the authors state that MSCs exist only in skeletal lineages i.e. bone, cartilage, stroma, and marrow adipocyte tissues [16]. Nevertheless, cells fulfilling the International Society for Cellular Therapy (ISCT) MSC criteria [17] (summarized in Fig. 3.1) have been isolated from tissues outside the skeletal linages, and whether or not these cells have the same regenerative potential as the skeletal stem cells remains to be investigated. However, several studies have demonstrated that MSCs from tissues other than bone marrow have immune-regulatory and regenerative properties that might be beneficial for treatment of severe lung disorders. In this chapter, we provide a comparison of the potential of MSCs obtained from different cellular sources, and how they can be used as therapeutic agents to treat lung diseases.

3.2 The Mesenchymal Stromal Cell Source and Their Endogenous Functional Role

3.2.1 Bone Marrow-Derived Mesenchymal Stromal Cells

In 1968, Alexander Friedenstein et al. discovered the adult non-hematopoietic stem cell, today referred to as mesenchymal stromal/stem cell, in the bone marrow. These bone marrow cells adhered to plastic, had a fibroblast-like morphology, demonstrated multi-lineage differentiation

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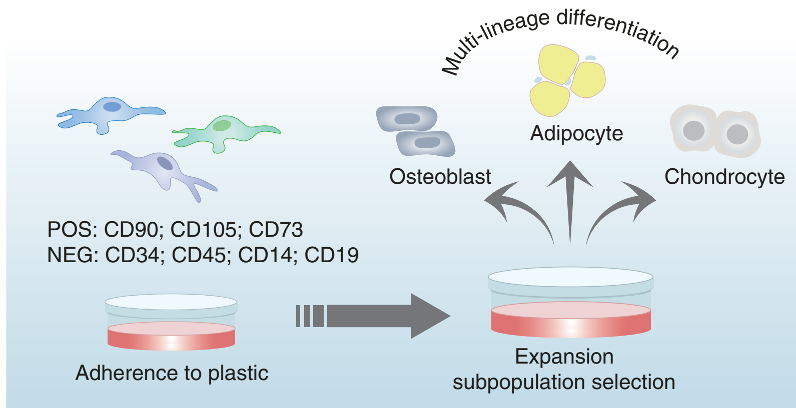

Fig. 3.1 A schematic illustration that summarizes the minimal criteria determined by ISCT to define cultured human MSCs. Abbreviations: *ISCT* the International Society for Cellular Therapy, *MSCs* mesenchymal stromal cells, *POS* positive expression, *NEG* negative expression. (This figure is adapted from the doctoral thesis Mesenchymal stromal cells in lung tissue [18])

capacity, and colony-forming potential [19, 20]. In 1991, Caplan published an article focusing on the multi-lineage capacity of the MSCs, and in that same paper he introduced the term mesenchymal stem cell for the first time [21]. During the next decades, the number of publications in the MSCs field increased rapidly, but so far, most of the studies have been performed using culture-derived MSCs. Cultured MSCs are heterogeneous and contain functionally different subpopulations [22]. The phenotype and function of primary MSCs is much less known than the culture-derived population. In contrast to culture-derived MSCs, primary MSCs are quiescent, noncycling cells [23].

The exact functional role of MSCs in the bone marrow is at present not completely known. However Mendes et al. demonstrated that MSCs could be isolated from fetal hematopoietic sites before they became colonized with hematopoietic stem cells. It is therefore likely that MSCs are involved in organization of the bone marrow niches [24]. Moreover, Sacchetti et al. demonstrated that MSCs isolated from human bone marrow can generate hematopoietic stroma in vivo [25]. In addition, MSCs have also been demonstrated to support homeostasis by promoting proliferation and survival of hematopoietic stem cells [26, 27]. Méndez-Ferrer et al. developed a mouse model where they investigated the role of MSCs expressing Nestin in vivo. This study nicely describes how Nestin-expressing MSCs are localized in close connection to the hematopoietic stem cells, within a maximum of five cell diameters. When Nestin positive cells were depleted, the number of hematopoietic stem cells and progenitor cells was reduced [28]. Interestingly, Nestin positive cells from human adult and fetal bone marrow displayed similar properties as the mouse-derived Nestin positive cells. Human Nestin positive MSCs showed a relative undifferentiated state when grown in mesenspheres, which indicates an increased self-renewal. In addition, human Nestin positive cells were also able to support hematopoietic stem cells from cord blood [29]. Furthermore, it is well known that MSCs, at least culture-derived MSCs, interact with cells of both adaptive and innate immune systems, thereby displaying both anti-inflammatory and pro-inflammatory effects [30, 31].

3.2.2 Lung-Derived Mesenchymal Stromal Cells

In 2003, In't Anker et al. demonstrated for the first time that MSCs were present in fetal lung tissue (second-trimester). These fetal lung-derived

MSCs possessed a similar surface marker profile and differentiation potential as MSCs isolated from fetal bone marrow [6]. Later, Sabatini et al. reported that a subset of adult human bronchial fibroblast displayed similar MSC properties as In't Anker et al. reported [8]. Interestingly, today MSCs can be isolated from bronchoalveolar lavage (BAL) fluid, which is a less invasive procedure compared to lung biopsies [7, 9]. However, whether the phenotype and function of BAL fluid-derived MSCs is the same as the phenotype of tissue-derived MSCs remains to be determined. In parallel with the findings of MSCs in human lung, Summer et al. reported that MSCs could be isolated from murine lungs using flow cytometry. They reported that the MSC population could be found within the CD45-/CD31- side population fraction [32]. This was the beginning of a new era, and in the following years the number of publications on this topic increased dramatically [33–36]. In similarity with bone marrow MSCs, lung-MSCs have mostly been studied in an in vitro system. However, primary MSCs can be isolated from lung tissue utilizing flow cytometry cell sorting (FACS) [37]. Interestingly, primary lung-MSCs, in similarity with bone marrow MSCs, differ from in vitro expanded MSCs [37].

The origin of lung-MSCs has been debated, where one of the hypotheses has been that MSCs are released from the bone marrow, and enter the lungs through the circulation [38, 39]. This hypothesis has been supported by in vivo results, demonstrating that GFP-labeled MSCs systemically administered into bleomycin-treated mice were found in the recipient lungs [38–40]. However, these studies infused cells prior to the evaluation of the origin of the lung cells. One disadvantage using this approach is that infused cells very easily become trapped in the lungs, which could mean that the cells were not actively recruited. The second hypothesis is that MSCs are tissue-resident cells, but due to technical limitations, this has been difficult to prove. Interestingly, utilizing the gender mismatch in lung transplanted patients, lung-derived MSCs have now been proven to be tissue-resident lung cells and that they do not migrate from the bone marrow to the lung [7, 37] (Fig. 3.2).

Although tissue-resident MSCs have been identified within the lung, the biological function of these cells is not well studied. In conformity with MSCs isolated from bone marrow, the lung-derived MSCs have a perivascular localization [37]. Therefore, it is likely that lung-MSCs interact and influence endothelial cells by paracrine factors as well as through cell–cell contact. In coculture systems, MSCs have been demonstrated to improve the vessel-like structures as well as the differentiation, proliferation, and sprouting of endothelial cells [41, 42]. Moreover, lung-MSCs have been suggested to communicate with epithelial cells by e.g. secretion of epidermal growth factors (EGF) and via gap junctions. MSCs are known to secrete keratinocyte growth factor (KGF), a growth factor that is important for epithelial cell proliferation and differentiation [43–45]. Furthermore, some reports on the potential of MSCs to differentiate into epithelial cells in vitro have been published [46, 47]. However, this has not yet been demonstrated in vivo, and whether or not MSCs possess this capability is still debated. Finally, lung-MSCs have, in conformity with MSCs within the bone marrow, immunomodulatory properties [48, 49].

Lung-resident MSCs share many properties with the well-characterized bone marrow-derived MSCs such as in vitro differentiation potential, expression of MSC surface markers (positive for CD73, CD90, and CD105 and negative for CD45 and CD31), and colony-forming potential. However, lung-resident MSCs display lung-specific properties such as lack of proper in vivo bone formation capacity, lower expression of osteopontin, increased proliferation rate, and increased colony-forming potential. Moreover, differences in gene expression, secretome, and proteome profiles between lung- and bone marrow-derived MSCs have been demonstrated [7, 37, 48, 50]. The lung-specificity of resident MSCs is important to study further, especially when thinking of MSCs as a cell therapeutic drug for various lung diseases.

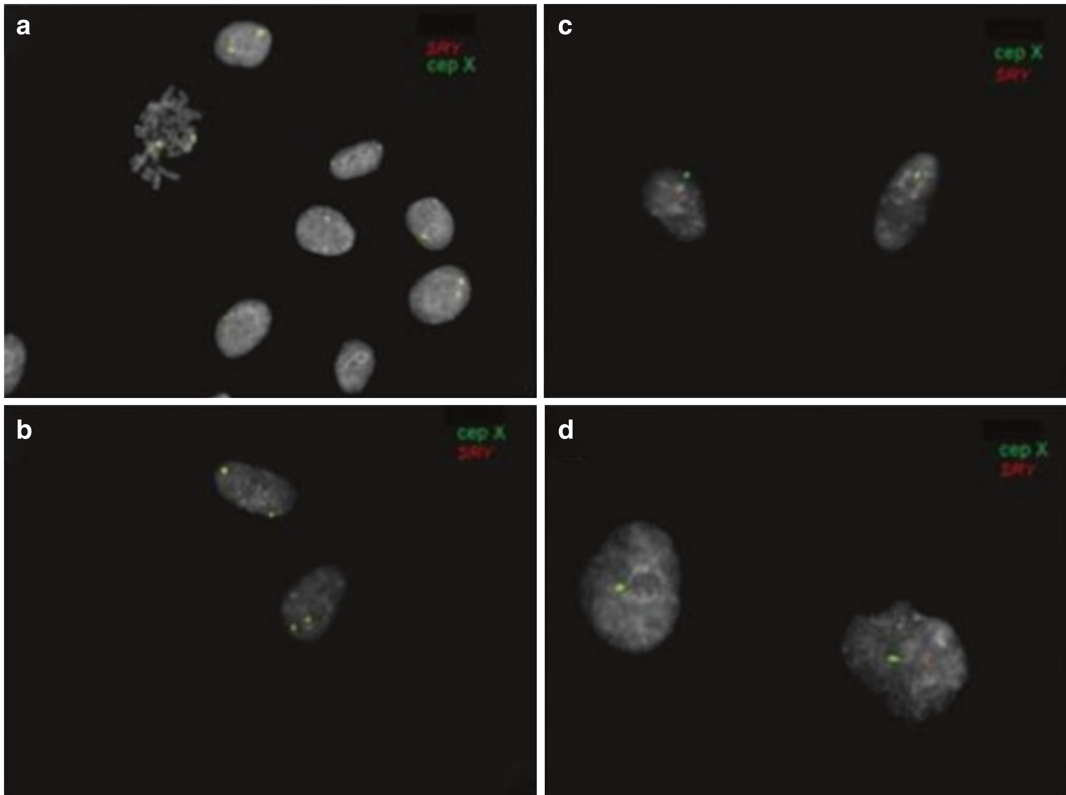

Fig. 3.2 Micrographs of XY chromosome analysis of lung-resident mesenchymal stromal cells isolated from central (**a** and **c**) and peripheral transbronchial (**b** and **d**) biopsies. The presence of Y and X chromosomes are indicated by the red and green signal, respectively. (This figure was originally published in BMJ Open Respir Res [37])

3.2.3 Adipose Tissue-Derived Mesenchymal Stromal Cells

Adipose tissue has emerged as an attractive MSC source, serving as one of the main alternatives to bone marrow, mainly because it is a less invasive method compared to bone marrow aspirates. MSCs were isolated from adipose tissue for the first time by Zuk et al. in 2001, and today it is known that MSCs comprise about 2% of mononucleated cells in lipoaspirates [4, 5], which is a high number compared to bone marrow aspirates (0.01–0.001%) [1]. Adipose-derived MSCs express a typical MSC surface marker profile, but a proportion of the cells is positive for CD34. CD34 is a cell surface sialomucin that is expressed by hematopoietic stem cells; however, its exact function is not fully known [51]. Furthermore, adipose-derived MSCs have been demonstrated to be more morphologically and genetically stable in a long-term population compared to bone marrow-derived MSCs [52]. Adipose-derived MSCs have been shown to differentiate towards fat, cartilage, and bone [53–55]. However, the differentiation capability of adipose-derived MCSs compared to bone marrow-derived MCSs is being debated. Some studies have reported that adipose-derived MSCs display decreased differentiation potential towards osteoblasts compared to bone marrow-derived MSCs [53, 56, 57]. On the other hand, others have reported that adipose-derived MCSs are equally good at differentiating towards bone in vitro [54, 55, 58].

In similarity with MSCs isolated from other tissues, adipose-derived MSCs are a heterogeneous cell population, with phenotypical variations among the different subpopulations [59]. Currently, several different protocols are being

used for the isolation process and the in vitro expansion. In an attempt to select a MSC phenotype with the best therapeutic properties, Najar et al. focused on the stem cell marker aldehyde dehydrogenase (ALDH). ALDH is a group of enzymes that is involved in stem cell differentiation, cellular expansion, and self-protection. When Najar et al. isolated adipose-derived MSCs based on their ALDH activity, they found that the ALDH expressing adipose-derived MSCs displayed a consistent hematopoiesis-supporting capacity. Furthermore, the ALDH expressing MSCs demonstrated significantly higher expression of genes implicated in stemness properties such as NANOG and OCT4 compared to adipose-derived MSCs that were negative for ALDH [60].

In the human body, adipose-MSCs reside in a microenvironment with low oxygen or hypoxia, approximately 1–5% O_2. Following isolation, adipose-derived MCSs usually are expanded in vitro at much higher levels of oxygen [61]. Previously, Choi et al. demonstrated that both adipose-derived MSCs cultured at 2% O_2 and at 21% O_2 retain their characteristics such as morphology, differentiation capacity, and surface marker profile. However, the proliferation rate was significantly higher in MSCs cultured at 2% O_2 compared to 21% O_2. In addition, the lower oxygen level also enhanced the viability of the cells and reduced DNA damage compared to MSC grown at 21% O_2. Xia et al. performed a secretome analysis on conditioned medium collected from adipose-derived MSC culture grown under hypoxic and normoxic conditions. It was found that hypoxia enriched the cytokines VEGF, CCL-20, and TIMP-1. In the same study, Xia et al. noticed that the conditioned medium from MSC cultured under hypoxic conditions enhanced the migratory effect, as well as the viability of gastric mucosal epithelial cells [62].

3.2.4 Umbilical Cord-Derived Mesenchymal Stromal Cells

Already during the fifth week of gestation, the human umbilical cord starts to develop, and it continues to grow until it is 50 cm in length [2]. MSCs can be isolated from different parts of the umbilical cord, including Wharton's jelly, cord blood, and the perivascular region [2, 3]. The human umbilical cord is a promising source of MSCs. First, the process to collect the umbilical cord-derived MSCs is a painless procedure, and banking of umbilical cord blood is common worldwide. In addition, because of this noninvasive collection procedure there is very low risk of infection [2]. Second, because MSCs are isolated from extra-embryonic tissue that is obtained after birth there are fewer ethical considerations compared to other MSC sources [63]. MSCs derived from umbilical cord are believed to be more primitive compared to MSCs isolated from more mature tissues. Early passages of Wharton's jelly-derived MSCs grow much faster compared to bone marrow-derived MSCs, with a cell doubling time that is almost two times shorter [14, 15]. Furthermore and in similarity with lung-derived MSCs, Wharton's jelly-derived MSCs have a greater ability to form colony-forming unit-fibroblasts colonies in vitro compared to the well-characterized bone marrow-derived MSCs [3, 48, 64]. Notably, MSCs isolated from Wharton's jelly show high expression of embryonic genes and pluripotent/stem cell genes such as NANOG, OCT4, LIN28, DNMT3B, REX1, and GABRB3 [3, 65]. Importantly, MSCs isolated from Wharton's jelly share the basic MSC criteria, which are used to define adult-MSCs, such as in vitro multi-lineage differentiation potential, and expression of the surface markers CD73, CD90, CD105, lack of the surface markers CD45, CD34, and HLA class II, and plastic adherence [3].

In addition to the umbilical cord, MSCs could also be isolated from the amniotic fluid at term of scheduled caesarean section deliveries [66, 67]. Moraghebi et al. showed that following in vitro expansion, MSCs isolated from the amniotic fluid showed a typical MSC surface marker profile, adhered to plastic, and had the ability to differentiate into adipocytes and osteoblast cell lineages [67]. Furthermore, Moraghebi et al. evaluated the gene expression profile of MSCs isolated from amniotic fluid and compared it to the gene expression profiles

of adult MSCs isolated from bone marrow aspirates and adipose tissue. The authors found that the gene expression profile of amniotic fluid-derived MSCs was very similar to the gene expression profiles from adipose-derived and bone marrow-derived MSCs [67].

3.2.5 Heart-Derived Mesenchymal Stromal Cells

For many decades, the heart was considered a fully differentiated organ with low regenerative capacity [68]. However, an accumulating body of data suggests that the cardiac tissue contains several resident populations of stem cells and/or progenitor cells with regenerative capacities. In 2002, Hierlihy et al. demonstrated that post-natal myocardium contained a side population of cells that possess stem cell-like properties [69]. These results were verified by Martin et al. who also demonstrated that the side population was able to proliferate and differentiate in vitro [70]. By comparing the side population identified in the adult heart with the side population identified in the bone marrow, Pfister et al. demonstrated that the cardiac side population was phenotypically distinct from the bone marrow-derived cells [71]. In 2004, a population of stem/progenitor cells were also identified in the adult human heart. Messina et al. reported that cardiac stem cells could be isolated from atrial or ventricular biopsies from human adults, and following in vitro expansion these cells were shown to be clonogenic with in vitro differentiation potential [72]. Today, it is known that the cardiac tissue contains resident cells fulfilling the MSC criteria and they are hypothesized to have cardiac regenerative properties during for example myocardial infarction mediated injuries. However, the most extensively studied stem and/or progenitor cell in the heart today is the c-kit expressing cardiac stem cell (CSC), which possess self-renewal capacity and differentiation potential [73]. The CSC has been shown to form cells from three cardiac lineages: the cardiomyocytes, smooth muscle cells, and endothelial cells [74]. Although, a few researchers consider cardiac stem cells and MSCs as two names of the same cell type [75], this needs to be further studied.

The frequency of stem/progenitor cells in the heart is very low, and therefore it has been hypothesized that nonresident MSCs such as umbilical cord-derived MSCs could be a better alternative for regeneration of the diseased heart. The umbilical MSCs share many similarities with heart-derived MSCs; however, the umbilical-derived MSCs possess higher capacity for proliferation [75]. Furthermore, an important feature for regeneration of the heart is the electrophysiological properties and both the heart- and the umbilical cord-derived MSCs have been shown to express ion channels, such as K^+, Ca^{2+}, and N^+-channels, on mRNA levels [75]. Also, in an aging perspective the use of umbilical cord-derived MSCs could be beneficial compared to using bone marrow-derived MSCs as the quantity and qualities of bone marrow-derived MSCs are found to decline significantly with age [76, 77]. It is also important to keep the homeostasis between normoxia and hypoxia, and MSCs have been described to keep their stemness and quiescence during hypoxic conditions. In the myocardium, however, MSCs have been described to be hidden in the hypoxic niches, in which they can promote myocyte regeneration after activation by stem cells factor. Another factor that could affect the differentiation of the cardiac-derived MSCs to endothelial cells in vitro is the mediator VEGF [78], a growth factor that might be important to consider in engineering the heart [79].

3.2.6 Dental Pulp-Derived Mesenchymal Stromal Cells

Adult teeth possess a repair process that acts to restore the dentine following injury [80], and MSCs have been suggested to play an important role in this repair and homeostatic turnover processes [81]. The dental pulp-derived MSC was first discovered by Gronthos et al. and today it is known that the adult teeth contain several subpopulations of MSCs [82, 83]. Notably, not all subgroups of dental pulp MSCs are equal in their functional properties and phenotype. For

example, variations in mineral capacity of different dental MSCs have been demonstrated [84]. In similarity with lung-derived MSCs, dental MSCs share many properties with bone marrow MSCs, such as a perivascular localization, colony-forming potential, immune-regulatory effects on immune cells, and multi-linage differentiation capacity [83, 85–87]. However, when dental MSCs were transplanted into immunocompromised mice they formed dentin and pulp-like structures. Moreover, dental MSCs displayed a higher proliferation rate in vitro, and the expression of dentin phosphoprotein and dentin sialoprotein mRNA were altered compared to bone marrow-derived MSCs [83]. In addition, dental pulp-derived MSCs express higher levels of neurogenic genes such as Nestin and SOX1 compared to bone marrow-derived MSCs. Following cultivation in neural induction medium, dental pulp MSCs exhibit neural cell morphology (round cell body with dendrite- or axon-like processes) and a small percentage of the dental pulp-derived MSCs displayed voltage-dependent K^+ and Na^+ currents that evoked action potentials [88]. A considerable amount of research has shown that dental pulp-derived MSCs could be beneficial for therapeutic approaches in for example ischemic stroke [89–91] and peripheral nerve injury [92].

3.2.7 Mesenchymal Stromal Cells Derived from Other Species

MSCs have been identified in several species apart from humans including horse [13], dog [14, 93], cat [94], cow [95], sheep [14], and mice [10–12]. In these animals, organ-specific MSCs have been found and isolated from the endometrium, adipose tissue, bone marrow, cord blood, umbilical cord, amnion fluid, and Wharton's jelly [13, 93–97]. Cross-species comparison of surface markers and functions of MSCs suggest that MSCs from different species are similar but not identical. For example, adipose tissue-derived MSCs from monkeys and horses showed differences in doubling time and surface marker expression compared to human adipose-derived MSCs. In line, MSCs isolated from rodents (mice and rats) show similar morphology and differentiation capacity [98]. However, the full characterization of MSCs in different species is sometimes not fully compatible, hindered by the variability in species-specific properties [14]. Xenotransplantation, i.e. transplantation of cells or tissues between different species, has been proposed as a possible solution to the shortage of available donor organs for transplantation. Blanco et al. transplanted human bone marrow-derived MSCs into a rabbit model of critical-size bone defects and found increased bone regeneration after the MSC injection [99]. Furthermore, Cruz et al. demonstrated that MSCs derived from human bone marrow were more potent to mitigate airway hyperreactivity and lung inflammation in an acute asthma mouse model compared to mouse-derived bone marrow MSCs [100, 101].

3.3 The Functional Role of Mesenchymal Stromal Cells in Lung Homeostasis and Disease

3.3.1 The Potential In Vivo Role of MSCs Within the Lung

Despite an increased interest in using MSCs for clinical approaches in severe lung disorders, the biological function of MSC in vivo is not completely known; in particular, when considering MSCs extracted from other tissues than bone marrow aspirates. In conformity with MSCs isolated from bone marrow aspirates, the lung-resident MSCs have a perivascular localization [37]. It is therefore likely, that lung-resident MSCs influence endothelial cells by cell–cell interactions as well as through paracrine factors. Coculture systems with MSCs and endothelial cells have demonstrated that MSCs improve formation of vessel-like structures. Furthermore, it has been reported that MSCs influence proliferation, differentiation, and sprouting of endothelial cells [41, 42]. Interestingly, MSCs have also been suggested to interact with epithelial cells during homeostasis and tissue repair by e.g. secretion of cytokines and via gap junctions. Badri et al. nicely

demonstrated that MSCs were able to establish gap junctions to alveolar and bronchial epithelial cells. In addition, they reported that MSCs could produce and secrete keratinocyte growth factor (KGF), a cytokine known to be important for epithelial cell proliferation and differentiation [43]. Another study found MSCs secretion of IL-6, via a STAT3-dependent pathway [102] (Fig. 3.3).

3.3.2 The Immune Modulatory Properties of Lung-Resident MSCs

A large body of evidence describes that the main function of MSCs is to provide support to other cells, and today it is known that MSCs interact with cells of both the adaptive and innate immune systems. The ability of MSCs to interact and affect cells within the innate immune system has been demonstrated both in vitro and in vivo. For example, MSCs have the ability to reprogram macrophages and thereby play a crucial role in inflammatory responses and tissue regeneration. This reprograming of macrophages involves several different mechanisms, the secretion of paracrine factors, such as prostaglandin E2 (PGE2) and TNF-stimulated gene 6 (TSG6), but also through cell–cell interactions. MSCs promote the differentiation of pro-inflammatory M1 macrophages into M2 macrophages, a phenotype that promotes the resolution of inflammation and tissue repair [30, 31, 103, 104]. Neutrophils are other important cells within the innate immune system, being crucial for the antimicrobial response. Upon stimulation, MSCs secrete paracrine factors such as macrophage migration inhibitor factor (MIF), IL-6, and IL-8, which attracts neutrophils to the site of interest where they receive pro-survival signals and gain proinflammatory capabilities [105]. In the adaptive immune system, MSCs are known to suppress proliferation of T-lymphocytes that have been stimulated with either alloantigens or mitogens. This inhibition of T-lymphocyte proliferation is due to cell cycle arrest by keeping the lymphocytes in the G0/G1 phase. Most likely, both paracrine factors, such as PGE2, indoleamine 2,3-dioxygenase (IDO), TGF-beta, and nitric oxide (NO), as well as cell–cell interactions are involved in these mechanisms [31, 106–110]. Focusing on lung-resident MSCs, preclinical studies have demonstrated

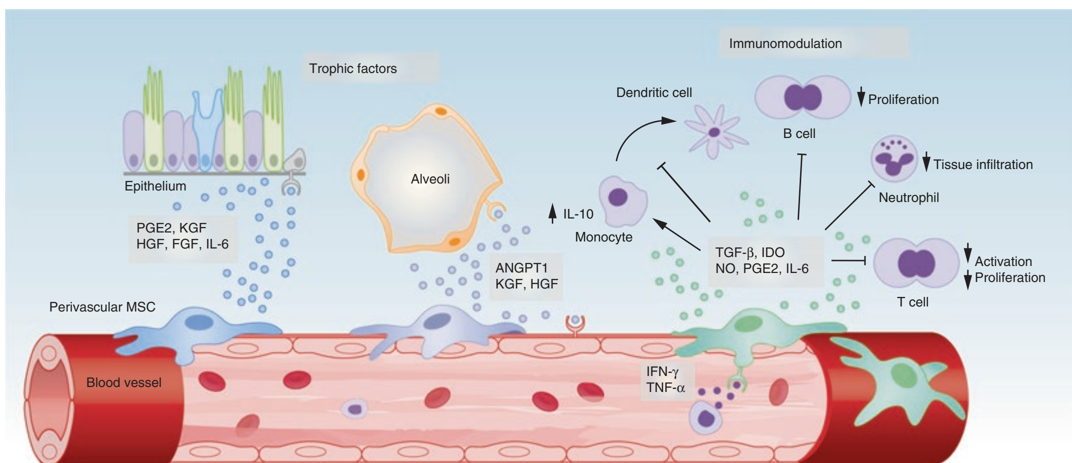

Fig. 3.3 A schematic illustration of the potential functions of resident MSCs within the lung. Abbreviations: *MSCs* mesenchymal stromal cells, *PGE2* prostaglandin E2, *KGF* keratinocyte growth factor, *HGF* hepatocyte growth factor, *FGF* fibroblast growth factor, *IL-6* interleukin-6, *ANGPT1* angiopoietin 1, *IL-10* interleukin-10, *TGF-beta* transforming growth factor beta, *IDO* indoleamine 2.3-dioxygenase, *NO* nitric oxide, *IFN-gamma* interferon gamma, *TNF-alpha* tumor necrosis factor alpha. (This figure is adapted from the doctoral thesis Mesenchymal stromal cells in lung tissue [18])

that lung-derived MSCs have similar potential to mediate immunological responses as bone marrow-derived MSCs [48, 49]. In similarity with bone marrow-MSCs, the soluble mediator PGE2 has been implicated to play a crucial role in the inhibition of T-lymphocyte proliferation [49]. However, if this capability is altered during different lung diseases remains to be investigated.

3.3.3 The Antimicrobial Activity of MSCs

Antimicrobial peptides are small effector molecules that are a part of the innate immune system. Human antimicrobial peptides have different mechanisms of action leading to the elimination of the target. Some of these peptides are expressed as a response to an infection or inflammation, while others are constitutively expressed [111]. It has been demonstrated that MSCs can produce and secrete antimicrobial peptides and proteins. Krasnodembsbskaya et al. reported that the expression of LL-37 in bone marrow-derived MSCs was significantly increased following *E. coli* and *P. aeruginosa* infection. However, when the authors blocked LL-37, the antimicrobial activity of MSCs was only partially reduced [112]. MSC-derived conditioned medium has also been demonstrated to slow down the growth rate of *P. aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* in vitro. In this study, the authors compared the bone marrow-derived and adipose-derived MSCs and found that both MSC populations possessed antimicrobial effectiveness but the adipose-derived MSCs were more potent [113]. In conformity with Sutton et al., Wood et al. observed an inhibitory effect of adipose-derived MSCs on *Staphylococcus aureus* and *P. aeruginosa*. Moreover, in this study the authors reported that adipose-derived MSCs engulfed the *Staphylococcus aureus*, leaving pores in the MSC membrane. Interestingly, this was not seen in the *P. aeruginosa* cultures. Furthermore, Wood et al. demonstrated that biofilm formation was significantly inhibited in the presence of MSCs [114]. Interestingly, an improved bacterial clearance has also been demonstrated in vivo in both MSC-treated ALI-mice, septic mice, and in ex vivo perfused human lung [103, 115–117]. Despite the demonstrated antimicrobial effects of MSCs both in vitro and in vivo, and the role of the antimicrobial peptide LL-37, other further mechanisms of action remain to be discovered.

3.3.4 The Paracrine Action of MSCs in Lung Injury Prevention and Tissue Repair

In addition to the immune regulatory properties of MSCs, a large body of literature suggests that MSCs mediate tissue repair by releasing a spectrum of soluble mediators. As indicated by in vitro studies following activation, MSCs produce and secrete cytokines and growth factors to influence surrounding resident cells in order to modulate and repair damaged tissue. The MSC secretome has attracted much attention, and today it is known to consist of an array of molecules important for their regenerative and protective abilities [48, 50, 118–122]. Utilizing conditioned medium from MSCs cultures, Kennelly et al. demonstrated that soluble molecules produced and secreted by MSCs had protective effects in a preclinical model of emphysema. One of the key mediators they identified was hepatocyte growth factor (HGF), a growth factor suggested to have protective properties in lung diseases such as chronic obstructive pulmonary disease (COPD). Furthermore, by knocking down HGF Kennelly et al. showed that HGF was required for this protective effect of MSC-derived conditioned medium on COPD [118]. Another interesting cytokine found in the MSC secretome was vascular endothelial growth factor (VEGF). Yang et al. reported that MSC-derived conditioned medium decreased the LPS-induced endothelial and transcellular permeability. This decrease of permeability was significantly inhibited when VEGF and HGF were blocked using blocking-antibodies [119]. MSC-derived conditioned medium has been used in preclinical studies for treatment of different lung diseases with good results, confirming the hypothesis that MSC exert

their therapeutic affect mainly through paracrine mechanisms. Treating lung injury with MSC-derived conditioned medium resulted in a significantly decreased lung inflammation and vascular permeability [123]. Interestingly, Lee et al. demonstrated that when treating acute lung injury in ex vivo perfused human lung with MSC-derived medium they observed a decreased vascular permeability and a restored alveolar fluid clearance. Furthermore, Lee et al. demonstrated by using a siRNA knockdown approach that keratinocyte growth factor (KGF) was a crucial mediator for this effect [120]. MSC-derived conditioned medium has also been used for treating other lung diseases in vivo such as experimentally induced bronchopulmonary dysplasia and lung fibrosis with good results [121, 122]. In addition to cytokine and growth factor secretion, MSCs are also known to produce and secrete extracellular matrix proteins. Utilizing quantitative in-depth mass spectrometry, the proteome of lung-resident and bone marrow-derived MSCs was compared. The authors found that both lung- and bone marrow-derived MSCs produce ECM proteins, however significant differences were found between the two MSC subpopulations. For example, tenascin-C, a glycoprotein known to be a critical component in the bone marrow, was significantly higher in the bone marrow-derived MSC samples compared to lung-MSCs. On the other hand, HGF was reported to be highly expressed by lung-derived MSCs [50]. Significant differences in the secretome produced by MSCs from different tissues and donors have been reported [50, 124, 125], and the question is whether or not that will affect the therapeutic properties of MSCs.

3.3.5 Extracellular Vesicle and Mitochondrial Transfer by MSCs to Target Cells

In addition to the secretion of soluble factors, MSCs have the capacity to communicate with other cells by releasing extracellular vesicles (EVs) [126, 127]. EVs are a heterogeneous group of membrane-bound vesicles that can contain different cargoes such as DNA, RNA, and microRNA [128]. The content, but also the amount, of EVs released by MSCs have been reported to vary significantly based on the local microenvironment they reside in, and different extracellular stimuli such as inflammation are known to have an impact on the EV cargoes. However, the precise mechanism by which the EV content is modulated by the local environment remains to be discovered [126, 129]. In similarity with MSCs, MSC-derived EVs have the potential to suppress T-lymphocyte proliferation [130, 131]. Interestingly, MSC-derived EVs have been shown to be as potent as the parent cell. For example, MSC-derived EVs have been used for treatment of acute lung inflammatory diseases such as acute lung injury [132, 133] and asthma [101] where they have been demonstrated to mitigate factors such as protein permeability, lung inflammation, and airway hyperresponsiveness. Even if the mechanism of action has not been fully discovered, the usage of this cell-free product remains promising.

Increasing data suggests that MSCs, in addition to the release of EVs, have the capability to transfer mitochondria to their neighboring cells. Spees et al. were able to demonstrate that bone marrow-derived MSCs could restore aerobic respiration in epithelial cells lacking functional mitochondria by transferring mitochondria and mitochondrial DNA to the damaged cells [134]. MSCs have also been shown to have the potential to rescue injured endothelial cells via mitochondrial transfer [135]. Moreover, compelling preclinical data demonstrate that MSCs can transfer mitochondria in vivo. Islam et al. demonstrated that MSCs attached and formed connexin 43-containing gap junction channels with epithelial cells. Following the formation of gap junctions, the MSCs were able to transfer mitochondria to the epithelium and increase the survival in LPS-induced ALI. The mitochondrial Rho-like GTPase (Miro1) has been suggested to regulate the amount of mitochondrial transfer. Ahmad et al. reported that enhanced expression of Miro1 increased the mitochondria transfer from MSCs to epithelial cells [136]. Recently, Mahrouf-Yorgov et al. demonstrated that MSCs engulf and degrade mitochondria

that have been released from damaged cells. As a result, the expression of the cytoprotective enzyme heme oxygenase-1 (HO-1) increases, leading to an enhanced mitochondrial biogenesis and MSC mitochondrial transfer [137]. Interestingly, Paliwal et al. recently demonstrated that MSCs isolated from different tissues had different mitochondrial transfer abilities. The authors observed that bone marrow-derived MSCs and adipose-derived MSCs had higher capacity to transfer mitochondria, but lower mitochondrial bioenergetics and less robust respiratory capabilities compared to dental pulp-derived MSCs and Wharton's jelly-derived MSCs [138].

3.4 The Important Cross Talk Between Mesenchymal Stromal Cell and the Microenvironment

The extracellular matrix (ECM) is a three-dimensional network composed of noncellular structures, which is a key player in stem cell microenvironments, or stem cell niches. Except for the most obvious role, to give physical support, it has a unique role in regulating cell activity and providing cells with positional information [139] (Fig. 3.4). Stem cells, and cells in general, are regulated by a combination of intrinsic mechanisms including transcription

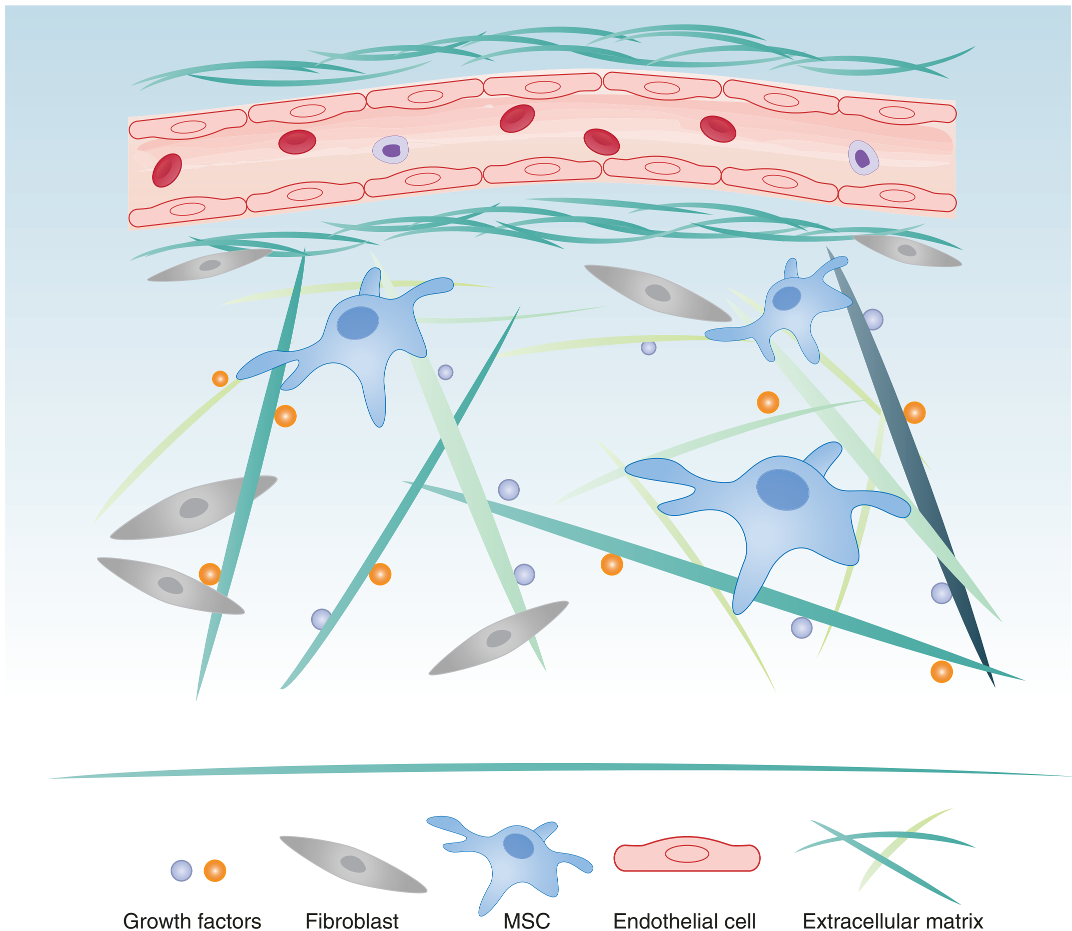

Fig. 3.4 A simplified overview of the interactions between MSCs and the microenvironment (extracellular matrix and other cell types such as fibroblasts and endothelial cells). (This figure is adapted from the doctoral thesis Mesenchymal stromal cells in lung tissue [18])

factors expressed by the cell itself, and extrinsic mechanisms consisting of signals provided by the local niche [140]. A correct microenvironment is essential for proper stem cell function by maintaining the appropriate balance between self-renewal, differentiation, and survival [141]. Several reports have demonstrated that MSCs are key players in the hematopoietic system, providing the correct stem cell environment for the hematopoietic stem cells [28, 142]. An intact and functioning bone marrow stroma is critical for maintenance of hematopoiesis, and several studies have shown that MSCs have the potential to generate the hematopoietic supportive stroma within the adult bone marrow [24, 143].

The composition of ECM has been demonstrated to play an important role, especially with regard to stiffness. A softer ECM has been shown to favor MSCs to differentiate towards adipocyte-lineage, whereas a stiffer matrix favored osteoblast differentiation [140, 144]. In addition, by varying the collagen cross-linking, using different concentrations of chemical cross-linkers, it is possible to affect the stem cells' behavior. For example, using a lower concentration of the cross-linker resulted in lower collagen anchoring leading to an increased MSC differentiation capability [145]. Furthermore, the topography of the matrix can affect the cellular behavior [146]. Not only the cross-linking and topography affects the ECM stiffness, but also the levels and ratios between different fibrillary ECM proteins, such as collagen and fibronectin, play an important role [147]. The stiffness of the ECM within the lung is directly related to the biomechanical properties of the organ, and the ECM composition have been demonstrated to be altered during different pulmonary diseases [148]. For example, an enhanced degradation of ECM, with increased levels of circulating ECM protein fragments have been reported during COPD exacerbations [149]. On the other hand, patients with idiopathic pulmonary fibrosis (IPF) have excessive amounts of ECM protein, leading to an increased ECM stiffness and a decline in lung function [150]. In which way differences in stiffness influence the in vivo anti-inflammatory and anti-fibrotic capacities of MSCs is at present not known, and needs to be further studied.

Another important function of ECM, is to bind and store soluble factors such as cytokines and growth factors, both in homeostasis but also during disease. Unresolved chronic inflammation is frequently observed in patients with acute respiratory distress syndrome, cystic fibrosis, asthma, and COPD [151]. Therefore, MSCs might encounter an inflammatory environment when used for cell therapeutic approaches in these diseases. During inflammation, MSCs are exposed to pro-inflammatory cytokines such as interferon (IFN)-gamma, transforming growth factor (TGF)-beta, and interleukin (IL)-10 [152]. Several studies have demonstrated that MSCs exposed to pro-inflammatory cytokines have a more efficient immunomodulatory activity compared to MSCs not exposed to an inflammatory environment [152]. IFN-gamma exposed MSCs have an increased induction of indoleamine-pyrrole 2,3-dioxygenase (IDO) activity, an enzyme known to inhibit T-cell proliferation, compared to MSCs not exposed to the pro-inflammatory cytokine [107, 153]. Furthermore, IFN-gamma induced expression of HGF and TGF-beta by MSCs [154]. Another interesting small protein that is upregulated during inflammation is tumor necrosis factor (TNF)-stimulated gene-6 (TSG-6). TSG-6 has been shown to have important and diverse anti-inflammatory and tissue protective properties. Despite the protective properties, TSG-6 has been shown to play a role in lung disease pathology. Upon stimulation with inflammatory cytokines such as TNF, MSCs express TSG-6, which mediates several of its immunomodulatory properties [155].

Finally, the composition of ECM is unique to every tissue type as well as the developmental age, and changes in both stiffness and composition drastically affect cell fate and function. In order to meet the shifting needs of the tissue, the ECM undergoes constant remodeling [147]. However, during pathological conditions, the remodeling process can become altered and in which way these alterations affect the MSC behavior needs to be further studied. Moreover, how MSCs from other tissue sources, such as bone marrow or adipose tissue, will interact with

the local microenvironment in the recipient lung is still unknown. An increased understanding in the cross talk between MSCs and the environment they encounter will provide us with better understanding of the biological function of MSCs as well as improve clinical strategies to treat lung disorders.

3.5 Preclinical and Clinical Applications of Mesenchymal Stromal Cell in Lung Disorders

MSC have become promising candidates for cell therapy for lung diseases, in particular because systemically administered MSCs initially lodge in the lung microvasculature [156, 157]. In addition, MSCs can also be administered locally into the trachea [158]. Early studies suggested that MSCs engrafted in the lung tissue after administration [159]. Today, however, it is known that MSCs do not engraft, rather data demonstrate that the majority of MSCs tend to be cleared from the lungs within a few days [160–163]. These results suggest that infused MSCs tend to have fast, short acting, paracrine effects in the lung, rather that engraftment and differentiation towards other cell types.

A growing body of preclinical data demonstrates that administration of MSCs improve lung function and increase survival rate in severe lung disorders such as COPD, IPF, asthma, and acute lung injury, mainly through paracrine mechanisms [12, 100, 164–168]. Gu et al. reported that intratracheally infused bone marrow-derived MSCs restored lung function in an emphysema rat model through inhibition of cyclooxygenase-2 (COX-2) and PGE2 expression [164]. In concurrence with previous results, Li et al. demonstrated a reduction of the lung inflammation in cigarette smoking-induced emphysema after systemic administration of bone marrow-derived MSCs. The authors reported that MSCs significantly modulated the inflammatory cells infiltrating the injured lungs [165]. The ability of MSCs to affect CD4 T-lymphocyte differentiation has also been demonstrated in a mouse model of allergic airway inflammation [12, 100, 166–168]. Goodwin et al. demonstrated that administration of both syngeneic and allogenic bone marrow-derived MSCs decreased the lung inflammation and the number of inflammatory cells. The authors found that MSCs inhibited the allergic airways inflammation by influencing the CD4 T-lymphocyte differentiation, partly through an IFN-gamma-dependent mechanism [12]. Similar to bone marrow-derived MSCs, adipose-derived MSCs and their EVs have displayed comparable effects on lung inflammation in an allergic asthma mouse model. Both adipose-derived MSCs and EVs decreased collagen fiber deposition, the level of TGF-beta expression, and the numbers of eosinophils in lung tissue [167]. Adipose-derived MSCs have also been used in a smoke-induced emphysema model with good results. Hong et al. found that conditioned medium from cultures with adipose-MSCs pretreated with pioglitazone, improved the morphology in an elastase-induced emphysema model. Furthermore, the authors suggested that VEGF played an important role in this repair process [169]. Interestingly, Cappetta et al. demonstrated that lung-derived MSCs were able to restore lung elasticity and alveolar architecture in a model of elastase-induced emphysema through the release of HGF [170]. Moreover, Hoffman et al. demonstrated that lung- and bone marrow-derived MSCs both reduced elastase injury to the same extent; however, lung-derived MSCs had enhanced lung retention after intravenous transplantation [171].

Growing amount of data suggests that pretreating MSCs before administration enhances their therapeutic effects. An increase in the immunomodulatory properties of MSCs was observed after exposure to IFN-gamma before administration [172, 173]. For example, pre-stimulation of MSCs led to an increased expression of IDO and iNOS, and at a functional level, IFN-gamma-exposed MSCs were more potent to inhibit lymphocyte proliferation [172–174]. IFN-gamma stimulation also appears to inhibit myeloid differentiation [175]. Furthermore, Goedhart et al. demonstrated that IFN-gamma treatment altered the cytokine secretion profile produced by MSCs, and increased levels of IL-6 and stem cell factor (SCF) was observed [175]. Moreover, Yang et al. screened 1402 FDA-approved bioactive

compounds for pre-stimulation of MSCs and found the compound tetrandrine, which is a substance known to activate secretion of PGE2. Interestingly, Yang et al. found that tetrandrine pre-stimulated MSCs possessed the ability to suppress immune responses both in vitro and in vivo [176]. In an attempt to mimic the in vivo situation after administration of MSCs into a diseased lung, Bustos et al. stimulated MSCs with serum obtained from patients with ARDS. MSCs pretreated with the patient serum demonstrated an increase in plasma IL-10, and a decrease in plasma TNF-alpha and IL-1beta [177]. In a recent study, Abreu et al. demonstrated that MSCs stimulated with serum from asthmatic mice induced macrophage polarization and increased expression of anti-inflammatory cytokines compared with MSCs stimulated with serum from healthy control mice. Interestingly, MSCs stimulated with asthmatic serum displayed a higher percentage of apoptotic cells. Similar results were seen when stimulating MSCs with BAL-fluid from the asthmatic mice [11]. Taken together, MSCs could be activated by external stimulation such as bioactive substances, inflammatory cytokines, and biological disease -specific mediators. However, in what way this will affect the MSC viability and their therapeutic effects remains to be explored.

Despite promising preclinical data indicating that MSCs as a treatment for severe lung disorders improve the survival rate and decrease or inhibit inflammation, clinical studies have not been able to reproduce these results. Today, MSC-based cell therapies on patients with severe lung disorders have been demonstrated to be safe and nontoxic, but no significant improvement in outcome has been observed [178–180]. However, current clinical trials have been underpowered and strategies to enhance the therapeutic potentials of MSCs needs to be improved.

3.6 Conclusion

In recent years, the number of publications using MSCs for treatment of different severe lung disorders has increased enormously. Predominantly, most studies have used bone marrow-derived MSCs, however MSCs with immune-regulatory and regenerative properties can be isolated from many different tissues such as lung tissue, adipose tissue, and placenta. Importantly, it has become clear that the knowledge regarding the biological function of endogenous MSCs and MSCs after either local or systemic administration is limited. In addition, several studies demonstrate that MSC function changes depending on the microenvironment they encounter, and an unresolved chronic inflammation and/or fibrosis are frequently observed in patients with severe lung disorders. The composition of the microenvironment is unique to every organ and tissue, and during pathological conditions the remodeling process can become altered and affect the MSC behavior. An increased understanding in the cross talk between MSCs and the environment they encounter will provide us with better understanding of the biological function of MSCs as well as improve clinical strategies to treat lung disorders.

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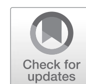

4 The Potential of Factors Released from Mesenchymal Stromal Cells as Therapeutic Agents in the Lung

Fernanda Ferreira Cruz
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4.1 Introduction

Mesenchymal stromal/stem cells (MSCs) have been widely investigated in a variety of biomedical disciplines. In the last decade, the International Society for Cellular Therapy proposed core criteria to define MSCs. First, MSCs must be plastic-adherent when kept in standard culture conditions. Second, MSCs must express CD105, CD73, and CD90, and lack expression of hematopoietic markers such as CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules. Third, MSCs must be able to differentiate to osteoblasts, adipocytes, and chondroblasts in vitro [1].

Investigators were first interested in MSCs due to their plasticity. MSCs were then understood as a class of cells from mammalian bone marrow and other sources that could be isolated and expanded in culture while maintaining their in vitro capacity to be induced to form a variety of mesodermal phenotypes and tissues [2]. Focusing on the in vitro multipotential capacities of MSCs, several preclinical and clinical trials have explored their regenerative ability, which was mainly believed to be derived from their incorporation into diseased tissue, followed by differentiation into the native cells. After years of research, several groups showed that MSCs home in on sites of injury and secrete bioactive mediators that have immunomodulatory, trophic, and repairing effects [3, 4]. In this context, some authors even support changing the name mesenchymal stem (or stromal) cells to “medicinal signaling cells,” a term that would indeed reflect their action in repairing tissues through secretion of medicinal factors that can act on the host’s own site-specific and tissue-specific resident stem cell population to construct new tissue, as well as on mature cells [5].

4.1.1 Lung Repair

Studies testing the administration of MSCs for treatment of lung diseases have evolved rapidly in recent years [6]. A very relevant aspect in terms of implications for cellular therapy in respiratory diseases was the finding that, in animal models, the intravenous infusion of labeled cells led to a markedly stronger signal in the lungs compared to several other organs such as the heart, liver, spleen, and kidney [7]. The lungs are believed to function as a filter, exerting a mechanical barrier on the passage of intravenously administered cells [8, 9]. On the other hand, there is a body of consistent experimental evidence showing

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migration of MSCs to the lungs, especially if this organ has been subjected to any type of trauma or injury [10]. It is believed that, under these conditions, the cells are attracted to the lung by chemotaxis, due to the release of local inflammatory cytokines and chemokines, combined with increased endothelial permeability at the site of injury [10]. Thus, although there is no consensus regarding the efficiency of MSC migration, experimental evidence consistently supports the possibility of using MSCs as therapy for pulmonary diseases [6, 11].

Many studies have demonstrated the efficiency of intratracheal or systemic administration of MSCs obtained from various sources, such as bone marrow, adipose tissue, cord blood, or placenta [6, 11–13]. MSC administration has been tested in an ever broader spectrum of lung injury models in animals, including the acute respiratory distress syndrome (ARDS), asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), pneumonia, pulmonary arterial hypertension (PAH), silicosis, and mechanical ventilation-induced lung injury (VILI), as well as pulmonary lesions associated with sepsis, burns, autoimmune diseases, hemorrhagic shock, and pancreatitis [6, 11, 12]. Cell therapy can also be used to treat tumors, since MSCs are able to migrate to neoplastic areas, and can even be used for delivery of antitumor or chemotherapeutic agents to cancer cells [13]. Several studies have focused on identifying optimal conditions to achieve the benefits of cell therapy, such as the best cell source, quantity, and number of passages; the impact of freezing; and the effect of sera derived from animals, among others [6, 11, 12].

The molecular mechanisms by which MSCs reduce inflammation and lung injury are not fully understood, but several beneficial effects have been described following their administration. Studies have shown that MSCs stimulate pulmonary endogenous progenitor cells to proliferate and differentiate into alveolar epithelial cells, promoting tissue repair [14]. MSCs are perhaps most recognized for their immunomodulatory ability and capacity to interact with elements of innate and adaptive immunity [3]; they are able to detect particular conditions in the microenvironment and to reverse the underlying inflammatory process [4]. In the context of acute lung injury induced by endotoxin, MSCs are able to inhibit the Th1 inflammatory response by releasing mediators such as IL-10, KGF, and angiopoietin [15]. In contrast, administration of MSCs in allergic asthma models improves airway hyperresponsiveness by reducing inflammation mediated by Th2/Th17 lymphocytes, by specific mediators, and by inhibiting proliferation of regulatory T lymphocytes [16–19]. MSCs have both direct and indirect antimicrobial activity. Directly, they promote the killing of infectious agents through the secretion of antimicrobial peptides or proteins, such as cathelicidin (LL-37), hepcidin, and defensins, [20]; these have a variety of mechanisms of action, including disruption of microbial membrane integrity, inhibition of DNA, RNA, or protein synthesis, and interference with certain intracellular targets. Indirectly, MSCs exert antimicrobial effects by stimulating innate and adaptive immune cells [20, 21].

MSCs can recover the ATP stores of monocytes, epithelial cells, and endothelial cells, enhancing energy metabolism through mitochondrial transfer and cellular respiration enzymes [22–28]. MSC therapy promotes improved pulmonary function and reestablishment of gas exchange by stimulating the production and secretion of surfactant by type II pneumocytes, promoting fluid resorption in the alveolar space by preventing synthesis of ion channels and aquaporins on the luminal surface of alveolar cells, decreasing vascular permeability by stimulating tight junctions in an acute lung injury model [29], and reducing the deposition of collagen fibers in the lung interstitium through release of metalloproteases and reduction of protein synthesis by fibroblasts [30]. In these animal experiments, these effects have been shown to result in functional improvement and reduced mortality [6, 16–19, 21, 29, 30].

4.1.2 Mechanisms of Action

Several studies have shown that the therapeutic potential of MSCs is not exclusively related to

their direct differentiation into specific cell types but rather their ability to modulate the physiology of cells in injured tissue through paracrine or endocrine stimuli [30]. MSC effects are believed to derive from their interaction with other cell types by cell-to-cell contact; by the release of soluble mediators; by the release of extracellular vesicles (EVs) containing biological mediators; or by transfer of organelles such as mitochondria (Fig. 4.1) [31].

A growing body of research suggests that administration of conditioned medium or extracellular vesicles derived from MSCs is sufficient to mimic many of the beneficial effects resulting from the administration of MSCs themselves in different models of lung injury [18, 31]. In part, this may reflect the action of cytokines, chemokines, growth factors, exosomes, or microvesicles containing mRNA, microRNAs, and long noncoding RNAs released by MSCs [31].

4.1.3 Conditioned Media

The hypothesis that MSCs act through secretion of soluble mediators has prompted several groups to investigate the content of the MSC secretome and MSC-conditioned media (CM), in the hope that these can be administered to animals or humans instead of the cells themselves. MSCs are known to release a myriad of soluble factors that have angiogenic, antiapoptotic, antifibrotic, antioxidative, microbicidal, immunomodulatory, and proliferative effects (Table 4.1) [31].

MSC-CM has been used in experimental trials, and is considered the least processed cell-free product from MSCs. Depending on the source of MSCs, culture condition, duration, and collection method, different amounts and types of secreted factors may be detected in the CM, thus leading to different experimental or therapeutic outcomes [32]. The process of obtaining CM can be summarized as follows. Generally, after reaching confluence during MSC culture, serum-containing growth media are replaced with fresh serum-free media [32–34]. Supernatant is then collected, after an incubation period of 24–48 h; centrifuged to remove cellular debris; and concentrated using ultrafilters. At this point, the cell-free product is named MSC-CM. There are several approaches to determine the yield of administered MSC-CM. One of the most common approaches is based on the number of MSCs from which the CM was derived [19, 35, 36]. Other parameters, including total protein content and the concentration

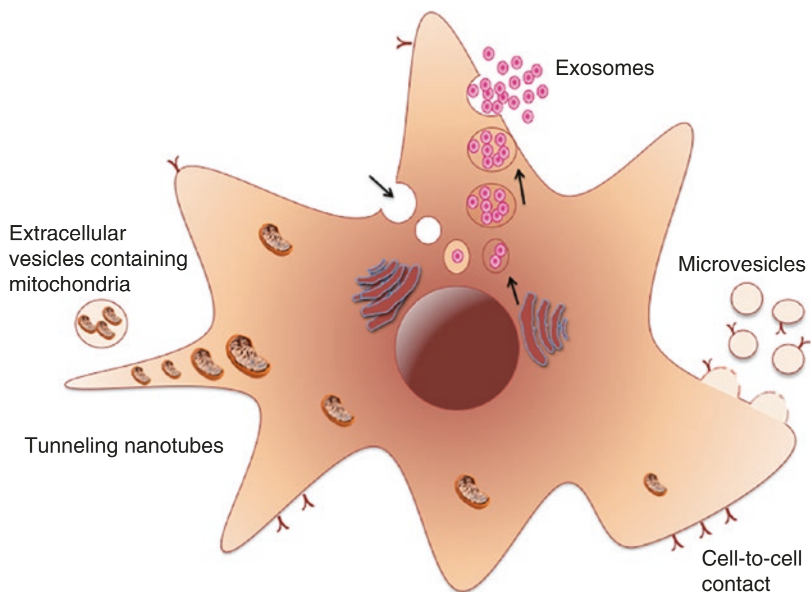

Fig. 4.1 Mechanisms of action of MSCs. MSCs can act through cell-to-cell contact, mitochondrial transfer, secretion of soluble mediators, and extracellular vesicles (classically exosomes and microvesicles)

Table 4.1 Bioactive factors in MSC-conditioned media and extracellular vesicles

Angiogenesis	Ang1	FGF	HGF	IGF-1	IL-6	MCP-1	PDGF	VEGF		
Antiapoptosis	FGF	GM-CSF	HGF	IGF-1	IL-6	STC-1				
Antifibrosis	Ang-1	FGF	HGF	KGF	MMPs	TIMP-1				
Antioxidation	HO-1	IL-1β	STC-1							
Chemoattraction	CCLs	CXCLs	G-CSF	LIF	M-CSF	MCP-1	SDF-1			
Immunomodulation	HO-1	IDO	IL-1ra	IL-6	IL-10	LIF	PGE2	STC-1	TGF-β	TSG-6
Microbicide	β-defensin	Hepcidin	Lactoferricin	Lipocalin	LL-37	Lysozyme				
Proliferation	FGF	HGF	IGF-1	KGF	PDGF	VEGF				

Ang-1 angiopoietin 1, *CCL* chemokine ligand, *CXCL* chemokine (C-X-C motif) ligand, *FGF* fibroblast growth factor, *GM-CSF* granulocyte monocyte colony stimulating factor, *HGF* hepatocyte growth factor, *HO-1* hemeoxygenase 1, *IDO* indoleamine 2,3-dioxygenase, *IGF-1* insulin like growth factor 1, *IL* interleukin, *IL-1ra* IL-1 receptor antagonist, *KGF* keratinocyte growth factor, *LIF* leukemia inhibitory factor, *LL-37* human cathelicidin, *MMP* metalloproteinase, *MCP-1* monocyte chemoattractant protein 1, *PDGF* platelet-derived growth factor, *PGE2* prostaglandin E2, *SDF-1* stem cell-derived factor 1, *STC-1* stanniocalcin 1, *TIMP-1* tissue inhibitor of metalloproteinase 1, *TGF-β* transforming growth factor beta, *TSG-6* tumor necrosis factor-stimulated gene 6, *VEGF* vascular endothelial growth factor

of certain soluble bioactive mediators, have also been considered for characterization and dosage determination [32–34].

As mentioned before, MSCs respond to microenvironmental changes and stress signals from injured tissues by changing phenotype and secretome. This means MSCs can be exposed to different stimuli, including hypoxia or proinflammatory cytokines, to promote the secretion of certain therapeutic molecules in vitro [37, 38]. In line with these findings, studies revealed that stimulation of MSCs with different insults and inflammatory cytokines, including lipopolysaccharide (LPS), eicosapentaenoic acid (EPA) [39], tumor necrosis factor alpha (TNF-α), IL-1β, interferon gamma (IFN-γ), and serum from animals with allergic asthma [40] or patients with ARDS [41], can induce the secretion of various anti-inflammatory mediators, such as TNF-stimulating gene 6 protein (TSG-6), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), IL-10, and IL-1 receptor antagonist (IL-1ra) [42, 43].

4.1.4 Extracellular Vesicles

In addition to their soluble factors, MSCs secrete different types of EVs, contributing to the overall therapeutic response [31]. In 2014, seeking to standardize the nomenclature, the International Society for Extracellular Vesicles (ISEV) published a definition of EVs and minimal experimental requirements for research about them [44]. EVs were defined as "small membrane vesicles," which includes exosomes, microvesicles, and apoptotic bodies. They are distinguished by specific membrane markers, origin, and size (exosomes, 40–150 nm; microvesicles, 0.1–2 μm; apoptotic bodies, 1–4 μm). The ISEV has since published a new guideline for EV definition [45] stating that, unless authors can establish specific markers of subcellular origin that are reliable within their experimental system(s), authors should consider use of operational terms for EV subtypes that refer to either: (a) physical characteristics of EVs, such as size ("small EVs" [sEVs] and "medium/large EVs" [m/lEVs], with ranges defined, for instance, as <100 nm or <200 nm [small] or >200 nm [large and/or medium]) or density (low, middle, high, with each range defined); (b) biochemical composition (CD63+/CD81 + -EVs, annexin A5-stained EVs, etc.); or (c) descriptions of conditions or cell of origin (MSC-EVs, hypoxic EVs, IFN-MSC-EVs) in the place of terms such as "exosome" and "microvesicle," which are "historically burdened by both manifold, contradictory definitions and inaccurate expectations of unique biogenesis." If confirmation of EV identity cannot be achieved according to the minimal requirements of the ISEV2018 guidelines, use of other terms such as extracellular particle (EP) is recommended as potentially more appropriate [45].

EVs are considered mediators of intercellular communication, as they contain several proteins, microRNAs, mRNAs, long noncoding RNAs, lipid mediators, and even organelles with biological relevance [31, 32]. Assessment of isolated EVs is mainly done by determination of concentration and size distributions using different methods, such as nanoparticle tracking analysis (NTA), transmission electron microscopy, and flow cytometry. In addition, the amount of total proteins in EVs is commonly assessed and used to select EV dosage for in vitro and in vivo studies. As for CM, the concentration of EVs or their total protein can be normalized to the number of MSCs from which they are derived. A variety of techniques may be used to isolate and concentrate different fractions of EVs from CM; these include ultracentrifugation, filtration and chromatography, precipitation, and immuno-affinity [46]. Each method provides different degrees of purity and enrichment; however, none can completely separate the different types of EVs from each other or from non-EV fractions (e.g. soluble proteins, cell-free nucleic acids, and membrane fractions). Combinations of two or more methods may be used to improve the purity of certain fractions of EVs [47].

MSC-derived EVs can be an important tool for obtaining the clinical benefits of MSC treatment [31]. Several groups have demonstrated that stem cell-derived vesicles are also immunosuppressive, probably through the transfer

of mRNA, miRNA, lnRNA, lipid and protein mediators carried by EVs [48–52]. Different strategies have been utilized to potentiate EV effects, including MSC exposure to hypoxia or to inflammatory mediators, such as interferon (IFN). EVs derived from MSCs exposed to normoxic or hypoxic conditions are efficiently engulfed by macrophages, triggering their switch from M1 to M2 phenotype [48]. EVs derived from IFNγ-primed MSCs increase macrophage phagocytosis and bacterial killing [53]. In parallel, it has been shown that the miR-146a, a well-known anti-inflammatory microRNA present in MSC exosomes, promotes M2 polarization and reduces mortality in septic mice [54]. EV-microRNA transfer to LPS-primed human monocytes has been shown to restore intracellular ATP, reduce levels of pro-inflammatory mediators, and greatly increase their phagocytic properties [27, 55]. Moreover, treatment with MSC-EVs induces a tolerogenic profile in T lymphocytes by promotion of regulatory T cells and increase in levels of the immunosuppressive cytokine IL-10 [50, 56].

EVs are also taken up by other structural cell types. Injured alveolar epithelial cells take up MSC-derived EVs through the CD44 receptor [28]. Umbilical cord-derived MSCs inhibit STAT3 signaling of hypoxic vascular endothelial cells [57]. Furthermore, EVs have potent anti-apoptotic and pro-proliferative effects in vitro [58, 59]. Finally, MSC-derived EVs have been implicated in the tissue-restoring effects of MSCs, including wound healing [60], antioxidant and antitumor effects [61], and microbicidal activity [20]. In summary, MSC-EVs, which can be rapidly isolated by ultracentrifugation and filtration, exhibit anti-inflammatory properties, decrease oxidative stress, increase ATP, reduce alveolar edema, and can promote bacterial clearance. These properties suggest they could be safely and easily used for therapy of lung diseases.

Stem cell-derived EVs have been tested in experimental lung injury, including models of asthma, ARDS, COPD, idiopathic pulmonary fibrosis, pneumonia, pulmonary artery hypertension, and silicosis.

4.1.5 Mitochondrial Transfer

Dysfunctional mitochondria can lead to cellular damage and apoptosis. Thus, a rescue mechanism wherein healthy mitochondria can be transferred to a diseased or injured host has tremendous therapeutic potential [62]. In the past few years, mitochondrial transfer was demonstrated as one of the main mechanisms through which MSCs can regenerate and repair injured cells in lung disorders and acute respiratory disorders [62]. Some groups have shown that mitochondrial transfer mechanisms can occur through nanotubules, gap junctions, cell fusion, and via EVs [62, 63]. MSCs transfer mitochondria to recipient cells after stress signals, only a few of which have been described, such as release of damaged mitochondria, mitochondrial DNA, and elevated reactive oxygen species levels [63]. However, cell signaling pathways that lead to mitochondrial transfer from healthy cells are still under investigation, and the changes that contribute to restoration of mitochondrial bioenergetics in recipient cells remain largely elusive [62]. Interestingly, mitochondrial transfer has been studied mostly in lung diseases (Table 4.2).

Human epithelial cells that had lost their mitochondrial activity, when cocultured with healthy MSCs, recovered the ability to perform mitochondrial respiration through MSC-mitochondrial transfer. This was shown by time-lapse photomicrography demonstrating presence of DsRed2-tagged MSC-mitochondria in epithelial cells. The same study also confirmed that uptake of mitochondria was facilitated by active and not passive transfer [22].

Islam and colleagues reported mitochondrial transfer in an in vivo study. Intratracheally instilled murine and human bone marrow-derived (BM)-MSCs were able to transfer mitochondria to pulmonary alveolar cells, increasing ATP concentration, repairing mitochondrial bioenergetics, and exerting protective effects in a murine model of LPS-induced acute lung injury [23]. Mitochondria were observed in the alveolar epithelium over a period of 24 h, and connexin 43 mediated mitochondrial transfer through formation of nanotubes and

Table 4.2 In vitro and in vivo studies of mitochondrial transfer

	Recipient cell	Mitochondrial transfer mode	Effect of mitochondrial transfer	Reference
1	A549 Cell line	Cellular contact and cytoplasmic projections	Rescue aerobic respiration in mammalian cells with nonfunctional mitochondria	Spees et al. [22]
2	Mice Alveoli epithelial cells	Connexin 43 alveolar attachment and microvesicles	Protective effects in acute lung injury model restituting alveolar bioenergetics	Islam et al. [23]
3	Rat airway epithelial cells	Tunnel tubes	Protective effects in cigarette smoke-induced injury rescuing bioenergetics of airway epithelial cells	Li et al. [24]
4	Murine bronchial epithelial cells	Tunnel tubes and miro1	Protective effects in models of asthma and bronchial epithelial cell injury	Ahmad et al. [25]
5	Macrophages	Extracellular vesicles	Regulation of mitochondrial dynamics and mitophagy	Phinney et al. [26]
6	Macrophages	Tunnel tubes	Increase phagocytosis and microbicide activity, as well as protective effects in acute lung injury model	Jackson et al. [27]
7	Macrophages	Extracellular vesicles	Polarization into M2 phenotype, increase microbicide activity and protective effects in acute lung injury model	Morrison et al. [28]

Mitochondrial transfer from bone marrow-derived mesenchymal stromal cells to different recipient cells in experimental lung diseases

microvesicles in a calcium-dependent manner. Following this study, Ahmad et al. showed the role of Miro1, a mitochondrial Rho-GTPase, in mitochondrial transfer from MSCs to lung epithelial cells through connecting nanotubes in a murine rotenone-induced lung injury model; further confirmation was obtained in an asthma model [25]. The group reported that overexpression of Miro1 enhanced mitochondrial transfer, whereas Miro1 knockdown reduced both mitochondrial transfer and MSC rescue efficacy. Mitochondrial transfer was also observed in a rat model of emphysema induced by cigarette smoke exposure. Li et al. reported that bone marrow-derived MSCs reduced lung damage by transferring mitochondria to lung epithelium through tunnel tube formation after 24 h of administration, restoring epithelial ATP levels [24]. However, further research along these lines is warranted, as the reasons for differences are largely unknown and a comparative analysis of mitochondrial transfer abilities of stem cells from different sources has yet to be performed.

Li et al. also suggested that mitochondrial dysfunction is observed in cases of prolonged inflammation, and that stem-cell mitochondrial transfer capacity can be used as an indicator of their rescue potential by promoting anti-inflammatory effects [24]. In parallel, MSCs have been shown to help fight infection by transferring mitochondria through nanotubes to macrophages, increasing their bioenergetics; this leads to augmented phagocytic ability, serving as an important mechanism in preclinical models of ARDS and sepsis [27, 28]. It has also been reported that noncontact transfer of mitochondria through microvesicles or exosomes significantly enhanced mitochondrial transfer and the phagocytosis index of macrophages, suggesting mitochondrial transfer by secreted extracellular vesicles as another critical mechanism for cellular therapy efficacy [28]. Another study by Phinney et al. has also shown that mitochondria are transferred through microvesicles to macrophages, where they support paracrine and immune reaction [26]. Further investigations to better understand the role of mitochondria in antimicrobial effects and immune modulation should provide better insights into the reparative contributions of mitochondria to the maintenance of cellular health after transfer to recipient cells [62].

4.1.6 Impact of the MSC Secretome and EVs in Lung Diseases

4.1.6.1 ARDS

Therapy with MSC-derived CM has already been tested and shown to lead to improvement of acute lung injury in mice [35, 36]. Human MSC-derived EVs have been shown to reduce lung permeability and edema in a murine model of LPS-induced acute lung injury [64, 65]. EVs also reduced neutrophil infiltration and macrophage inflammatory protein-2 levels in bronchoalveolar lavage fluid (BALF) by 73% and 49%, respectively, indicating a reduction in inflammation. Silencing KGF via siRNA pretreatment of MSCs partially abolished the therapeutic effects of the secreted EVs, suggesting that KGF plays an important role in the underlying mechanism [65]. In recent ARDS research, mitochondria secreted in MSC-EVs were shown to promote an anti-inflammatory and highly phagocytic macrophage phenotype, which depended critically on oxidative phosphorylation [27, 28]. Finally, EVs from IFN-γ–primed human umbilical cord MSCs more effectively attenuated *E. coli*-induced lung injury compared with extracellular vesicles from naïve mesenchymal stromal cells, potentially via enhanced macrophage phagocytosis and killing of *E. coli* [53].

4.1.6.2 Asthma

There is a growing experience demonstrating the benefit of MSC-derived secretome therapy in experimental asthma [19, 66, 67]. When administered systemically, both CM and, in particular, EVs isolated from human and murine BM-MSCs at the onset of antigen challenge in previously sensitized mice were as potent as MSCs themselves in mitigating Th2/Th17-mediated allergic airway inflammation in a mouse model of severe refractory clinical asthma. Human MSCs (hMSCs), conditioned media, and EVs were effective in this immunocompetent mouse model, ameliorating *Aspergillus* hyphae extract-provoked increases in airway hyperreactivity, lung inflammation, and the $CD4^+$ T-helper 2 (Th2) and Th17 phenotypes. Notably, both CM and EVs from hMSCs were generally more potent than those from mouse MSCs (mMSCs) in most of the outcome measures [19]. When both soluble mediators and EV secretion were blocked by the cross-linking agent 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, the observed effects of hMSCs were fully abolished, whereas with the administration of mMSCs, they were partly ameliorated. These results demonstrated potent xenogeneic effects of CM and EVs in an immunocompetent mouse model of allergic airway inflammation [19].

A recent study assessed the effects of systemically administered adipose tissue-derived MSCs and their extracellular vesicles. Both had beneficial effects in ovalbumin-induced allergic asthma, acting on the inflammatory process and reversing tissue remodeling [67]. While the effects of each were largely similar, differences were observed in outcome assessment of lung mechanics and inflammation: MSCs and EVs had different effects on eosinophil cell counts, levels of eotaxin, IL-4, and IL-13 in lung parenchyma, $CD3^+CD4^+$ T cells in BALF, and lung mechanics [67]. This highlights the importance of in-depth studies of the differential mechanisms by which MSCs versus EVs might act in respiratory diseases.

4.1.6.3 Chronic Obstructive Pulmonary Disease

In the past decade, conditioned media obtained from bone marrow-derived MSCs protected a rodent model of emphysema induced by cigarette-smoke exposure, reducing inflammation and histological damage within the broncho-alveolar airspace and lung parenchyma. The authors have shown the conditioned media may recover lung fibroblasts from cigarette smoke-induced damage, possibly through inhibition of apoptosis, induction of proliferation, and restoration of lung fibroblast repair function, which are mediated in part by the PI3K/Akt pathway [68].

Recently, therapy with extracellular vesicles derived from adipose-derived MSCs and therapy with artificially generated nanovesicles from the same cell source was tested in a murine model of emphysema induced by elastase. Nanovesicles were generated by using sequential penetration through polycarbonate membranes, displayed

a size (100 nm) and spherical shape resembling those of natural exosomes, and expressed both exosomal and stem-cell markers [69]. Nanovesicles can be obtained in large scale, differently from exosomes, that are naturally released only in very small amounts [70]. The proliferation rate was increased in lung epithelial cells treated with artificial nanovesicles compared with cells treated with natural exosomes; lower doses of nanovesicles compared to natural exosomes were able to induce similar regenerative effects. Taken together, these data indicate that lower doses of ASC-derived artificial nanovesicles may have beneficial effects similar to those of higher doses of ASCs or ASC-derived natural exosomes in experimental emphysema, suggesting that artificial nanovesicles may have economic advantages and be clinically applicable to emphysema patients [69].

4.1.6.4 Idiopathic Pulmonary Fibrosis

The secretome obtained from bone marrow-derived MSC inhibits the proliferation of fibroblasts and enhances HGF-mediated lung epithelium wound repair in vitro. Furthermore, significantly enhanced numbers of MSC were grown from lung tissue obtained from patients with fibrotic lung diseases as compared to lung tissue obtained from patients with normal lung. Thus, a significant anti-fibrotic effect of the MSC-secretome in vitro was demonstrated. The in vitro anti-fibrotic properties of the secretome make it an interesting candidate to be tested as a novel therapeutic approach for patients with IPF and further in vivo studies are needed to consolidate such an approach [30].

In parallel, some authors have tested the effects of extracellular vesicles in animal models of lung fibrosis. Tan et al. [71] showed that purified exosomes from amniotic MSCs directly polarized and increased macrophage phagocytosis, reduced neutrophil myeloperoxidases, and suppressed T cell proliferation. Intranasal instillation of exosomes one day following bleomycin challenge reduced lung inflammation, while treatment at day 7 improved alveolar collapse and reduced fibrosis [71]. However, a contradictory result suggested that MSC-isolated microvesicles reduced pulmonary fibrosis, and that MSCs have a better treatment effect than microvesicles. Unfortunately, these authors did not reveal the amount of MSCs used for isolating the MV, and the result is also lacking in dosage effectiveness [72]. Finally, an in vivo efficacy study demonstrated that intravenous delivery of extracellular vesicles derived from hMSC in a murine model of fibrosis induced by intratracheal injection of bleomycin significantly downregulated α-smooth muscle actin expression and decreased histopathological fibrosis, indicating therapeutic effects of these vesicles for established lung fibrosis through modification of the myofibroblast phenotype [73].

4.1.6.5 Pneumonia

MSC's antimicrobial potential is particularly interesting, as these cells are involved especially in dynamic coordination of the pro- and anti-inflammatory elements of the immune system or in increasing phagocyte activity, as well as directly by secretion of antimicrobial peptides and proteins (AMPs) [20]. AMPs are evolutionarily conserved, gene-encoded small effector molecules that interact with different molecular targets either on the cell surface or within cells. Importantly, in some specific cases, AMPs can be active against pathogens that are resistant to conventional antibiotics (e.g., multidrug-resistant bacteria). In this context, MSCs from different sources or origins have shown ability to reduce the burden of pathogens in different preclinical models of pneumonia, regardless of the route, dose, or timing of administration [74]. Interestingly, treatment with MSC coadministered with antibiotic therapy presented better microbicide response in chronic skin infections associated with biofilms [75]. Antibiotic-activated MSC were found to accumulate around injured tissue, where macrophages assumed an M2 phenotype, compared to untreated infections which contained predominately M1 macrophages. Bacterial killing by MSC was found to be mediated in part by secretion of cathelicidin in the conditioned media and it was significantly increased by antibiotics. Studies in pet dogs with spontaneous chronic multidrug-resistant wound infections demonstrated clearance of bacteria and tissue repair. Thus, systemic

therapy with activated MSC may be an effective new, non-antimicrobial approach to treatment of chronic, drug-resistant infections [75].

Administration of hMSC-derived EVs decreased the influx of inflammatory cells, cytokines, protein, and bacterial load, resulting in higher survival rates of mice with bacterial pneumonia, in a mechanism partially dependent on keratinocyte growth factor secretion. The antimicrobial effect of BM-MSC-derived EVs was partly attributed to enhancement of monocyte phagocytosis of bacteria while decreasing inflammatory cytokine secretion, as well as to increased intracellular ATP levels in injured alveolar epithelial type 2 cells. The therapeutic effects of released EVs could be further enhanced by pre-stimulation of BM-MSCs with a TLR-3 agonist before isolation [76].

4.1.6.6 Pulmonary Arterial Hypertension

In experimental models of pulmonary arterial hypertension (PAH), some authors have previously shown that the MSC-derived cell-free conditioned media afforded superior protection compared to MSCs themselves in preventing alveolar damage [77]. Administration of MSC-derived extracellular vesicles in HAP animals improved histology and cardiovascular function [78]. EVs suppressed the hypoxic activation of signal transducer and activator of transcription 3 (*STAT3*) and upregulated lung expression of miR-204, a key microRNA, which is normally decreased in human PAH. Finally, EVs inhibited vascular remodeling and consequent pulmonary hypertension [78].

A few years ago, a group sought to determine which EV subpopulation plays the major role in the reversal of PAH in mice. They found that the exosome fraction of EVs isolated from mMSCs (MSC-EXOs) prevented and reversed PH in a monocrotaline-induced model of PAH. Furthermore, MSC-EXOs contain increased levels of miRNAs that blunt angiogenesis, inhibit proliferation of neoplastic cells, and induce senescence of vascular smooth muscle cells and endothelial progenitor cells. EXOs isolated from hMSCs were just as effective as those from mMSCs in reversing pulmonary hypertension in mice [79]. Together, these findings suggest that exosomes play a prominent role in mediating the pulmonary vascular remodeling of PAH and may be a promising modality for its treatment. More information regarding the effects of MSCs in HAP can be read in Chap. 8.

4.1.6.7 Silicosis

MSC-derived EVs are able to reduce neutrophil and lymphocyte accumulation in BALF and reduce collagen deposition in lung parenchyma in silicotic mice [26, 80]. Research by the same group showed that MSCs manage intracellular oxidative stress by targeting depolarized mitochondria to the plasma membrane via arrestin domain-containing protein 1-mediated microvesicles [26]. The resulting vesicles are engulfed by macrophages and reutilized to enhance their bioenergetics. Simultaneously, MSCs shed exosomes enriched with micro-RNAs that inhibit macrophage activation, desensitizing macrophages to the ingested mitochondria. In parallel, administration of both adipose tissue-derived MSCs and EVs, intratracheally delivered, ameliorated fibrosis and inflammation, but dose-enhanced EVs yielded better therapeutic outcomes in this model of silicosis [80]. These studies provide evidence of a mechanistic link between MSC survival and macrophage function [26].

4.2 Conclusion

MSCs promote beneficial effects in lung diseases through paracrine effects. Soluble mediators, extracellular vesicles, and mitochondria play important autocrine/paracrine roles in intercellular communication. EVs package proteins, mRNA, microRNA, lnRNAs, and lipid mediators, which have the ability to transfer biological information to recipient cells in the lungs [81]. Conditioned media and EVs derived from MSCs have been found to promote therapeutic activities that are comparable to those of MSCs themselves. Recent animal studies suggest that MSC-derived EVs have significant potential as a novel alternative to whole-cell therapies.

Compared to their parent cells, EVs may have a superior safety profile, and can be stored without losing function. MSC-derived EVs suppress proinflammatory processes and reduce oxidative stress, pulmonary fibrosis, and remodeling in several in vivo inflammatory lung disease models by transferring their components [82]. However, there remain significant challenges to translate this therapy to clinical trials, such as the decision of the best moment to begin cell therapy, the best dose, frequency, and route of administration, as well as MSC source and culture conditions.

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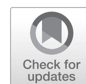
Part II

Preclinical Evidence and Clinical Applications in Chronic Lung Diseases



5 Preclinical Evidence for the Role of Stem/Stromal Cells in COPD

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5.1 Introduction

An estimated 330 million people worldwide suffer from Chronic Obstructive Pulmonary Disease/COPD, and it is the third leading cause of death killing around 2.9 million every year [1]. It is believed that the increased use of tobacco (a known risk factor for COPD), especially in less-developed countries, will further increase the mortality within the next decade [2]. COPD is predicted to be the third largest cause of global death by 2030 [3].

Clinically, COPD is associated with cough, sputum production, and dyspnea, but the symptoms and severity of symptoms are highly variable [4]. Patients commonly experience decreased quality of life and progressive limitations in day-to-day activities [4]. Symptoms are manifestations of chronic inflammation in the airways and lung parenchyma as well as emphysema, but the clinical phenotype varies with some patients having predominantly bronchiolitis, whereas others mainly have emphysema [5]. Importantly, comorbidities are very common in COPD. In one study, almost 98% of the patients had at least one comorbidity [6]. Some of the most common comorbidities are cardiovascular disease, metabolic disorders, osteoporosis, lung cancer, gastrointestinal diseases, and cognitive impairment [7].

COPD is categorized into four groups (A-D) according to guidelines prepared and published by the Global Initiative for Chronic Obstructive Lung Disease (GOLD). Revised guidelines were published in 2017 taking risk (airflow limitation), exacerbation history, and symptoms into account when assigning patients to one of four categories (A-D) (From the Global Strategy for the Diagnosis, Management and Prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017. Available from: https://goldcopd.org.). The new guidelines have been refined to allow symptoms and exacerbations alone to assign the A-D category, minimizing the need for spirometry. Spirometry is needed for a diagnosis, but not for monitoring and adjustment of medications. Furthermore, a more personalized disease management regimen is encouraged in the new guidelines, where symptoms and future risk of exacerbations act as the main determinants of treatment strategy in stable disease.

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The single most important risk factor for COPD is cigarette smoke; however, a minority of cases cannot be attributed to smoking. A meta-analysis [8] that investigated COPD risk factors other than smoking found strong evidence linking development of COPD with genetic predisposition as well as occupational exposure to dust, fumes, and gases. Biomass smoke, outdoor pollution, and secondhand smoking have also been identified as risk factors for COPD. In developing countries, a large proportion of COPD occurs in never-smokers, especially women who cook over fire stoves fueled with biomass such as wood or animal dung [9]. The particulates generated by this burning are similar to cigarette smoke, and live fire in an enclosed space may create a noxious environment triggering processes similar to inhaling cigarette smoke.

Similar to many other chronic lung diseases, COPD therapy focuses on relieving symptoms and prevention and management of exacerbations to keep patients in a stable condition and prevent deterioration. The only curative therapy is lung transplantation, which is not an option for many patients due to age or comorbidities as well as shortage of donor lungs. The need for alternative, novel and effective therapeutic options is thus obvious.

Despite extensive research efforts on COPD, the exact pathology is still not fully elucidated, and the greatest mystery is why some individuals develop disease, whereas others do not. If investigated thoroughly, most individuals who have smoked for a number of years are likely to display some symptoms of COPD, such as bronchitis or slightly decreased lung function. However, in many cases the symptoms are not severe enough to prompt a clinical visit, thus a large number of people unknowingly have mild COPD. Longitudinal studies have shown that only around 50% of the patients who are diagnosed with COPD actually display a progressive and accelerated decrease in lung function [5]. It has been suggested that susceptibility to COPD relates to regenerative capacity and abnormal aging [10]. In COPD, features of accelerated and aberrant aging are present, such as increased cell senescence, increased oxidative stress, alterations in extracellular matrix (ECM) and stem cell depletion and are believed to contribute to pathology [11].

5.2 Risk Factors

Risk factors related to COPD include an interrelationship between genetic factors and exposures to different types of pollutants in various environments. Additionally, the patient may be affected by comorbidities that play a role in the development of COPD [12]. The most relevant genetic factor linked to COPD is deficiency of serine protease α1 antitrypsin (A1AT), which affects about 1–3% of COPD patients. Low α1 antitrypsin concentrations in combination with other environmental factors, such as smoking or other type of exposures, raise the risk of panlobular emphysema [13]. A number of other genes have been implicated in COPD, including those for transforming growth factor β1 (TGF β1) [14] and tumor necrosis factor α (TNF α) [15].

5.2.1 Smoking as a Risk Factor

Overall, tobacco smoke is the main environmental risk factor associated with the development of COPD. According to a WHO estimate, 70–75% of COPD mortality has a connection with smoking in more-developed countries, while in less-developed countries the indices are around 40% [16]. However, there is a clear relationship between genes and susceptibility to disease, since up to 50% of smokers develop COPD [12, 15, 17, 18]. In addition, smoking during the gestational period can adversely affect proper development of fetal lungs and may contribute to the emergence of lung diseases later in life [19, 20].

The use of marijuana has been related to respiratory symptoms caused by the pyrolysis of the burning of the drug but to date there are insufficient data that correlate the use of the substance to the development of clini-

cal COPD [21–23]. However, there are a few case reports and small cohort studies where pathologic changes (e.g. airway inflammation and bullous emphysema) in the lung have been observed in marijuana smokers [24–26]. Furthermore, there is recent evidence in animal models that marijuana smoke can induce features of COPD [27].

5.2.2 Occupational Dust, Vapors, and Fumes

Exposure to many types of materials that become suspended in the atmosphere such as dust, chemicals, and fumes in the workplace can also introduce a significant risk for the development of COPD. In less-developed countries, where occupational exposure to dust and fumes may be even higher than in developed countries due to less stringent workplace regulations, occupational exposures may play a prominent role as a risk factor. Patients with a diagnosis of COPD or chronic bronchitis recall exposures to previous gases, dust, vapors, or smoke in the workplace at a rate of twice that of patients without a diagnosis [28].

5.2.3 Indoor Air Pollutants

Worldwide, one of the major risk factors for the development of COPD is exposure to biomass fuels, such as coal, straw, animal manure, crop residues, and wood, which are typically used to keep houses warm, as well as for cooking. In less-developed countries, many homes have inadequate ventilation which compounds the problem. According to WHO estimates, in the least developed countries, exposure to internal smoke from biomass fuels was a key factor in about 35% of people with COPD [16]. Reports from China have shown that the prevalence of COPD in women who have never smoked is 2–3 times higher in a rural area where women are exposed to smoke from biomass burning in contrast to urban women without such exposure [29].

5.2.4 Air Pollutants

The threat posed by external pollutant exposures for the development of COPD is thought to be lower than exposure to indoor pollutants [8, 30, 31]. Air pollution has also been shown to be related to increased rates of lower respiratory infections and acute exacerbations and cardiopulmonary events, which in turn may also be important in the development and progression of COPD [31, 32]. There is also evidence linking air pollution with the worsening of COPD. The recognition of negative health outcomes at high levels of pollution has led governments to develop legislation that encourages a drastic reduction in atmospheric pollutants, particularly emissions from industries [33]. Unfortunately, despite the decrease in the emission of these traditional, industrial pollutants, there has been a concomitant increase of air pollutants from motor vehicle traffic in some parts of the world such as China and Brazil. There is strong evidence relating adverse health effects and the amount of pollutants dispersed due to vehicle pollution [33], but legislation to curb this form of pollution has been challenging to pass and enforce across all regions worldwide.

The negative effects observed from pollution are strongly related to ozone levels [34]. Significant increases in ozone are known to be capable of triggering a series of damaging lung effects, ranging from decreased pulmonary function, worsening of preexisting respiratory diseases, to increased hospital admissions as well as death. Several North American and European studies have shown increased risk of hospitalization for exacerbation of COPD related to the presence of high concentrations of ozone [35].

5.2.5 Summary of Risk Factors

There are a number of risk factors related to COPD and this includes a combination of genetic and external factors such as exposure to pollution and smoking that increase the chances of development of the disease. However, despite these known associations from epidemiological data,

the exact origin of COPD and its interconnection with specific gene candidates remains elusive, with increased difficulty in these analyses due to the heterogeneity of the disease. The timing and duration of exposure, in combination with genetic factors, is thought to be a key determinant in the onset of clinical symptoms and the development of disease. COPD takes several decades to develop following exposure or injury [36]. Patient age at the time of exposure and in combination with exposure type and duration is critical as to whether disease develops. Short-term exposure during development (i.e. 6–9 months) or early life infections has been shown to have dramatic lifelong consequences on clinical parameters such as rate of decline in forced expiratory volume in 1 s (FEV1) over time and later development of COPD [37]. Patients with early life exposure may remain largely asymptomatic for much of their early adulthood and only first present when chronic disease has set in. However, a similar exposure timeframe of the same type of damaging agent (e.g. pollution, smoking, infection) during adulthood does not seem to have the same longitudinal effects. The great challenge for the clinic is the identification of early pathological changes before the development of symptoms, since this is the most likely time window for effective therapies.

5.3 Disease Pathogenesis

The pathological changes in COPD are characterized by chronic inflammation in the airways (chronic bronchitis/bronchiolitis) as well as the lung parenchyma, in addition to the destruction of alveolar parenchymal tissue (i.e. emphysema) [38, 39]. Importantly, the clinical phenotype varies, where some patients predominantly have bronchiolitis whereas others mainly have emphysema [5]. Additionally, tissue remodeling such as small airway fibrosis is an early event in the development of COPD and commonly precedes emphysematous changes [40]. The specific correlation between remodeling and inflammation is not yet fully elucidated, although both play significant parts in the pathogenesis. A number of different inflammatory and resident cell types have been implicated in COPD pathogenesis—both airway and parenchymal remodeling. Structural cells such as epithelial cells, endothelial cells and fibroblasts are deranged in COPD and have also been shown to release inflammatory mediators which may enhance inflammation, highlighting the close connection between inflammation and remodeling [5, 40].

Inflammatory cells are recruited from the blood into the lungs of patients with COPD. This recruitment is orchestrated by chemotactic factors which are released locally. The inflammatory response includes both innate and adaptive immune responses. The innate response is characterized by the presence of eosinophils, neutrophils, macrophages, mast cells, natural killer cells, γδ-T cells, innate lymphoid cells (ILCS), and dendritic cells (DC) while the adaptive response is associated with T lymphocyte activity and B-cells [41].

In general, macrophages and neutrophils have both been well characterized in terms of their contributions to COPD pathogenesis [42]. There is an increase in the presence of macrophages in the airways, pulmonary parenchyma, bronchoalveolar lavage (BAL), as well as in the sputum of COPD patients. Markers of proinflammatory M2-type macrophages are elevated in BAL of COPD patients, and macrophages from patients with COPD release greater amounts of inflammatory mediators (e.g. IL-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α)) [43, 44], as well as elastolytic enzymes, including matrix metalloproteinases (MMP) -2, MMP-9, and MMP-12 and cathepsins K, L, and S in comparison to patients without COPD [45]. A subgroup of M2-type distorted macrophages may possibly contribute to the defective remodeling process in COPD [46].

Inflammatory proteins are regulated in the macrophages of COPD patients via several different factors such as nuclear factor transcription factor—κb (NF-κb) [47]. Alveolar macrophages of patients with COPD become highly activated during exacerbations [48]. In addition, macrophages additionally release chemokines for CD8 + cytotoxic T cells (Tc1) and CD4 + Th1 cells [49].

Characteristically, inflammation in COPD is described as an increase in the number of activated neutrophils in the sputum and BAL, which correlates with disease severity. There is a low number of neutrophils in the airway wall and in the parenchyma as compared to the sputum and BALF. Neutrophils are recruited to the respiratory tract by several neutrophil chemotactic factors, which are elevated in the airways of patients with COPD. Neutrophils secrete proteases which in turn contribute to alveolar destruction and additionally may play a role in mucus hypersecretion observed in COPD. Hypersecretion of mucus occurs through the following proteases: NE (neutrophil elastase), cathepsin G, and proteinase-3 being potent stimulants of mucus secretion from the submucosal glands and goblet cells [50].

The occurrence of high levels of mast cells in patients with COPD with centrilobular emphysema has been described; however, their potential contribution to disease has not yet been definitively determined [51]. In an animal model of cigarette smoke-induced COPD, tryptase-expressing mast cells were shown to be required for the development of murine COPD [52]. However, other studies have shown decreased amounts of mast cells in the small airways, parenchyma and blood vessels of COPD patients with more severe disease [53]. Thus, further studies are needed to clarify the exact role of mast cells in COPD.

Increased numbers of B- and T-lymphocytes are present in lungs with COPD compared to healthy lungs, and a relation to disease severity, level of alveolar damage and airflow obstruction [49, 54]. B cells become established in the lung parenchyma and airways and are organized into lymphoid follicles [54]. T lymphocytes are also in higher concentration in the pulmonary parenchyma and airways in patients with COPD, with a greater presence of CD8 + T cells in relation to CD4 + T cells [49, 54]. The amount of T cells is linked to the level of alveolar damage and the level of airflow obstruction.

Th17 cells, which secrete IL-17A and IL-22, are in higher concentrations in the airways of patients with COPD and possibly play a role in the modulation of neutrophilic inflammation [55, 56]. Innate lymphoid cells (ILCs) also have a role in the regulation of pulmonary immunity and can be regulated by signs of danger and cell damage [57]. In COPD cases, there is a concentration of ILCs cells that are similar to Th17 innate cells that release IL-17 and IL-22 and possibly play a role in the induction of neutrophilic inflammation [58].

5.3.1 Oxidative Stress

In COPD, environmental oxidative stress as well as endogenous lung and systemic oxidative stress play essential roles in the onset and development of the disease [59]. Smoking, the recognized major risk factor for the onset of COPD, induces oxidative stress in the airways and epithelium of patients with COPD [59]. There is also decreased expression of antioxidants in the lung of COPD patients, particularly in the epithelium and alveolar macrophages. Many antioxidants are regulated by the transcription factor "Nuclear factor (Erythroid-derived2)-like 2" (Nrf2), which is normally activated by oxidative stress. However, in patients with COPD, there is a reduction in Nrf2-responsive genes as compared to normal patients, despite the fact that there is a high level of oxidative stress in the lungs of patients with COPD [60]. Thus, the normal negative feedback loop which exists to regulate oxidative stress appears to be impaired in patients with COPD.

In addition, oxidative stress is capable of hindering antiprotease functions, such as α 1—antitrypsin, and thus enhances the degradation of elastin in the lung parenchyma.

5.3.2 Protease–Antiprotease Imbalance

There is a large body of evidence that suggests that patients with COPD have an imbalance between proteases and antiproteases [61]. Neutrophil elastase (NE) and other proteases, such as matrix metalloproteinases (MMPs) are known to play a role in COPD pathogenesis. MMPs are a family of more than 20 linked endopeptidases with potential for degradation of all

the elements that make up the ECM of the lung parenchyma, including elastin, collagen, proteoglycans, laminin, and fibronectin. MMPs are synthesized and released from neutrophils, alveolar macrophages, and airway epithelial cells [62]. The enzymatic conversion of the proteolytically inactive pro-form of the MMPs to proteolytically active MMPs can occur through intracellular processing or in the extracellular space through interaction with a variety of neighboring cell types (e.g. airway smooth muscle cells, fibroblasts) [63–65]. MMP1, 9, and 12 have all been found to be increased in COPD and play a role in ECM degradation [66, 67].

Defective antiprotease levels also play a key role in the development of COPD. A1AT is the most well-characterized antiprotease with regards to COPD development and it normally functions to neutralize neutrophil elastase activity. In addition, other serum protease inhibitors such as serpins and elafins, can similarly neutralize elastolytic activity in the lung [68].

5.3.3 Systemic Inflammation and Comorbidities

Systemic inflammation is common in COPD patients (up to 70%), mainly in patients with severe disease and during exacerbations, where the levels of circulating cytokines, chemokines, and acute-phase proteins are elevated [69, 70]. Systemic inflammation is related to negative clinical outcomes and greater decline in lung function. It is currently unknown whether this represents extravasation of the peripheral pulmonary inflammatory process, or if it is a concomitant abnormality associated with diseases which are comorbid with COPD (e.g. cardiovascular diseases, diabetes, and lung cancer) [71–73].

5.3.4 Inflammatory and Structural Consequences

Mucus hypersecretion is a hallmark of COPD where it contributes to small airway obstruction, and exerts effects on ciliary function and mucociliary clearance [50]. Submucosal gland hyperplasia is present in the large airways and there is a greater amount of goblet epithelial cells in COPD. Both NE and serine proteases are increased in COPD and produced by neutrophils (described earlier to be increased in COPD) are powerful mucus secretion stimulants and have been shown in in vivo animal models to induce goblet cell metaplasia [74], which may explain the connection between mucus hypersecretion (chronic bronchitis) and neutrophilic airway inflammation in normal smokers and patients with COPD [75].

Peribronchiolar fibrosis commonly occurs in COPD, and is believed to be the result of activation of fibroblasts in the small airways by fibrogenic mediators, such as TGF-β and connective tissue growth factor (CTGF), both of which have been shown to be produced by epithelial cells and macrophages [76] as well as the fibroblasts themselves, in addition to the endothelins which have been associated with a variety of lung cell types [77]. Fibrosis of small airways may be an early injury in COPD and usually precedes the onset of emphysema [40, 78].

5.3.5 ECM Deregulation

The ECM composition is known to change with age [79, 80], and similarly, the ECM also undergoes changes in COPD [81, 82]. In COPD, the most prominent changes involve deranged expression of ECM proteins, such as collagen, fibronectin, and laminin [41], changes in the structural organization of collagen, such as collagen fiber disorganization [83] and loss of elastic fibers [84].

Imbalances between proteases, such as matrix metalloproteinase 12 (MMP12), neutrophil elastase, and antiproteases, such as A1AT and tissue inhibitor of metalloproteinase (TIMP) 1–4, leads to parenchymal tissue remodeling (i.e. loss of alveolar spaces) [85]. In addition to the loss of alveolar tissue, ECM fragments generated from these proteases have also been shown to play a role in the development of the pathogenesis of COPD. One such example is the degradation products of elastin. Elastin fragments instilled into the trachea of mice have been shown to be

sufficient to cause emphysematous changes in the animals due to their proinflammatory effects [86–88]. Several other ECM fragments have been shown to be elevated in the serum of patients with COPD as compared to normal patients and increased amounts of collagen fragments were associated with increased mortality [89, 90]. These fragments have been shown to induce inflammation in other contexts (e.g. laminin and fibronectin fragments are chemotactic to human neutrophils and monocytes), but fragments from ECM components other than elastin have not yet been mechanistically linked to COPD development [82, 91]. Ongoing proteolysis of ECM generates fragments that may perpetuate inflammation even after smoking cessation and further drive disease progression.

It was previously believed that in COPD lung repair capacity is impaired; however, new evidence suggests that the COPD lung is attempting to repair itself, but that this process is deranged. Many COPD patients have been identified to have high expression of genes that participate in elastogenesis, such as fibulin-5 (FBLN5), microfibril-associated protein 4 (MFAP4), latent transforming growth factor binding protein 2 (LTBP2), and elastin (ELN) [92].

It is not yet clear whether the presence of these components is beneficial or potentiates the pathogenesis of the disease, since these ECM proteins have the potential to interfere with different cell signaling pathways [93, 94]. Additionally, another intriguing aspect of COPD is the change in pulmonary fibroblasts. These cells are directly related to homeostasis and repair of ECM in the lungs and several studies have shown differences in the production of ECM components between fibroblasts from COPD patients as compared to fibroblasts from non-COPD controls, indicating a potential role in dysfunctional repair [95].

5.3.6 Exhaustion of Stem Cells and Senescence

There is growing evidence that COPD may be due in part to acceleration of pulmonary aging. There is evidence of accumulation of senescent cells, including epithelial cells of the airways and alveolar regions, endothelial cells, and fibroblasts [11, 96, 97]. Senescent cells produce a range of inflammatory proteins, such as: TNF-α, IL-1β, IL-6, TGF-β, and MMP-9 and ROS. This phenotype is known as the senescence-associated secretory phenotype (SASP), which amplifies and propagates senescence [98].

Inflammatory proteins, including those in the SASP, are elevated in the lungs of patients with COPD. The SASP may be a mechanism driving COPD and COPD-associated disorders (e.g., cardiovascular disease, chronic kidney disease), which are also diseases associated with accelerated aging. There is strong evidence that the SASP is diffused from cell to cell through the release of extracellular vesicles [98], which may represent a mechanism of systemic transfer of inflammatory proteins from one organ undergoing accelerated aging to a distant organ.

It is generally understood that adult lung tissue normally remains in a quiescent state; however, the lung has a great diversity of cells, including stem and progenitor cells, which help to maintain homeostasis and lung structure and function [99]. Following lung injury, lung tissue can be repaired by the activation of stem cells capable of promoting tissue repair through proliferation and transdifferentiation of endogenous stem and progenitor cell populations to replace the damaged cell types [100].

Endogenous stem cell populations of the lung have been most extensively characterized in the murine lung and to date several different regional populations of cells with regenerative capacity have been identified in the adult lung such as the basal airway cells, located in the trachea, bronchi, and small airways, as well as the alveolar type II cells. These cell types are less characterized in the adult human lung as compared to the murine lung due to a limited number of tools available to explore their regenerative potential. Basal cells have the capacity for self-renewal and can differentiate into secretory, ciliated, and neuroendocrine cells [101]. Alveolar type II cells [102] are present in the distal lung with the capacity to reestablish cells of the same type and to transdifferentiate in to alveolar cells type I, thus ensuring maintenance

of the gas exchange surface [103]. Several studies have also demonstrated the presence of lung-resident mesenchymal stromal cells (MSCs), a multipotent cell type which can proliferate in vivo and ex vivo. This cell type has been shown to assist in the recovery of damaged lung tissue by suppressing inflammatory processes when administered exogenously following ex vivo expansion, but their role in normal lung physiology and, furthermore, in COPD remain unclear [104, 105].

COPD has been associated with a decreasing number of circulating hematopoetic and endothelial progenitor cells, indicating a potential loss in the capacity for repair from cells recruited into the lung [106]. Smoking, a significant risk factor for the development of COPD, has been shown to reduce the potential for repair of endothelial and epithelial progenitor cells [106, 107] and mesenchymal stem cells present in the bone marrow, in turn hampering homing and cellular proliferation [108].

Consequently, stem cell depletion may play a role in the pathogenesis of COPD, decreasing the endogenous potential for lung replacement and repair by local and recruited cells. In line with this, basal progenitor cells have been shown to have a lower regenerative capacity in COPD [101] and ATII cells from COPD patients show different transcriptomes as compared to ATII cells derived from normal patients [109]. Thus, replacement or stimulation of endogenous stem cell populations may present a therapeutic target.

5.4 Animal Models of COPD

Although widely studied, COPD is still a disease with enormous negative effects on human health causing alarming mortality rates globally. Unfortunately, the available treatments are not very effective for the cure of a disease with different aspects and numerous risk factors.

Animal models of the disease allow greater understanding of disease onset and potential progression and additionally provide an experimental avenue for the identification and analyses of mechanisms that may provide new therapeutic targets for the treatment of the disease. The use of animal models allows us to improve our understanding of some of the known hallmarks of the disease such as the inflammatory events, airway remodeling, and the development of pulmonary emphysema.

Several different species and strains of animals have been used in combination with different damaging agents to study COPD in animals. There is no animal model which completely recapitulates the human disease, but rather, the models aim to mimic different aspects of both acute and chronic phases of COPD such as exacerbations (i.e. acute inflammation), bronchitis, and emphysema. Much like the human epidemiological data suggests, there are several different agents that can result in similar phenotypic changes (i.e. chronic exposure to cigarette smoke or exposure to elastase results in emphysematous changes). Tables 5.1 and 5.2 briefly summarize some of the known animal models and species

Table 5.1 Animal models with acute pulmonary inflammation

Species	Strain	Agent and application	Frequency and amount	Phenotype	References
Mouse	Balb/cJ	TNBSA (IT)	15 µL 2.5% TNBSA	Inflammation	[110]
Mouse	C57BL/6 J	d0: PPE (IT) d21: LPS (IT)	PPE (4.2 U) LPS (1 mg/kg)	Emphysema	[111]
Rat	S-D	LPS	0.1 mL LPS (100 µg/mL)	Pulmonary inflammation-bronchoconstriction	[112]
Rat	S-D	d0–14: CS d14: Nac (oral[a])	25 c/d 1% Nac	Bronchitis	[113]
Rat	S-D	HT (IV) + O_3 (WBE)	0.05 ppm O_3 for 4 h	Airway hyperresponsiveness	[114]

IT intratracheal, *TNBSA* 2,4,6-trinitrobenzene sulfonic acid, *PPE* porcine pancreatic elastase, *LPS* lipopolysaccharide, *S-D* Sprague-Dawley, *CS* cigarette smoke, *c/d* cigarettes/day, *Nac* N-acetylcysteine, *HT* intravenous 5-hydroxytryptamine, *O_3* ozone, *WBE* whole body exposure
[a]Drinking water contained 1% Nac

Table 5.2 Animal models with chronic COPD-like lung disease

Species	Strain	Agent	Frequency and amount	Phenotype	References
Mouse	B6C3F1/Crl	CS	WBE, 6 h/d, 5d/wk. for 7, 13 months	Emphysema	[115]
	BALB/C, C57BL/6,	CS	NOE, 2c/d, 5d/wk. for 1–12 weeks	Emphysema, airway remodeling	[53]
	C57BL/6 J	CS	WBE, 3c/d, 5d/wk. for 7 months	Emphysema	[116]
	DBA/2 J	CS	WBE, 3c/d, 5d/wk. for 7 months	Emphysema/fibrosis	[116]
	A/J	CS	WBE, 6 h/d, 5d/wk. for 15 weeks	Emphysema	[117]
	A/J	E-CS	WBE containing 18 mg/ml nicotine, 5d/wk. for 4 weeks	Airway hyperreactivity/airspace enlargement/cytokine, protease expression	[118]
	C57BL/6 J	E-CS	WBE, 1,5 h/2×/d, 2 weeks	Inflammation, impaired bacterial and viral defenses	[119]
Rat	F344	CS	WBE, 6 h/d, 5d/wk. for 7, 13 months	Emphysema	[115]
	S-D	PPE + MCh	PPE for 4 weeks and (MCh) for 30-s, 3 min between each inhalation	Emphysema	[120]
Guinea pig		CS	NOE, 5c/d, 5d/wk. for 13–16 weeks	Emphysema	[121]
		CS	10 cigarettes for 1–12 months	Emphysema	[122]
Hamsters		CS	Cigarettes for 59–80 weeks	Laryngeal cancer/metaplasia/accumulation of alveolar macrophages	[123]
		LPS	Twice a week for up to 5 weeks	Emphysema/bronchial mucus cell hyperplasia (BMCH)	[124]
Canine	Beagle	CS	Inhalation for 6-month or 1-year periods	Impaired mucociliary transport/central airway and bronchiolar walls lesions/tracheal epithelial basal cell hyperplasia	[125]
Ferret		CS	60 min of smoke from 3R4F research cigarettes, twice daily for 6 months	Chronic bronchitis and bronchiolitis	[126]

CS cigarette smoke, *WBE* whole body exposure, *NOE* nasal only exposure, *LPS* lipopolysaccharide, *PPE* porcine pancreatic elastase, *PPE + MCh* Intratracheal instillation of PPE (1 IU/g body wt in 1 ml saline) and inhaled methacholine (MCh), *S-D* Sprague Dawley

and strains used for them. The models listed in Tables 5.1 and 5.2 are the most well-characterized and widely used models for evaluating potential therapies for COPD. However, in the clinical scenario, there is a genetic component to disease, and the models in Tables 5.1 and 5.2 do not take this into account, beyond susceptibility of the strain. Several other animal models exist to emphasize the genetic component (Table 5.3).

Interestingly, there are several animal models where COPD spontaneously develops as the animal ages. These models are distinctly different from animals born with enlarged airspaces (Table 5.4) and these models may be interesting to use to explore potential new therapies. Many of these transgenic animals have altered levels of ECM and ECM-assembly components (e.g. Emilin 1, Fibulin-5) which supports

Table 5.3 Genetic models (Strain of mice naturally predisposed to develop increased air space and emphysema)

Strain	Mutation	Evaluation timeframe	Finding	References
Tight-skin (*tsk*) mouse	Fibrillin-1	4 days to 16 months	Emphysema	[127–129]
Beige (Bg)	5 kb deletion in Lyst	1, 12 and 24 months	Adult lungs: Generalized enlargement of air spaces	[129]
Blotchy (*Blo*)	Menkes disease gene (Mnk)	26–192 days	Defective collagen and elastin cross-linking, enlarged air spaces and effacement of alveolar septa	[130]
Pallid (pa)	Serum α1-antitrypsin	2–16 months	Progressive increase of elastase and decreased expression of elastin from 2 months of age	[128, 129]
Osteopetrotic (Op/Op)	Deficient in macrophage colony stimulating factor	20–120 days	Increased levels of MMPs, abnormal elastin deposition, and spontaneous development of emphysema	[131]
Klotho	Deficient in klotho	2–9 weeks	Enlargement of air spaces, destruction of normal alveolar architecture	[64]

Table 5.4 Developmental defects and spontaneous development of COPD features from transgenic and knockout mice

Transgenic	Phenotype	References
Platelet derived growth factor A (PDGF-A) −/−	Absence of tropoelastin expression and failed alveolar septation	[132]
Fibroblast growth factor receptor (FGFR) 3 and 4 −/−	Mice lacking both FGFR 3 and 4 (but not FGFR 4 alone) are normal at birth but do not form secondary septae or alveoli	[133]
Fibulin-5/DANCE −/−	Increased elastin deposition subsequent to alveogenesis, growth retardation/abnormal distal airway development and alveogenesis due to defective development of elastic fibres	[134]
Elastin −/−	Fewer and dilated distal air sacs at birth and arrested terminal airway development (the mice die from obstructive arterial disease due to subendothelial cell proliferation and a reorganization of smooth muscle	[135]
Retinoic acid receptor (RAR) γ −/−	Increased alveolar size is worsened in RARγ−/− by co-deletion of retinoid X receptor-α	[136]
Forkhead box F1 (Foxf1) transcription factor +/−	Severity of the pulmonary abnormalities correlates with the levels of Foxf1 mRNA, those with lowest levels have defects in alveolarization and vasculogenesis	[137]
Tumour necrosis factor- α converting enzyme (TACE/ ADAM−17) −/−	Lungs fail to form normal saccular structures, fewer peripheral epithelial sacs, deficient septation, and thick-walled mesenchyme	[138]
POD−1 (Tcf21, capsulin, epicardin) −/−	Hypoplastic lungs, abnormal lung branching, lackingalveoli and type II pneumocytes. Mice die in perinatalperiod	[139]
Tissue inhibitor of metalloproteinases(Timp)-3−/−	Progressive air space enlargement evident at 2 weeks	[140]
Surfactant protein D −/−	Progressive pulmonary emphysema from 3 weeks of age	[141]
Wnt2/2b−/−	Hypoplastic lungs with relatively normal airway development	[142]
Wnt4−/−	Lung hypoplasia and tracheal abnormalities, reduced mesodermal proliferation in the lung bud	[143]
Wnt5a−/−	Truncated trachea, overexpansion of distal airways, thickened intersaccular interstituim (knockout)	[144]
SPC-Wnt5a overexpression	Smaller lungs, reduced number of alveolar sacs with dilated alveoli, lobation abnormalities (transgenic)	[145]
Conditional knockout of Wnt7b in Sox2-expressed embryo	Hypoplastic lungs with normal patterning and cell differentiation, proportionate decrease in the replication of epithelial and mesenchymal progenitors	[146]

Table 5.4 (continued)

Transgenic	Phenotype	References
Conditional knockout of β-catenin in Shh-expressing cells	Defective bronchiolar epithelial cell differentiation and marked ectasis of the developing and adult airway (Shh-β-catenin-conditional knockout)	[147]
Emilin1−/− Emliin1−/− and Fibrillin-1 mutant	Emilin1 loss effects early vessel morphogenesis; airspace enlargement in Emilin1−/− and exacerbated in Emliin1−/− and Fibrillin-1 mutant	[148]
Taz knockout	Enlargement of alveolar space at birth due to abnormal lung development	[149]
Haptoglobin-case	Enlarged air spaces	[150]
CC-10-IL-11	Abnormal air space development	[150]
SP-C-PDGF-B	Enlarged air spaces, fibrosis, inflammation	[151]

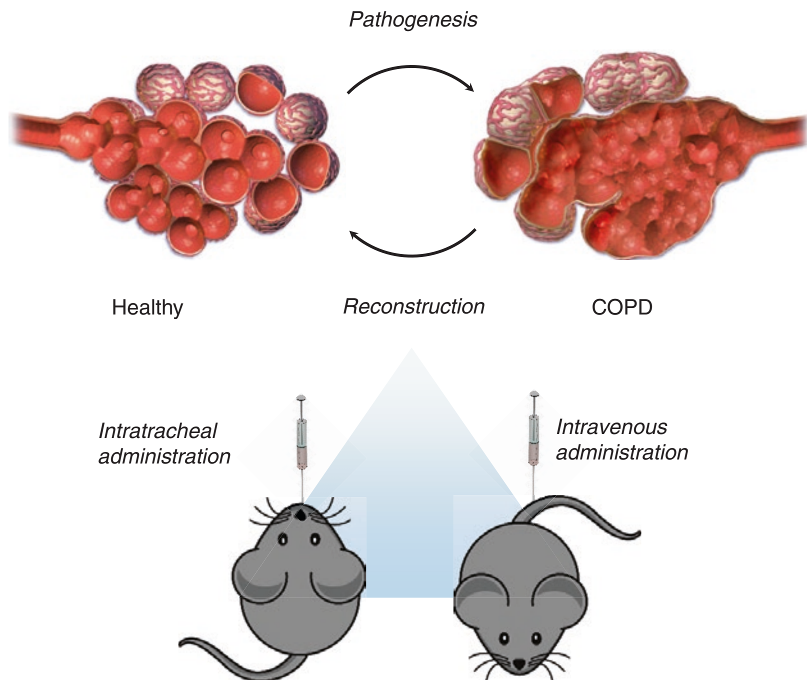

Fig. 5.1 Schematic showing an overview of current preclinical in vivo cell therapy approaches for COPD

recent work indicating that the matrix itself may be a driver of COPD [93, 152, 153].

5.5 Stem Cell Therapies in Animal Models

One potential therapeutic approach being explored for COPD is cell therapy. Cell therapy is based on administration of cells or stem cells into injured or diseased tissue/organs as a regenerative therapeutic approach and has been recently explored for COPD treatment [154]. A wide variety of cell types have been used preclinically for COPD cell therapies and these range from bone marrow mononuclear cells, mesenchymal stromal cells (MSCs) derived from various sources (e.g. adipose and bone marrow) to embryonic stem cells (ESCs) (see Fig. 5.1, Table 5.5 and ref. [104]).

Most studies to date have tested the efficacy of cell therapies in animals treated with elastase or CS exposure, with a few that exploited the use of papain instillation as a COPD model prior to cell therapy

Table 5.5 Preclinical studies with stem cell therapy for COPD treatment

Cells used		COPD model			Cell therapy			
Species	Types	Species	Agent	Exposure time	Administration time	Route	No of cells	References
Mouse	BM-MSC	Mouse	Elastase	Day 0	Day 14	IT	5×10^5	[155]
Mouse	BM-MSC, AD-MSC, L-MSC	Mouse	Elastase	Days 0, 7, 14, 21	Day 21	IT/IV	1×10^5	[156]
Mouse	BM-MSC	Mouse	Elastase	Day 0	Day −1, 0, 1, 21	IT IV	5×10^5 1×10^5	[157]
Mouse	BM-MSC	Mouse	Elastase	Day 0	Day 14	IV	NA	[158]
Mouse	ESC	Mouse	Elastase & Irradiation	Day 0 Day 2	Day 2	IT/IV	1×10^6	[159]
Guinea pig	ASC	Guinea pig	CS	Days 0–90	Day 90	IT/IV	1×10^6	[160]
Guinea pig	ASC	Guinea pig	CS	Days 0–90	Day 90	IT/IV	1×10^6	[161]
Rat	ASC	Rat	Elastase	Day 0	Day 7	IV	5×10^7	[162]
Rat	BM-MSC	Rat	Irradiation & Papain	Day 0 Day 0	Day 0	IV	4×10^6	[163]
Rat	BM-MSC	Rat	Papain	Day 0	Day 0	IV	4×10^6	[164]
Rat	BM-MSC	Rat	CS	Day 0–60	Day 60	IV	6×10^5	[165]
Rat	ASC	Rat	Elastase	Day 0	Day 7	IV	2.5×10^6	[166]
Rat	BM-MSC	Rat	CS	Day 0–77	Day 49	IT	6×10^6	[167]
Rat	AF-MSC	Rat	CS & LPS	Day 0–84 Days 28 and 56	Day 84	IT	4×10^6	[168]
Rat	BM-MSC	Rat	CS & LPS & Irradiation	Day 0–60 Days 0 and 14 Day 90	Day 90	IV	4×10^6	[169]
Rat	BM-MSC	Rat	CS & LPS	Day 1–34 Days 0 and 14	Day 35	IV	5×10^6	[170]
Rat	BM-MSC	Rat	CS	Day 0–84	Day 49	IT	6×10^6	[171]
Rabbit	BM-MSC	Rabbit	Elastase	Day 0	Day 1	IB	1×10^8	[172]
Human	CB-MSC	Mouse	Elastase	Day 0	Day 7	IV	Various	[173]
Human	T-MSC	Mouse	CS & Irradiation	Day 0–75 Day 60–75	Days 60 and 67	IP/IN	1×10^6	[174]
Human	ASC	Mouse	CS	Day 0–168	Days 56, 70, 84, 98	IV	3×10^5	[175]
Human	iPS-MSC, BM-MSC	Rat	CS	Day 0–56	Day 29 and 43	IV	3×10^6	[176]

AD-MSC adipose-derived MSC, *AF-MSC* amniotic fluid-derived MSC, *ASC* adipose tissue-derived stromal cells, *BM-MSC* bone marrow-derived MSC, *CB-MSC* cord blood-derived MSC, *CS* cigarette smoke, *ESC* embryonic stem cell, *IB* intrabronchial, *IN* intranasal, *iPS-MSC* induced pluripotent stem cell-derived MSC, *IT* intratracheal, *IV* intravenous, *L-MSC* lung-derived MSC, *T-MSC* tubal MSC

in rats [163, 164]. Cells have been introduced through both intratracheal and intravascular routes, with both showing efficacy [156]. Preclinical studies have used syngeneic, allogeneic, and xenogeneic models, including the use of human cells in rodent models of COPD [173–176]. Despite the fact that there is a large variation in the type and number of cells used, in general, cell administration has resulted in a reduction of inflammation as well as reduction in histological emphysema and a corresponding increase in gas exchange [162].

Interestingly, in the majority of studies, cell engraftment does not appear to be the mechanism driving improvement [155]. Furthermore,

cell-derived products such as conditioned media, which contains extracellular vesicles secreted from cultured cells, extracellular vesicles alone and extracellular nanovesicles synthetically derived from adipose stem cells have been shown to have ameliorating effects in animal models of COPD [165, 177]. Therefore, in many preclinical studies, the goal of many cell therapies is immunomodulation and/or reduction of apoptosis, rather than engraftment and a contribution of these cells to structural repair of the lung.

Alternatively, embryonic stem cells (ESCs) were tested in a recent study for pulmonary regeneration in an elastase model of COPD [159]. In this study, the authors hypothesized that increased engraftment would correlate with an increase in the efficacy of cell therapy and an improvement in lung function and restoration of structure. In order to improve engraftment, the authors pre-sensitized the animals with irradiation, which has been previously shown to result in higher cell engraftment efficiencies in conjunction with naphthalene administration [178]. This effect was also confirmed with the ESCs resulting in improved cell engraftment after irradiation of the elastase-treated mice with emphysematous lungs [159]. However, in many studies which show a positive effect of cell therapy, no engraftment has been observed. Therefore whether or not engraftment is the end goal may be dependent on both the model (i.e. type of injury) and type of cell therapy (type and route of administration). See Table 5.5 for a summary of models, cell types, routes of administration, and dosing.

5.6 Activation of Endogenous Progenitor Cells Using Pharmacological Approaches

While COPD was once considered to be a predominantly inflammatory disease, there has been increasing evidence of late which suggests that COPD is also characterized by an impaired regenerative response of endogenous progenitor cell populations. It was long thought that the human lung contained little to no regenerative capacity, but recent case reports have challenged this paradigm and caused increased attention towards attempts at activating endogenous regeneration in the adult lung [179, 180]. Thus, while exogenous cell therapy, as discussed above, has received attention over the last decade, there has been renewed interest in identifying and exploring the potential of activating endogenous cell populations through pharmacological approaches in COPD. Both endothelial and epithelial cells have been identified to be deranged in COPD [5, 101, 106, 107, 181]. Approaches which seek to activate endogenous stem cell populations through pharmacologic approaches have been recently explored and may be an additional area of interest in the future [107]. In previous studies, pharmacologic activation of the Wnt pathway in both an in vivo and ex vivo model of emphysema, including ex vivo culture of human lung tissue from patients with COPD, promoted an increase in several distinct markers indicating the potential initiation of repair attempts. Surfactant protein C production and secretion was increased and was concurrent with increased expression of phenotypic markers of type I alveolar epithelial cells in ex vivo COPD lung tissue as well as deposition of new elastin, suggesting that the onset of stem cell-mediated repair in the COPD lung is feasible in vitro [182].

5.7 Considerations for Translating Effective Stem Cell Therapies into the Clinic

Development of successful treatment strategies for COPD requires bridging the gap between preclinical and the clinic. For this to succeed, a key aspect is awareness of opportunities and pitfalls, both when modeling disease and translating results to the clinic.

COPD is a complex disease that develops over many years, manifests as variable symptoms of airway/alveolar inflammation and emphysema, where diagnosis is primarily based

on spirometry. The pathological complexity as well as the time aspect makes COPD a difficult disease to model.

5.7.1 Modeling Human Disease in Animals

There is, as yet, no animal model which completely mimics clinical COPD. The majority of papers utilize the elastase model of emphysema or cigarette smoke. While these models both recapitulate the structural phenotype of COPD, occupational exposure and indoor air pollutants remain a major risk factor and there is a general lack of these models in the literature which may hamper the development of effective therapies for a broader patient population. However, despite these limitations, animal models can recapitulate symptoms similar to COPD, including emphysema and bronchitis. When comparing humans to research animals, some significant biological and physiological differences are found, which should be taken into account during translational studies. Several anatomical differences are present between humans and small rodents, such as the number of lung lobes (mice have four lobes in the right lung, whereas humans have three), number of generations of airways (13–17 in mice and 17–21 in humans), the presence of respiratory bronchioles in humans but not rodents, as well as the alveolar fraction of the total lung volume (18% in mice and 12% in humans). These differences may also be of importance in successful translation of promising therapies from rodents to humans.

In mice, the pulmonary circulation supplies the entire lung, including the intraparenchymal airways. In contrast, larger bronchi are supplied by branches from the systemic circulation in larger animals [183]. Furthermore, the pulmonary circulation is a low pressure system with less vascular musculature and reacts differently to hypoxia compared to the systemic circulation. These may be significant differences to take into account when seeking to model COPD.

Interestingly, the thickness of the respiratory membrane is around 0.3 μm in mice and rats, but 0.6 in humans [184]. This difference may be highly relevant in situations when the diffusion capacity is of importance. Moreover, despite the fact that the architecture of the pulmonary vasculature is relatively similar in mammals of different sizes, it is scaled to fit the size of the animal [185]. The number of capillaries per alveoli differs depending on size; humans have around 20 capillaries/100 alveoli, whereas rats only have 0.4. The degree of muscularization and the size of muscularized vessels vary between species (the pulmonary vascular system is extensively reviewed in [185]), and obviously simple factors such as distance from IV injection site to lungs may be highly variable from a couple of centimeters in a mouse to several decimeters in humans. These are all facts that may have significant effects on local and/or systemic administration of cells in cell therapy.

Another difference is posture, where the four-leg position of most research animals results in a different ventilation and perfusion profile of the lungs compared to the two-legged stance of humans. This is an important consideration for histology or when obtaining samples for analysis or determination of distribution of administered cells.

5.7.2 Translating Experimental Data to the Clinic

The translation of experimental data and protocols into clinical reality is rarely straightforward. Numerous studies have identified effective targets in animals, with no or marginal effect on human disease.

COPD is multifactorial and usually develops after many years of smoking, unless the patient has a genetic mutation such as α1-antitrypsin deficiency. COPD is a disease of the elderly and potentially also premature aging [10]. Studies have shown aging to be of vital importance in COPD (reviewed in [11]), yet most studies of COPD pathology are performed in young or young adult mice. Furthermore, elderly patients have experienced a lifetime of inhalational exposures, infections and normal lung matrix degen-

eration and remodeling, which may affect their susceptibility to damaging agents and disease progression rate, all of which are factors that cannot easily be mimicked in animal models. However, there is a wide variety of models available for studying cell therapies in animal models, yet the overwhelming majority have focused on elastase and cigarette smoke. Furthermore, cell therapy is performed during the acute or inflammatory phase of the model. Animal models which more closely mimic chronic lung disease may be more suitable. Alternatively, cell therapies should also be tested in the later phases of the models where inflammation has subsided, but major structural changes remain.

On the other hand, clinical studies mainly include patients during stable disease and not during an acute exacerbation. Therefore, the efficacy of cell therapy on an ongoing inflammation may differ significantly from the effect on stable disease or regeneration, and thus treatment may necessarily need to differ accordingly. Treatment of COPD patients undergoing acute exacerbations may more closely mimic the current preclinical data and may be an opportunity for clinical study.

A previous study investigated MSC administration to COPD patients, and found the regimen to generally be safe and to decrease CRP in patients with elevated levels at inclusion [186]. This is in accordance with the notion that MSCs have an anti-inflammatory role, and induce expression of genes associated with regulation of immune responses and oxidative stress [187]; however, there was no significant clinical effect observed. A second study administered autologous MSCs to eight patients undergoing lung volume resection surgery (LVRS) on two separate occasions. Patients received two autologous infusions, separated by one week. Three weeks after the second infusion, they underwent a second LVRS and tissue was collected for analysis. There were no adverse events and there was an increase in the endothelial marker CD31, indicating that the MSCs were able to exert a biological effect, even in patients with severe disease. However, further studies are needed to better understand whether there was any functional improvement. Despite no clear clinical evidence and legislation in place to prevent the administration of cells to patients outside of formal clinical trials, there has been an increase in the number of clinics offering unproven cell and stem cell therapies around the globe and COPD is one of the most commonly targeted lung diseases [188, 189]. Thus, it is important that properly conducted (scientifically and ethically sound) clinical trials are conducted moving forward.

In addition to local pulmonary manifestations, COPD also induces systemic inflammation which is believed to be related to known comorbidities such as cardiovascular disease [190]. Low-grade systemic inflammation affects the endothelial barrier and may over time result in endothelial dysfunction and remodeling [181]. Dysfunctional endothelial activation may have a profound effect on the localization of MSCs following systemic administration as activated endothelial cells express adhesion receptors to which MSCs may bind [191]. This may affect the number of viable cells ultimately ending up in the lung.

Another variable is introduced if/when comorbidities are treated pharmacologically, as the drugs may affect MSCs or other cell therapies directly, or the niche where they are supposed to reside. Furthermore, decreased kidney function, altered pulmonary circulation, or a change from laminar to turbulent blood flow may have a significant impact on the effectiveness of the experimental treatment, as they affect blood flow and perfusion. Thus, animal models which have multiple comorbidities may be interesting to include as preclinical models.

For rare diseases or specialized methodological approaches, it is nowadays common to perform multicenter studies. This allows a study to be run in parallel at different locations, instead of sequentially in one or two locations, which has several advantages but also poses some unique challenges. To be comparable, the execution of the study needs to be identical at all sites. For example, the thawing procedure of frozen cells, the size and type of needle used to infuse cells IV and whether patients on oxygen therapy or continuous positive airway pressure (CPAP) are to be included. CPAP may be critical for local cell administration

as well as IV administration, but is not taken into account when animal experiments are performed. Differences in cell viability following thawing have been observed to play a role in a recent study which administered MSCs in patients with ARDS [192]. Therefore, improved understanding of the technical and methodological details important to successful cell therapy are important to better understand and present an opportunity for further investigation in animal models.

From a more technical point of view, xenogeneic cell administration (e.g. human cells into rodents) has been shown to induce a specific response, but one cannot assume that human cells administered to humans will induce the same results, due to species mismatch. It is well known that inflammation differs both between species and strains of the same animal [193]. Key players such as cytokines will differ between species and may contribute to the interspecies incompatibility which has been described [194]. The use of human tissue such as precision cut lung slices or decellularized lung tissue from humans recellularized with primary patient cells may provide an opportunity for further investigation [93, 153, 182].

Due to differences in body size, and thus number of cells needed for administration, expansion in vitro may be needed to obtain a sufficient number of cells to administer. In similarity to other cells, culturing MSC in vitro may induce changes in proliferation rate and cell activity [195], as well as methylation status of the cells [196], all of which may have significant effects on the outcome. These are all areas which should be explored further.

5.8 Conclusion

A model that strictly mimics all features of COPD in a laboratory animal is not yet feasible, for practical, economic, and ethical reasons. Models should be chosen depending on the specific aims and goals of a study. In addition, scaling up results obtained from experiments performed in mice or rats cannot be assumed to be directly applicable to humans. Understanding biological and anatomical differences between species are crucial for the successful translation of promising preclinical therapies from models to man.

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6 Clinical Application of Stem/Stromal Cells in COPD

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6.1 Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive life-threatening disease that is significantly increasing in prevalence. The World Health Organization (WHO) predicts that COPD will become the third leading cause of death worldwide by 2030 [1–3]. There is currently no cure for this disease, and smoking cessation remains the most prominent intervention [4]. Because of the lack of effective curative pharmaceutical options and the increase in prevalence, extensive efforts have been devoted to the development of new strategies for cell replacement and tissue remodeling in COPD. So far, most focus has been on mesenchymal stromal cell (MSC) therapy. MSC are theoretically ideal candidates for cell therapeutic approaches because of their low or absent constitutive HLA class I and II expression, allowing allogeneic administration of MSCs obtained from normal healthy volunteers, and their immunosuppressive and antibacterial properties [5, 6]. In this chapter, we will examine in detail the biological rationale for use of MSCs in COPD, clinical trials, and the current challenges for implementing this approach as a potential therapy for COPD.

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6.2 COPD—A Heterogeneous Lung Disease with No Curative Treatment Available

COPD is a progressive lower respiratory condition, which has a massive impact on public health worldwide. Increasing in prevalence, COPD is currently responsible for over 120,000 US deaths annually and is expected to become the third leading cause of death globally in the next few years [7]. COPD is most often associated with long-term smokers over the age of 40 and is thought to be driven by abnormal tissue response(s) to inhaled toxic particles over time. The life expectancy of continuous cigarette smokers is at least 10 years shorter than nonsmokers and the absolute risk of developing COPD among this population has been estimated to be 15–30% [8]; however, there is evidence for significant underdiagnosis [9, 10]. The most common symptoms of COPD are chronic bronchitis (persistent cough with chronic mucus production), dyspnea (shortness-of-breath), wheezing, and chest tightness. As a progressive disease, these symptoms get worse

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over time. Current treatments, most importantly smoking cessation, are part of a delay strategy to slow down the physiological disease progression. These physiologic changes all contribute to the impairment of efficient breathing and include: the gradual loss-of-elasticity of the lung tissue leading to collapse of airway and alveolar sacs, weakening-to-rupture of alveolar septal walls, enlargement of segmented airspace, loss of gas-exchange surface area, increased mucus production, airway plugging, and airway narrowing driven by swelling and fibrosis (Fig. 6.1). COPD is a complex pathology with a diverse spectrum of clinical phenotypes, comorbidities, and treatment profiles [11, 12]. The GOLD criteria have been widely utilized to help standardize the COPD definitions and treatment guidelines; however, they do not fully encompass the diversity of COPD phenotypes [13, 14].

The treatments available to patients diagnosed with COPD are not curative and cannot completely stop disease progression; however,

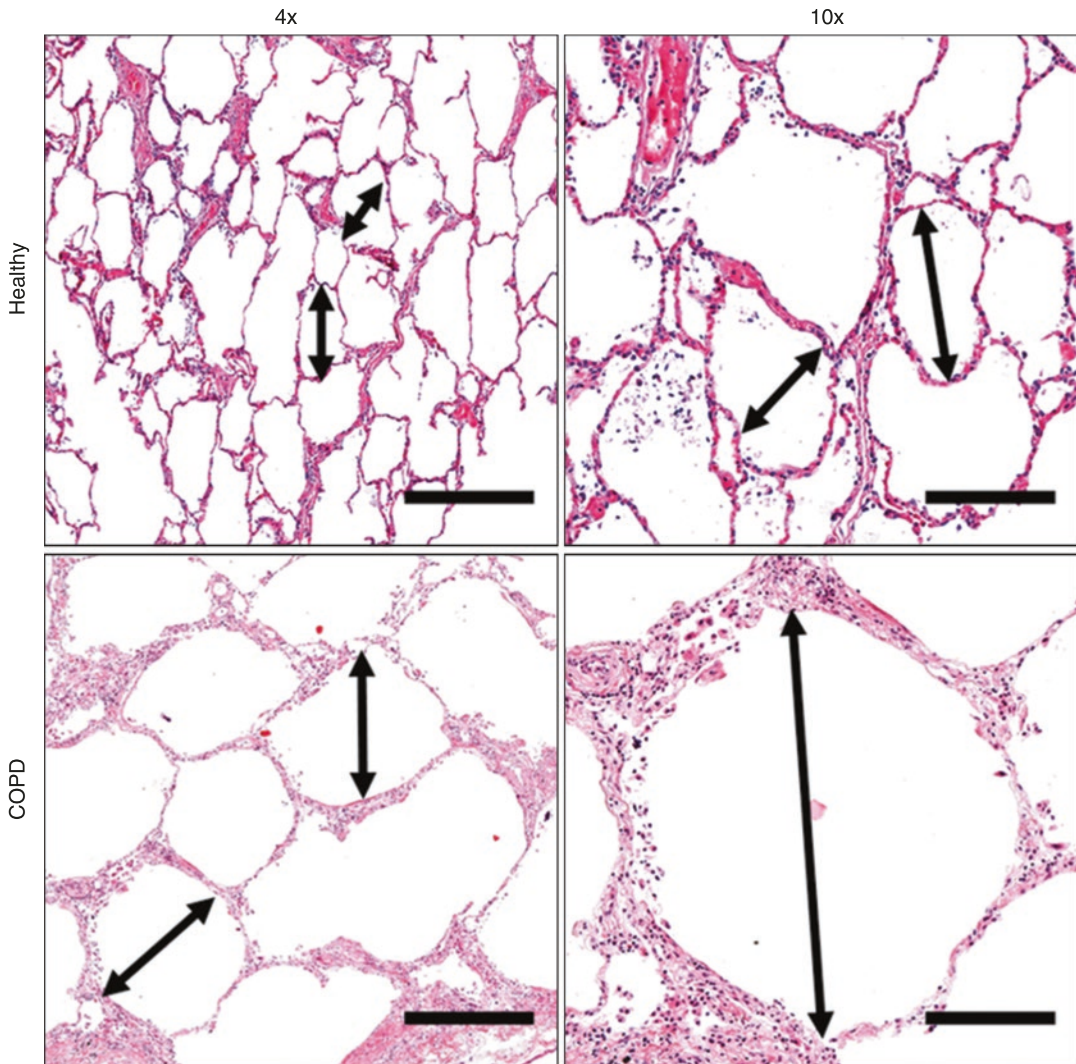

Fig. 6.1 Photomicrographs of lung tissue obtained from healthy and chronic obstructive pulmonary disease (COPD) subjects stained with hematoxylin-eosin. Arrows indicate alveolar space (side-to-side alveolar wall distance). Scale bars at 4× magnification represent 500 μm and at 10× magnification 200 μm

they are key to slowing disease progression and importantly to improve quality of life. The most important intervention at any stage is the cessation of smoking and/or limitation of exposure to other identified environmental risk factors. Symptomatic treatment throughout disease progression often relies on bronchodilators, which are inhaled beta-agonists or muscarinic antagonists. Early-stage individuals will most often be treated with short-acting bronchodilator therapies (SABA/SAMA); however, as the disease progresses treatment will need to incorporate long-acting drugs that affect these receptors (LABA/LAMA). Unfortunately, bronchodilators can only partially resolve lung hyperinflation in emphysema [15], becomingly increasingly less effective as the disease progresses. Inhaled corticosteroids (ICS), often used to treat acute respiratory exacerbations, work by interfering with the transcription pathways of key inflammation genes; however, this treatment does not always work and unfortunately can have little to no long-term benefits [16].

In addition to direct toxic effects of cigarette smoke on lung epithelial cells, there is increasing appreciation that altered or aberrant immune cell signaling significantly contributes to much of the irreparable tissue damage. Smokers with undiagnosed COPD normally experience low-level infiltration of inflammatory cells into the large airways and peripheral lung parenchyma and have what is increasingly recognized as early disease. In individuals with diagnosed COPD, the inflammatory process is amplified and prolonged leading to many of the tissue-remodeling events associated with chronic bronchitis and emphysema; hallmarks of COPD [17]. For example, in smoking-induced emphysema, chronically activated macrophages have been found to express upregulated levels of several proteinases and matrix metalloproteinases (MMPs) in both human smokers and in mouse models of cigarette exposure [18]. Macrophages also play a crucial role in triggering the initial immune response in responding to smoking induced inflammation. Alveolar macrophages are usually in a quiescent state and actually work to suppress the adaptive immune system in the healthy lung; however, in chronic inflammatory situations alveolar macrophages are the main source of proinflammatory amplification and play a significant role in causing an influx of other immune cells [19].

Ultimately, the disease progresses to a point where gas exchange is limited by the tissue damage and extent of hyperinflation. In many cases invasive surgical interventions are the only option; these include endobronchial valve insertion, bullectomy, lung volume reduction surgery, and lung transplantation [20]. Lung volume reduction surgeries can successfully address some issues with hyperinflation in selected patients by returning some of the mechanical advantage of normal breathing. However, invasive surgeries are associated with high morbidity and operative mortality [21–23], especially in late-stage COPD patients who are often poor targets for surgical intervention. For some patients with end-stage COPD, lung transplantation is the only option. However, this approach offers its own unique challenges including rejection risk, requirement for immunosuppression, and the limited supply of donor lungs. While transplanted lungs can certainly facilitate better gas exchange than severe COPD lungs, the benefits are balanced by the risks, as the 5 years survival of transplant recipients is only around 50% [24–27]. At present, there are no true curative treatments that can stop the progression of COPD, thus new therapeutic strategies are needed. Advances in cell-based therapies provide a platform for development of new therapeutic approaches in COPD. At this moment, much focus has been given to MSC cell-based therapies, mainly because of their immunomodulatory properties.

6.3 MSC-Based Therapy in Human Clinical Trials of COPD and Emphysema

The promising results in animal models have translated into clinical trials for treatment of COPD and emphysema. Searching on the ClinicalTrials.gov database for trials listed through November 28 2018, using the keywords “COPD” and “stromal cell”; “COPD” and “mesenchymal

stromal cell"; "COPD" and "mesenchymal stem cell"; "Emphysema" and "stromal cell"; "Emphysema" and "mesenchymal stromal cell"; and "Emphysema" and "mesenchymal stem cell", identified 18 studies of human clinical trials. So far, four of the studies have been completed and had their results published in the PubMed database, four are still in the process of recruiting patients, three of them are active but not recruiting patients, three have an unknown status, and four of them have been withdrawn [28]. This section will be focusing on the clinical studies that have been completed and for which results have been published (Table 6.1).

In 2011, Ribeiro-Paes et al. conducted the first clinical investigation evaluating the safety of using bone marrow-derived mononuclear cells (BMMC) in four patients with advanced-stage COPD (NCT01110252). Autologous BMMC were collected after 3 days of granulocyte colony stimulating factor (G-CSF) stimulation, and BMMC were isolated using Ficoll-Hypaque Premium™. The cells were further resuspended in albumin saline solution (ASS) at a final concentration of 1×10^8 mononuclear cells/mL, and intravenously administered directly to the patients without freezing or in vitro culture procedures. The patients were evaluated by several pulmonary function tests, including forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1), and partial pressure of carbon dioxide ($PaCO_2$). [29] Importantly, due to the small size of this study, lack of controls, and the lack of statistical analysis no clear conclusions can be drawn from these results. Furthermore, the cells used in this study were heterogeneous mononuclear cells isolated from bone marrow aspirates, and not MSCs, and therefore this study cannot be considered as the first MSC study for treatment of COPD patients.

In 2013, Weiss et al. performed a prospective, randomized, double-blind, placebo-controlled industry-sponsored trial evaluating the safety and the efficacy of intravenous allogeneic MSCs (NCT00683722). The study enrolled 62 patients (40–80 years of age), from six different centers, with moderate-to-severe COPD (GOLD II or III). The patients were randomized into two groups, where the first group received non-HLA-matched allogeneic MSCs and the second group

Table 6.1 Completed clinical trials investigating MSCs for COPD treatment

NCT number	Study design	No. patients	Route	No. of cells	Follow up (months)	Primary outcome	Comments
NCT01110252	Non-randomized Phase I Unicenter Single group Open label	4	IV	1×10^8/mL (30 mL total)	12	Safety FVC FEV1 VC	BMMC
NCT00683722	Randomized Phase II Multicenter Parallel assignment Placebo controlled	62	IV	100×10^6 BM-MSC/kg (four infusions total)	24	Safety	
NCT01306513	Prospective Phase I Unicentric Single group Open label	10	IV	$1–2 \times 10^6$ BM-MSCs/kg (two infusions total)	12	Safety Tolerability	Prior LVRS
NCT01872624	Non-randomized Parallel assignment Open label	10	IB	10^8 MSCs in 30 mL saline	4	Safety Absence of deficits	EBV insertion

received placebo (vehicle) treatment. MSCs were obtained from unrelated donors, expanded in vitro for a total of five passages in culture medium supplemented with 10% FBS followed by cryopreservation using human serum albumin and 10% DMSO. After thawing, MSCs or vehicles were systemically infused four times (at day 0, 30, 60, and 90) and the patients were followed for 2 years. The patients were evaluated by ECG, exacerbation records, FEV1, FVC, total lung capacity, dyspnea assessment (Borg scale), and 6-min walk test. Systemic inflammation was measured by circulating levels of inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interferon (IFN)-gamma, IL-10, and C-reactive protein (CRP). All 62 patients completed all four infusions, and 63% of the patients receiving MSC infusion and 84% of the patients in the placebo group completed the full protocol. The infusions were well tolerated and no severe or fatal adverse events were observed during the MSC or vehicle administration. No significant differences in FEV1, FVC, and total lung capacity were seen between the groups. Nor were differences in 6-min walk test or dyspnea assessment observed between the two groups. For most of the circulating inflammatory cytokines no significant differences were seen between the MSC-treated patients and the vehicle group. However, a decrease in the CRP level in patients treated with MSC compared to their baseline CRP levels was observed. The most important finding in this study was that MSC administration was safe in an older population of patients with moderate-to-severe COPD [30].

Stolk et al. performed a Phase I, prospective, open-label study (NCT01306513) where they aimed to assess the safety and feasibility of intravenously infused bone marrow-derived MSCs for ten patients with severe emphysema that had serial lung volume reduction surgeries (LVRS). During the first LVRS bone marrow was aspirated. MSCs were isolated from the bone marrow aspirates and expanded in vitro (passage 1–3) followed by cryopreservation. At three and four weeks prior to the second LVRS, MSCs were intravenously administered to the patients at two different occasions. Spirometry, gas transfer, lung volumes, and lung densitometry were evaluated at baseline and at the 12 months follow-up. Seven patients completed the full protocol. Three patients were withdrawn from the study due to problem aspirating bone marrow, no MSC growth, or persistent air leak after the first LVRS. No toxicity after the MSC infusions was observed and the patients did not report any symptoms that were considered related to the treatment. At 12 months follow-up, a significant increase in FEV1 and body weight was observed compared to baseline levels. However, if changes in FEV1 and body weight was due to MSC administration or to the surgeries remain unknown, since this study protocol did not include a control group. Importantly, no signs of increased pulmonary fibrosis were observed when lung tissue was evaluated by both histology and CT-derived lung density [31].

de Oliveira et al. combined MSC administration with one-way endobronchial valve (EBV) insertion [32]. This study was a prospective, patient-blinded, placebo (vehicle)-controlled, phase I study on ten patients with advanced heterogeneous emphysema (NCT01872624). de Oliveira et al. aimed to investigate the safety of combining EBV insertion with intrabronchial MSC administration. The authors hypothesized that combining intrabronchial MSC administration with EBV would reduce the inflammation, a common side effect of EBV placement. This study, however, was not designed to investigate MSC as a treatment for COPD, but rather specifically to investigate if MSC treatment would enhance EBV placement by reducing the underlying inflammation. Therefore, the secondary aim was to investigate if MSC administration reduced the systemic inflammation. Mononuclear cells (MNCs) were isolated from 60 mL bone marrow aspirate collected from the iliac crest of a single healthy donor using density-gradient centrifugation. MNCs were cultured at a density of 1×10^5 cells per cm^2 in Iscove's Modified Dulbecco's Medium supplemented with 15% fetal bovine serum, penicillin, and streptomycin at 37 °C, 5% CO_2 for generation of MSCs. MSCs were immunophenotyped and samples were taken for microbiological and cytogenetic testing. MSCs were harvested at passage three or four, diluted

in saline solution, and placed in infusion bags. Right before EBV insertion 10^8 MSCs (in 30 mL saline) were administered to five of ten patients using a video bronchoscope with a 2.8-mm instrument channel. The patients in the vehicle group received saline. In both groups, the infusions were performed in the region where the EBVs were supposed to be placed (the segmental or subsegmental bronchus of all branches of the target lobe). Immediately after the MSC administration or vehicle administration and EBV insertion, a chest radiograph was performed to confirm the EBV placement. For the following 2 days, the patients were evaluated for body temperature, blood pressure, oxygen saturation, heart, and respiratory rates. Arterial blood gas, complete blood count, urea, creatinine, glucose, and electrolytes were evaluated at day 0, 1, 7, 30, and 90. Chest CT scans were performed at day 0, 30, and 90. Circulating levels of inflammatory cytokines were assessed in serial blood samples obtained throughout the study period. Efficacy was evaluated as improvement from baseline in FEV1, FVC, FEV1/FVC, total lung capacity, single-breath carbon monoxide diffusing capacity, the body mass index, airway obstruction, dyspnea, exercise index, and health-related quality of life (St. George's Respiratory Questionnaire). All ten patients completed the full protocol. The MSC administration was well tolerated and all patients tolerated the EBV insertion but one, who developed pneumonia, pneumothorax, empyema, and respiratory failure. No severe adverse events were seen in the group receiving MSC, but 40% in the MSC group and 60% in the placebo group experienced adverse events during the study period, and importantly none of the adverse events was reported to be related to the MSC administration. No difference in toxicological or lung function parameters such as FEV1, FVC, and total lung capacity were observed between the groups. In accordance with data reported by Weiss et al. [30] the MSC treated group had significantly reduced levels in CRP at day 30 and 90 post administration. Patients receiving MSC infusions were reported to have a significant decrease in the St. George's Respiratory Questionnaire scores compared to the placebo group at day 90 post administration. The authors concluded that intrabronchial MSC administration in combination with EBV insertion appears to be safe in patients with severe heterogeneous emphysema. Furthermore, in this study MSC administration tended towards decreased circulating CRP levels; however, due to the low number of recruited patients and the limited follow-up period it was not possible to evaluate if MSC treatment altered the efficiency of the EBV placement or the subsequent clinical COPD course. [32].

Finally, Armitage et al. recently published a single site, phase I study that was not listed at the NIH ClinicalTrials.gov database, rather only in the Australian clinical trials registry (number 12614000731695), which aimed to investigate the distribution of intravenously infused MSCs into COPD patients. Nine patients with mild-to-severe COPD (GOLD I-IV) received infusion of low passage allogenic bone marrow-derived MSCs radiolabeled with indium-111, followed by a second infusion of unlabeled MSCs one week post the first administration. In similarity with the other clinical trials, all patients tolerated the MSC infusions well and no infusional or short-term adverse effects were reported. Following the first infusion, labeled MSCs were detected in the lungs within 30 min by computed tomography (CT) scan, and remained detectible 24 h after the infusion. After 24 h, indium-111 was detected in spleen, liver, and bone marrow up to 7 days after infusion. Moreover, 4 h after the first infusion the patients were assessed by single-photon emission computed tomography (SPECT) to evaluate MSC localization within the lungs. Furthermore, the amount of indium-111 positively correlated with the baseline FEV1 and the diffusing capacity of the lung for carbon monoxide. In addition, this study further aimed to investigate systemic inflammation following the MSC infusion. The authors were not able to detect IL-1 beta, IL-10, IL-12p70, or IL-17A; however, increased circulating levels of CRP were detected at 1 h and up to 2 days after MSC administration. Interestingly, this study suggests that MSC infusion shifted the balance towards a more anti-inflammatory profile, as the number of circulating regulatory T-cells were increased

7 days after MSC administration and the proportion of dendritic cells were altered, favoring plasmacytoid dendritic cells [33].

Current clinical trials that aimed to evaluate the effect of MSC administration in COPD patients differ in a wide range of factors such as routes of administration, number of MSC administered, number of administrations, use of fresh MSCs or culture-expanded MSCs. Furthermore, all the investigations discussed above, were phase I-II studies that were underpowered in order to detect potential efficacy and no improved pulmonary function or respiratory quality of life was observed. Although the primary end-point was safety and all studies reported that MSC administration was well tolerated and no toxicity was observed, further studies, both clinical and preclinical, are needed to better understand potential therapeutic efficacy of MSCs in COPD.

6.4 The Hurdles That Need to Be Overcome

Despite increasing number of preclinical studies demonstrating that MSC administration could prevent or treat experimental COPD and emphysema [34–50], clinical studies have not been able to reproduce the preclinical results, and to date no efficacy or significantly improved pulmonary function in COPD patients have been observed. In this section, we will be discussing some of the challenges in the field and the hurdles that need to be overcome in order to improve the efficacy of MSC therapy in COPD [51].

6.4.1 Standardization of MSC Cell Culture Conditions

MSCs are known to be a heterogeneous cell population [52, 53], containing subpopulations that have been demonstrated to be functionally different from each other [54, 55]. Many of the phenotypic and functional differences depend on differences in culture conditions, individual donors, the harvest site, and the tissue source [56–59]. This makes it difficult to compare results between different studies, both preclinical and clinical, and importantly it hinders progression in the field. Efforts should therefore be concentrated on developing standardized MSC isolation methods and culture conditions. In 2006, the International Society for Cellular Therapy published a position paper in order to address this issue. In this article, they defined minimal suggested criteria for cultured human MSCs [60]. Since this position paper by Dominici et al. was published it has been updated once in 2012 [6], but the MSC field has advanced and today MSCs are isolated from different organs and tissues and therefore these minimal criteria urgently need to be modified and updated.

To date, bone marrow-derived MSCs are the most widely investigated, but preclinical studies have demonstrated that MSCs with immune-regulatory and regenerative properties can be isolated from other tissues such as adipose tissue, umbilical cord, and lung [58, 61–66]. A large body of data demonstrates that MSCs execute their therapeutic effects through a spectrum of paracrine activities, and interestingly preclinical data suggest that MSCs isolated from different tissues have different secretome profiles [57, 67]. It is also important to realize that primary MSCs change phenotype when they are isolated from their native tissue and plated on a plastic culture dish [56]. The bona-fide MSC, which are thought to be small and quiescent, noncycling cells in vivo, changes phenotype into a spindle-shaped and active proliferating and secretory cell in culture [56, 68]. At early passages, MSCs have a high proliferation rate but as their time in culture progresses their proliferation rate declines and they finally enter a senescence stage [69–71]. Also the morphology is changed during culture expansion, MSCs in early passages have a thin spindle-shaped morphology, but at higher passages MSCs tend to become larger and more flattened cells with an irregular shape [56, 70]. Moreover, MSCs have been reported to accumulate DNA damage during in vitro expansion, which could potentially lead to tumorigenesis upon implantation [72, 73]. Importantly, tumor development was not observed in any of the clinical studies using MSC as treatment for

COPD and/or emphysema patients, although longer follow-up is necessary [29–32].

Furthermore, the biological properties of MSCs can also be strongly influenced by the cell culture medium. Cell culture media are often supplemented with serum, and most often fetal bovine serum (FBS) is used. The use of FBS has several disadvantages, especially in clinical settings. First, the possibility of contamination with pathogens such as prions and viruses and the potential immune reaction to bovine proteins. Second, lot-to-lot variation between different FBS batches might induce differences in MSC behavior such as proliferation rates and differentiation potential, and make it difficult to standardize methods and reproducibility of results [56, 74, 75]. Human platelet lysate (HPL) is as an alternative to FBS in clinical settings. HPL has the advantages of containing non-animal products and therefore no risk of xenogeneic infections and immune rejection. On the other hand, HPL is a human product and has the potential to transmit human diseases such as hepatitis B and C, and human immune deficiency virus (HIV). In similarity with FBS, HPL also brings the disadvantage of having lot-to-lot variation [75, 76]. A third option would be to use serum-free cell or synthetic culture media. These media are highly promising, but more studies are needed in order to have evaluate their utility for producing clinical grade-MSCs. Recently, Lensch et al. demonstrated that MSCs had a higher proliferation rate when growing in xeno-free medium which resulted in a greater viable cell yield compared to standard FBS containing culture medium [77]. Nevertheless, further studies are needed in order to evaluate if the in vivo biological properties of MSC are altered when expanded in the in vitro setting.

Another factor that may influence the biological function of MSCs is the freezing and thawing of cells before administrated to patients. The current model for allogeneic MSC use is to expand cells on plastic culture dishes following harvest and isolation from bone marrow or other source and cryopreserve the cells until usage. When it is time for administration, cells are thawed, washed, and directly administered to the patients most commonly through intravenous infusions [78]. However, a number of studies have demonstrated that MSCs that have been freeze-thawed have impaired functional properties. Francois et al. reported that cryopreserved MSCs had impaired immunosuppressive properties [78]. In accordance with these results, Moll et al. published an article where they demonstrated that freeze-thawed MSCs had a reduced responsiveness to proinflammatory stimuli and an impaired production of anti-inflammatory mediators [79]. Minor effects on gene expression of freeze-thawed MSCs compared to continuously cultured MSCs have been observed. However, the alterations in gene expression between different donors were larger than the effects of cryopreservation [80]. Although there is a practical need for expanding and cryo-banking cells for therapeutic use [79], most preclinical studies have been performed using log phase of growth MSC. There are studies, some of them discussed above, which demonstrated that freeze-thawing procedure alters the biological properties of MSC. Francois et al. found that during the thawing process a heat-shock stress response was initiated that was associated with the impaired immunosuppressive properties of MSC. Interestingly, this response was reversible and cells were recovered after 24 h of culture [78]. These results imply that cryopreservation and banking of cells might be possible, as long as the cells are allowed to recover in culture before use. The study by Cruz et al. further supports the potential of using freeze-thawed cells for clinical trials. In this study, the authors compared the therapeutic effect of continuously cultured versus freshly thawed bone marrow-derived MSCs in an *Aspergillus* hyphal extract (AHE) exposed asthma mouse model, and found no difference in therapeutic effect between the two groups [81].

Utilizing plastic culture dishes are by far the most traditional way of cultivating and expanding MSC; however, alternative culture systems have been developed that might mimic the in vivo situation more compared to the more traditional 2D cultivation on plastic. The use of alternative three-dimensional cell culture systems can hopefully contribute to narrowing the gap between preclinical and clinical research. Different groups

have studied the possibility to grow MSCs on plastic culture dishes coated with extracellular matrix molecules (ECM) such as collagen and fibronectin [82, 83]. ECM is a three-dimensional network composed of noncellular structures that play an important role within the lung, not only by providing structural support and adding stability but also as a bioactive environment that can influence cellular responses [84]. Engler et al. demonstrated that changing the elasticity of the ECM that MSCs were grown on significantly affected the MSC phenotype. MSCs grown on a stiffer ECM differentiated towards the osteoblast lineage, whereas MSCs grown on a softer ECM differentiated towards the adipocyte lineage [85]. The MSC differentiation potential could also be altered by changing the cross-linking of the collagen fibers [86]. In addition, modifications of the geometric shape, cell density, and cell size have been implicated in the differentiation potential of MSC [87, 88]. Interestingly, McMurray et al. developed a nanoscale surface that maintained the phenotype and multilineage potential of long-term cultured MSCs [89]. How the ECM environment affects the MSC therapeutic behavior, especially in a fibrotic or emphysematous COPD lung, is currently a largely untouched area that will most likely play a pivotal role in the development of successful MSC-based therapies.

A different approach of the three-dimensional cultures is the usage of the hanging drop model. In conformity with primary MSCs, culturing MSCs using the hanging drop method resulted in nondividing cells [90], but an increased potential to differentiate towards osteoblast and adipocyte lineages was also demonstrated [91]. Another strategy that has been used for MSC expansion relies on culturing MSCs in 3D scaffolds (decellularized lung tissue or synthetic scaffolds) [92–94]. In this system, cultivation on a plastic surface could be avoided, but a perfusion-based bioreactor system is required [56]. Studies have shown that MSCs cultured in lung ECM hydrogels have enhanced viability and increased expression of Sox2 and Oct4 compared to cells grown on plastic [95]. Furthermore, changes in secretion of cytokines including IL-1Ra, VEGF, G-CSF, FGF, and HGF have been demonstrated in MSCs grown in 3D culture compared to 2D [96, 97]. Taken together, the traditional way of cultivating MSCs as monolayer on a plastic surface may result in MSCs with a different phenotype compared to MSCs expanded in three-dimensional culture systems. However, whether cultivating MSCs on ECM coating, in scaffolds, or in hanging drops increases the beneficial effects when used for clinical settings remains to be evaluated and further studies are needed.

It is well known that oxygen levels can affect cell functions, such as differentiation, cytokine production, and proliferation [98–101]. Furthermore, it is also known that different adult tissues experience a wide range of oxygen levels [102] and that severe pathological inflammation can cause hypoxia, reduced pH, and oxidative stress [103, 104]. Nevertheless, MSCs tend to be cultured at atmospheric oxygen levels (20–21% O_2) which do not reflect the microenvironment they normally reside in, or the microenvironment they will encounter when administered into the diseased lung [102]. Culturing MSCs at oxygen levels that more closely represent their in vivo situation have a huge impact on MSC behaviors. Lennon et al. observed that MSCs grown at lower oxygen levels had a greater number of colony-forming cells and proliferated at a higher rate compared to MSCs grown at higher oxygen levels. Also, Lennon et al. demonstrated that MSCs cultured at 5% oxygen formed more bone structures in vivo, compared to MSCs grown in 20% oxygen [105]. Moreover, adipose-derived MSCs grown at low oxygen levels, secreted higher levels of cytokines such as VEGF and FGF compared to cells cultured at 20% oxygen [102]. Combining the low oxygen condition with growing the MSCs in 3D cultures has been shown to increase the expression of pluripotent genes such as Oct-4, Sox-2, Nanog, and Rex-1 compared to control [99, 106]. Beegle et al. reported that MSCs pretreated with hypoxia before administration enhanced survival rate and cell retention compared to cell grown at 20% oxygen. Taken together, these studies emphasize the importance of understanding the effects of differences in protocols, culture conditions, and oxygen levels in the context of culturing MSCs

for clinical trials for COPD where you have gas-exchange impairment, active immune response, and inflammation.

6.4.2 The Exact Mode of Action of MSC In Vivo Needs to Be Discovered

Despite an enormous interest in using MSCs for clinical settings, the exact in vivo function is not understood, especially not within the lung. A compelling amount of data now points towards that MSCs act by paracrine mechanisms rather than through engraftment [51, 107–116]. Tracking studies of intravenous injected MSCs reveal that most of the injected cells disappear after 24 h [33, 46, 110, 117], and since MSCs do not engraft it is unlikely that MSCs can remodel injured tissue by differentiating into other cell types. The mechanisms by which MSCs are the most likely to be involved in COPD and emphysema are discussed below.

Immunomodulation through paracrine actions is one of the main mechanisms of actions of MSCs and involves both the innate and the adaptive immune system [118–131] (Fig. 6.2). These effects include inhibition of T-cell [120, 121] and B-cell proliferation [127], macrophage polarization [119], and differentiation of T-cells towards T-regulatory cells [132–134]. The paracrine actions have been associated with several mediators such as hepatocyte growth factor (HGF), transforming growth factor beta (TGF-β), prostaglandin (PGE2), IL-10, IFN-gamma, TNF-stimulated gene 6 (TSG6), and indoleamine 2, 3-dioxygenase (IDO) [115, 118, 119, 135, 136]. In addition to the paracrine immunomodulatory effects, MSCs might activate the immune system by recognition of the immune cells. As mentioned, MSCs are rapidly cleared from the lung after infusion, which was recently demonstrated to be mainly through phagocytosis by monocytes [137]. The recognition of MSCs by monocytes results in a polarization of monocytes/macrophages towards an immuno-

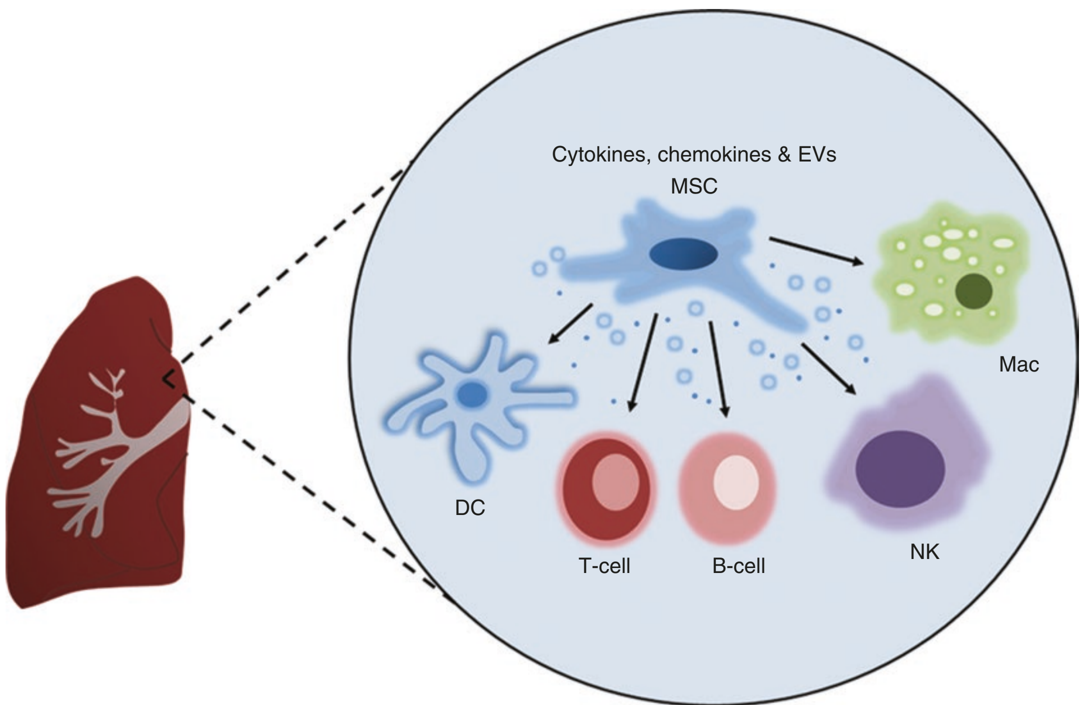

Fig. 6.2 A schematic illustration describing the potential immunomodulatory functions of MSCs. Abbreviations: *EVs* extracellular vesicles, *MSCs* mesenchymal stromal cell, *DC* dendritic cell, *NK* natural killer cell, *Mac* macrophage

suppressive phenotype that results in an immunomodulatory response [137, 138]. Similar results have also been demonstrated with heat-inactivated MSCs, suggesting that MSCs also can act in a passive immunomodulatory manner [139]. However the potency of apoptotic MSCs are controversial, apoptotic MSCs have been demonstrated to be completely ineffective when injected intravenously in mice [140]. MSC are also known to secrete antimicrobial proteins and polypeptides that are molecules responsible for bacterial killing. MSCs secrete the antimicrobial peptide, LL-37, following *Eschericia coli.* stimulation, which was subsequently found to be responsible for the antimicrobial activity in a model of *E. coli* Pneumonia [141]. In addition to its antimicrobial activities, LL-37 can also play an important role in inflammatory and immune modulatory actions [141, 142].

A growing body of data suggests that MSCs can form links with other cells, and that they have the potential to transfer components such as mitochondria [143–146]. Through mitochondrial transfer MSC have been demonstrated to be able to rescue epithelial cells with defective mitochondria [144]. The mitochondria transfer is thought to be via direct transfer by microtubules and tunneling nanotubes (TNT) [144, 147]. MSCs can also transfer mitochondria to macrophages resulting in an increased phagocytic activity [148]. Mitochondrial biogenesis is regulated by extracellular stimuli [149] and several lung diseases are associated with impaired mitochondrial biogenesis and dysfunctional mitochondria [150, 151]. However, beyond the mitochondria-derived reactive oxygen species (ROS), the contribution of mitochondria in the development of COPD is still under investigation [152]. In addition to mitochondria transfer through microtubules and TNT, mitochondria can also be transported via extracellular vesicles (EV) [144, 153–155]. It is also becoming increasingly clear that MSC-derived EVs can influence the behavior of surrounding inflammatory and structural cells. For example, EVs released from MSCs can stimulate bronchial epithelial cells and alveolar cells to secrete pro-inflammatory cytokines [156, 157]. Furthermore, MSC-derived EVs suppress the potential of lung fibroblasts to differentiate towards myofibroblasts [158]. It is not only mitochondria that could be transferred by MSC-derived EVs, also other components such as microRNA, proteins, lipids, DNA, and mRNA [159, 160]. EVs are taken up by other cells, and EVs derived from MSCs have been demonstrated to impact immune cells. EVs isolated from IL-beta pretreated MSCs induced macrophage polarization towards the anti-inflammatory phenotype (M2) [161]. MSC-derived EVs have also been associated with inhibition of T-cell proliferation, inducing apoptosis of activated T-cells and promotion of regulatory T-cells [162]. MSC-derived EVs have been tested in experimental COPD models, but further studies are needed [163].

6.4.3 The Beneficial Outcome Needs to Be Significantly Increased

It is now widely accepted that, following in vivo delivery, culture-derived MSCs respond to the microenvironment they encounter, which in COPD and emphysema could encompass everything from massive inflammatory environment to emphysematous tissue destruction. Therefore, it is important to consider several important aspects of the MSC preparation and administration used today.

The route by which MSCs are delivered into the patients most likely plays an important role in the MSC potential function. Despite the fact that several clinical trials has been performed using MSCs for severe lung disorders [29–32, 164–168], the best route of administration have not been determined. In preclinical studies, two main administration routes have been evaluated: systemic administration [34, 41, 43, 45, 47, 48, 50, 169, 170] and local administration [39, 41–43, 171, 172]. COPD is a systemic disease and therefore systemic administration might be better suitable for these patients. In addition, systemic administration is less invasive and has less contamination risks compared to local administration [51]. Not only has the route of administration been different in the different studies conducted to date but also the number of cells

administered with each injection and whether single or multiple injections were administered during the trial. According to Antunes et al. a wide range of MSC doses in preclinical settings have been used, from 10^4 up to 6×10^6 [51]. So far, bone marrow-derived MSCs are the most frequently used cell source for MSC-treatments, especially when used in human clinical trials. However, MSCs derived from other sources such as adipose-derived, umbilical cord-derived, lung-derived, and amniotic fluid-derived MSCs have been evaluated for treatment of COPD/emphysema models [36, 41, 50, 172].

Since it is known that the environment affects MSC function and viability, several preconditioning strategies have been tested. Some researchers have been focusing on the effect of the inflammatory environment and the cytokines that may be encountered in the diseased lung [124, 173–179]. Krampera et al. reported that MSCs stimulated with IFN-gamma, increased the levels of IDO produced and secreted by MSCs, leading to an increased suppressive effect on T-lymphocyte proliferation. Moreover, the authors were able to demonstrate that the inhibitory effects of MSCs on T-lymphocyte proliferation were completely abolished when adding an IFN-gamma blocking antibody to the culture system [124]. In an IFN-gamma knock out mouse model, Polchert et al. were able to demonstrate that endogenous IFN-gamma was required to initiate MSC efficacy. However, after pretreatment of MSCs with high doses of IFN-gamma they immediately became active [173]. Also pre-stimulating MSCs with a combination of inflammatory cytokines has been explored [174, 175]. Another interesting approach to mimic the microenvironment is to utilize patient samples such as serum and bronchoalveolar lavage (BAL) fluid from patients and pre-stimulate cells with such prior to the administration [180, 181]. Moreover, attempts to improve the beneficial effects of MSCs have utilized treatment with the toll-like receptor-3 ligand (poly(I:C)). The authors found that MSCs pretreated with poly(I:C) had improved immunosuppressive properties, an effect that was inhibited by addition of the microRNA miR-143 [182]. In addition to the inflammatory environment, others have studied the effect of pretreating MSCs with hypoxia and nutrient deficiency. During culture under hypoxic conditions, MSCs have been shown to have decreased expression of senescence-associated beta-galactosidase and an increase in the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL [183, 184]. By exposing MSCs to hypoxia the hypothesis is that the cells will adapt to the ischemic environment with oxidative stress, an environment they likely will encounter in the COPD lung. This might potentially enhance the time that MSCs can survive and exert their therapeutic paracrine actions in the recipient lung.

A different way of increasing the therapeutic effect by MSCs is to genetically manipulate the cells prior to administration [185–187]. For example, Jiang et al. demonstrated that after co-overexpressing the genes Ang-1 and Akt in MSCs, an increased cell survival and improved angiomyogenesis was observed in an experimental model of acute myocardial infarction [188]. In lung, MSCs overexpressing Ang-1 have been demonstrated to more potently decrease LPS-induced pulmonary inflammation and proinflammatory cytokine release into the BAL fluid [189]. In another study by McGinley et al., overexpression of heat shock protein 27 (HSP27) in MSCs led to decreased apoptosis and improved cardiac function [190]. Overexpression of manganese superoxide dismutase in adipose-derived MSCs, a gene strongly upregulated during hypoxia, increased the time that the MSCs were detectable in a matrigel plug implanted into a mouse model [191]. Moreover, He et al. transduced MSCs with angiotensin-converting enzyme 2 (ACE2), an enzyme that degrades angiotensin II and had previously been demonstrated to have a protective role against acute lung injury. The ACE2-transduced MSCs were demonstrated to reduce pulmonary vascular permeability, normalize the expression of eNOS, and improve the endothelial barrier integrity, when infused into an ALI-mouse model. Furthermore, the ACE2 overexpressing MSCs also displayed an improvement in the suppression of the inflammatory response [192].

Combination of different treatments could be another approach to enhance MSC efficacy.

This approach was used in two of the clinical trials discussed above. Stolk et al. combined MSC treatment with lung volume reduction surgery and de Oliveira et al. with a one-way endobronchial valve insertion [31, 32]. An alternative could be to pretreat the recipient tissue with pharmacological drugs in order to make the recipient site more accessible to the infused cells [104, 193–195]. In a cardiac disease model, pharmacological pretreatment of a vasodilator drug in the recipient site of transplantation resulted in an enhanced delivery of MSCs [193]. In a clinical trial using MSCs for treatment of chronic heart failure, the administration site was treated with a shock wave prior to the administration of the cells. In the group receiving both the shock wave pretreatment and the MSC infusion, the overall occurrence of major adverse cardiac events were significantly decreased compared to the control groups [194].These are all important observations for potential cell-based therapies for lung diseases and should be investigated further.

6.4.4 Is COPD the Best Disease to Treat with MSC-Based Cell Therapy and How Do We Foresee Which Patients Will Respond to the Treatment?

The lack of translating the encouraging preclinical data into clinically relevant effects in patients with COPD and emphysema brings up the following question: is COPD the most suitable pulmonary disease for MSC-based treatment? The animal models of COPD and emphysema used in the preclinical studies were optimized to detect the maximum therapeutic effects [196], and might therefore not reflect the in vivo situation that MSCs encounter when infused into patients with COPD and/or emphysema. COPD is characterized by tissue damage, structural changes, and inflammation, and as mentioned it is a heterogeneous disease with different degrees of fibrosis and emphysema [197]. COPD patients with different phenotypes might respond differently to MSC administration [198], and choosing patients that are more likely to respond to the treatment could be one way to improve the clinical outcome. Another possible way to improve the outcome could be the timing of the treatment. In animal studies, MSCs are frequently administered to the animals in close proximity to the induction of the disease [42, 48, 50, 172] or even at the same time or prior to the disease induction [43, 47]. Based on these preclinical findings, MSCs might be more beneficial earlier in the disease than in later stages of the disease. However, COPD patients tend not to seek medical attention in early stage of the disease [199]. One way to foresee which patients would most likely respond to the treatment could be to develop in vitro potency assays. Even if it is widely accepted that the therapeutic effect of MSCs is mainly mediated by paracrine effects, the exact mechanism of action is not determined. This makes it difficult to develop one single analytic or biological assay, and most likely, a combination of evaluating different mechanisms would be needed [200, 201]. Another potential way would be to develop biomarkers to indicate which patients have an active disease and therefore might benefit more from a MSC-based therapy. To date, several potential biomarkers, including circulating fragments of ECM proteins, have been shown to be increased in COPD patients with an active disease e.g. in relation to acute exacerbations [202–204]. Finally, Broekman et al. suggested that in addition to the optimization of MSC-treatment and potency assays, challenges such as improved outcome parameters needs to be addressed [196].

6.5 Stem Cell Tourism: A Growing Problem for the Field

In parallel with the growing interest of cell-based therapies for COPD and other lung diseases, an increased market for commercial stem cell therapies has developed both in the USA and globally [205]. This very unfortunate and problematic outcome might partly be due to an

increased visibility to desperate patients through the internet and open social media channels [206]. These unproven and often unsafe stem cell treatments can create a situation in which desperate patients easily can be misled into participating in very expensive treatments, which are not covered by insurance. Furthermore, the providers at the stem cell clinics often fail to prove safety and efficacy of their treatments failing to fulfill recognized biological and medical standards, exposing the patients to unnecessary risks and leaving the patient and their family with dashed hopes [205, 207]. These stem cell clinics have the potential to harm even more patients and their families, as well as bring the field into disrepute and hamper the progression of safe and effective MSC-based therapies. Therefore, organizations such as the International Society for Stem Cell Research (ISSCR) and the International Society for Cell and Gene Therapies (ISCT) have taken stances against these unethical cell-therapy clinics. Also, the Food and Drug Administration (FDA) is beginning to take actions against the stem cell tourism [205, 208]. In a review by Dominici et al., the authors discuss the importance of having proper communication between different players such as medical doctors, industry, patient organizations, and patients, in order to enhance credibility and patient welfare [205]. In an attempt to begin proactively addressing this issue, the American Thoracic Society (ATS) Respiratory Cell and Molecular Biology Assembly Stem Cell Working Group posted a statement online and several other related publications [207, 209–212]. This statement will help to translate new scientific findings into patient education in an unbiased way and to make the public aware of the limitations and potential risks associated with such therapeutic approaches [209, 212]. However, it is not only the patients that need education, many pulmonologists are also not familiar with the stem cell field, and the ATS Respiratory Cell and Molecular Biology Assembly Stem Cell Working Group has developed educational resources for this audience also [207].

6.6 Conclusions

MSC-based therapy for treatment of COPD and emphysema has demonstrated promising results in animal models; however, this has not translated into clinically relevant effects in patients to date. Current clinical trials have failed to demonstrate efficacy and improved lung function, but importantly they have uniformly demonstrated the MSC administration to be safe. The challenges ahead for this field are to standardize the isolation and culture conditions in order to have a cell product with high quality and reproducibility, to select the proper subpopulation of patients that is most likely to respond to the cell treatment, to develop appropriate potency assays, and to improve or develop new methods to measure outcomes. Furthermore, the usage of cell-free products such as EVs and conditioned medium, or pretreating MSCs prior to administration has demonstrated promising results. However, there is still a long way to go and many challenges are ahead before we have an optimal MSC-based treatment for patients with COPD and emphysema.

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7 Stem Cell Based Therapy for Lung Disease Preclinical evidence for the role of stem/stromal cells Clinical application of stem/stromal cells in lung fibrosis

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7.1 Introduction

Interstitial lung fibrosis may develop as a consequence of occupational or drug exposures, lung injury, or as the end stage of chronic intersititial lung disease. The pathogenesis of lung fibrosis remains elusive and controversial, but prevailing hypotheses assume an ineffective wound healing response to alveolar epithelial cell injury [1, 2] Injury magnitude and susceptibility appears to be related to aging and genetic predisposition, with subsequent innate immune system and fibroblast activation [1, 3, 4].

In the right clinical picture, and in the absence of other known causes of lung fibrosis, the diagnosis of IPF may be made by typical radiologic findings on high-resolution computed tomography (subpleural and basilar predominance of honeycomb cysts and reticulation). In cases where the diagnosis is not clear, lung biopsy may be necessary. Histologically, IPF is identified by the presence of the usual interstitial pneumonia (UIP) pattern with extracellular matrix deposition, phenotypic alterations of fibroblasts and alveolar epithelial cells, formation of fibroblastic foci, and regional and temporal heterogeneity characterized by scattered areas of aberrant wound healing interspersed with normal lung parenchyma [1, 2, 5–13].

Evidence suggests that areas of fibrosis seen in the lungs of patients with IPF share features associated with normal aging lung, such as genomic instability, telomere attrition, mitochondrial dysfunction, cellular senescence, and immune dysregulation [9, 14, 15] (Fig. 7.1). Because of this overlap, connections between IPF and diseases of premature aging have been postulated.

Partly due to the inefficacy of immunomodulatory and immunosuppressive agents in the treatment of IPF, the role of the immune system in the pathogenesis of IPF remains poorly understood [16–21]. However, a link between IPF and immune dysregulation is suggested by the presence of highly activated and proliferative CD4+ cells and functional impairment of T-regulatory cells in patients with IPF [9, 22, 23]. Pathologic

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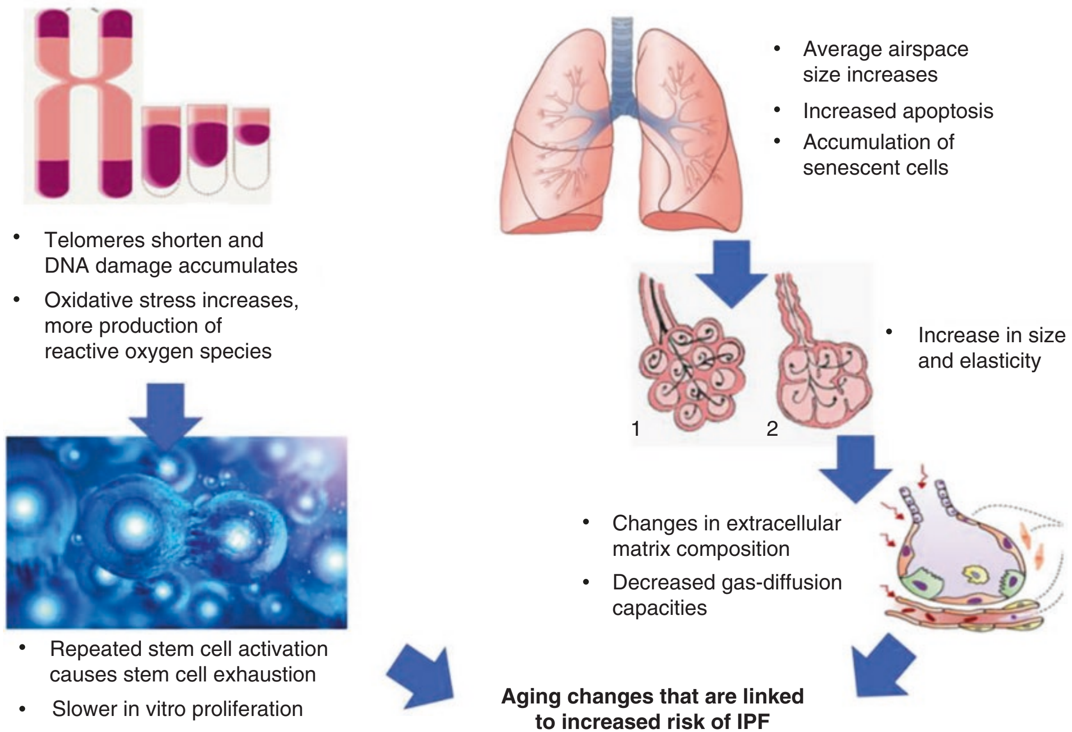

Fig. 7.1 Aged mesenchymal stem cells in aging lungs and IPF

features of the epithelium suggest that a dysregulation of progenitor cells may contribute to the IPF phenotype, with abnormal cell cycling resulting in dysfunctional repair [24].

Currently, pirfenidone and nintedanib are the only two FDA approved compounds for the treatment of IPF. Pirfenidone, an antifibrotic compound with an unknown mechanism of action, targets several molecules including transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interleukin-6 [25]. Nintedanib, a tyrosine kinase inhibitor, targets vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor (PDGFR) [21]. Pirfenidone was shown to slow the rate of decline in forced vital capacity (FVC) and 6-min walking distance in patients with IPF, and may improve mortality in select patients [3]. Nintedanib was also shown to slow the rate of decline in FVC with a trend toward reduced mortality [4]. However, neither of these compounds has been shown to ameliorate respiratory symptoms or improve acute exacerbation rates, and lung function has continued to decline in all trials completed to date. In addition, though pirfenidone and nintedanib have been shown to slow the progression of IPF [26–28], both compounds are associated with significant side effects [28–30]. The only definitive treatment for IPF at this time is lung transplantation. Morbidity and mortality from IPF remains high and thus there is a pressing need for alternative therapeutic options for this complex and devastating disease.

Ongoing clinical trials of other potential therapeutic targets include mofiers of connective tissue growth factor, IL-4 and IL-13, galectin-3, lysophosphatidic acid, the phosphoinositide 3-kinase pathway, and finally, mesenchymal stromal cells (MSCs).

7.2 Animal Models of Pulmonary Fibrosis

Spontaneous pulmonary fibrosis does occur in nonhuman animals, including ferrets, dogs, horses, donkeys, and cats. While the fibrotic

lungs of these animals share many characteristics with the lungs of humans with IPF, current veterinary classifications of fibrotic lung disease are not equivalent. The field of comparative oncology has set the stage for collaborations utilizing spontaneous models of progressive fibrotic lung diseases of mutual interest to veterinary and human medicine. The results of these kinds of studies promise to enhance the understanding of common factors important to disease development in a variety of species and to refine treatments for both humans and animals. Moreover, they may provide insights into unanswered questions involving naturally occurring models of pulmonary fibrosis. However, further studies in veterinary models of lung fibrosis are needed to define their relation to human disease and their potential use as models for the development of effective treatments.

Because no reliable spontaneous animal model exists, understanding the pathogenesis of IPF and other fibrotic lung disorders has primarily relied on research using animal models of induced lung fibrosis. Unfortunately, although some of these animal models exhibit progressive disease, none fully recapitulates the histological pattern of UIP. Traditional animal models of lung fibrosis have generated important insights into the pathobiology of lung injury, inflammation, and fibroproliferation [8]. Although it is appreciated that the spontaneous development of fibrosis in other species (e.g., ferrets, donkey, sheep, cats, horses, and dogs) [9–11] can be instructive, the most tractable models for studies of pathogenesis involve rodents. Traditionally, preclinical trials have utilized mouse models of bleomycin (BLM)-induced pulmonary fibrosis and studies of BLM-induced fibrosis in aged male mice remain the most clinically relevant model for preclinical studies of IPF because young mice treated with BLM may show recovery from pulmonary fibrosis, an event not appreciated in human fibrotic lung disease.

BLM is a chemotherapeutic antibiotic first identified as a pro-fibrotic agent after the development of pulmonary fibrosis in patients being treated for lymphoma. BLM has been studied in multiple species including mice, rats, sheep, guinea pigs, hamsters, dogs, and primates and in various modes of administration [20, 21], but the consensus view at this time is that the intratracheal murine BLM model is “the best-characterized animal model available for preclinical testing” of IPF [31, 32].

BLM acts by causing single- and double-strand DNA breaks thereby inducing apoptosis. BLM hydrolase, a BLM-inactivating enzyme, influences drug effects on a tissue-specific basis. Becuase the lungs maintain low levels of this enzyme, lung tissue is highly susceptible to BLM-induced injury. An overproduction of reactive oxygen species, due to chelation of metal ions and reaction of the formed pseudoenzyme with oxygen, leads to epithelial cell death (days 1–3), excessive inflammatory infiltrates (days 3–9, neutrophils found in the bronchoalveolar lavage fluid at day 3 and lymphocytes at day 6), and ultimately to fibroblast activation, extracellular matrix deposition, and development of fibrosis (days 10–21 with a peak around day 14). These changes are seen at both the molecular [23, 25, 26] and histologic [20, 23, 25, 27] levels.

The early molecular signature of BLM-induced injury appears to be most similar to the accelerated acute phase of IPF in humans [28]. Measurements of alveolar septal thickening, intra-alveolar fibrosis, increases in alveolar macrophages, and dilation of bronchioles and alveolar ducts demonstrates fairly uniform fibrosis [29]. Nevertheless, the BLM-induced lung fibrosis model is not perfectly representative of IPF. The rapidity of development of BLM-induced fibrosis, the marked inflammation preceding fibrosis, and the possibility of spontaneous resolution are signignificnt differences between the BLM model and human IPF.

C57BL/6J mice have been the predominant animal model, as this particular strain is highly susceptible to lung injury following intratracheal BLM administration [30, 33]. Conversely, the BALB/c or SV129 strains confer resistance to BLM-induced pulmonary fibrosis, presumably due to alterations in transforming growth factor (TGF)-β expression [33].

While BLM-induced lung injury has been studied via intratracheal, intraperitoneal, subcutaneous, intravenous, and inhalational delivery methods, the intratracheal route is most commonly

used because it best recapitulates the human phenotype which is limited to the lungs [20, 23, 26, 28, 29, 34–39]. Another issue identified in studies using the BLM mouse model is the wide range of dosing regimens used [40]. In mouse studies, weight-based dosing is most common [35, 36, 39] and slightly lower doses (2.0–2.5 U/kg) appear to provide the most effective model of lung fibrosis, while reducing sample loss due to high mortality [28]. With regards to the frequency of dosing, repetitive dosing in young mice was found to promote persistent fibrosis as evidenced by measures of hydroxyproline content and inflammatory cell infiltrates. In contrast, single-dose experiments have demonstrated spontaneous resolution in young mice [23, 41].

Animal models of IPF have yielded valuable insights, but studies using these models also have important limitations. Most studies of therapeutic interventions use a single dose of intratracheal BLM followed shortly thereafter by the administration of the therapy under investigation [35–38]. Because these thrapuetic agents are often administered within the first 1–7 days following BLM exposure, their observed effects may be due to prevention of the inflammatory cascade rather than reversal of fibrosis, thus limiting their applicability to human IPF [40]. More recent studies have begun to explore administration of drugs after 7 days [42, 43] and, to our knowledge, only two studies to date have evaluated repetitive BLM injury [44, 45]. Intriguingly, pirfenidone and nintedanib received approval to proceed to clinical trials based on preventive protocols or even therapeutic protocols targeting the inflammatory or the early-fibrotic phase of the BLM model [46–48].

In addition, most studies investigating BLM-induced pulmonary fibrosis have used young male mouse models, aged 8–12 weeks [28, 29, 34]. Young mice, however, have been shown to undergo spontaneous resolution of BLM-induced pulmonary fibrosis, a phenomenon not observed in aged mice [23, 41, 49]. Whether sex differences in mice parallel human IPF, which exhibits a tendency toward male predominance, has not been fully determined. However, the use of aged male mice may provide a more clinically relevant model of IPF [49]. Many of the hallmarks of aging, including genomic instability, telomere attrition, epigenetic alterations, deregulated cellular bioenergetics, and cellular senescence, are also seen in fibrotic lung disease [50–52]. Studies have shown that older mice are more susceptible than younger mice to pro-fibrotic stimuli including BLM [26]. This is of particular interest given that IPF is predominantly seen in older individuals. Transgenic deletion of senescence-related genes including RAGE and relaxin has been associated with spontaneous age-dependent development of lung fibrosis indicating a role for aging in disease susceptibility [31, 53, 54].

The preclinical efficacy of the majority of antifibrotic agents tested in animal models has utilized a single model, (most often BLM) and has measured histologic, not clinical, endpoints. In addition, the lack of blinding in most preclinical animal studies may contribute to evaluation bias in outcomes. Reproducibility issues arising from different experimental settings could also account for discrepancies in treatment effects and lack of generalizability. As with all studies, when using animal models, sample size must be balanced with the statistical power needed to generate robust data. Finally, insufficient reporting of experimental animal data or unpublished negative therapeutic results severely hamper the validity of experimental studies.

7.3 Rationale for Stem Cell Therapy

A stem cell is defined as an undifferentiated cell capable of self-renewal and multipotent differentiation potential. To achieve this remarkable task, stem cells undergo asymmetric cell division whereby one daughter cell is maintained as a self-renewing stem cell and the other becomes a precursor or progenitor cell capable of giving rise to futher differentiated cells. A progenitor cell shares the potential for differentiation into different tissue lineages, but has limited self-renewal capacity [6, 9].

Due to ethical issues and ecumenical directives, embryonic stem cells are not used in the research of human disease. Other stem cells used in research and disease treatment include tissue-specific stem cells, mesenchymal stem cells (MSCs), fetal stem cells, and cord blood stem cells. In addition, research has been directed at tissue-specific stem cells which reside in and give rise to mature parenchymal cells within a particular tissue or organ.

Mesenchymal stem cells (MSCs) have been shown to have immunomodulatory, antiproliferative, and anti-inflammatory effects. In addition, because of their migratory ability and immune-privileged state, much research has been aimed at understanding the therapeutic poetntial of MSCs. Among others, the therpuetic role of MSCs has been explored in cardiac ischemia, autoimmune disorders [10], severe graft-versus-host disease [11], chronic lung diseases [12–15], and acute lung injury [16–20].

While researchers, biologists, and bioethicists have rallied the medical community to improve our understanding of the biology and mechanism of action of stem cells, confusion over what exactly is a "stem cell" has led to a lack of standardization in production processes [7] and the indiscriminate commercialization of various cell products as purported treatments for patients [9].

7.4 The Role of Stem Cells in Lung Repair

Various stem/progenitor cells that function in lung repair reside in other areas of the body and are recruited in times of injury and inflammation. Cells from the bone marrow, blood, adipose tissue, placenta, and umbilical cord have been shown to structurally engraft in the airway [34, 49] as well as the pulmonary vasculature. In addition, evidence suggests that bone marrow-derived cells such as MSCs are recruited to areas of lung injury and exert their regenerative effects via a paracrine function [35, 41].

MSCs are multipotent and have a diverse but restricted differentiation capability. They appear to function in a paracrine manner with minimal engraftment, interact with the innate and adaptive immune systems [36, 37], and aid in lung repair and regeneration via secretion of cytokines and growth factors to restore alveolar epithelial and endothelial permeability [38–40, 42].

Other sources of stem cells have also been studied in lung disease. Endothelial progenitor cells (EPCs) appear to exert their therapeutic effects via direct differentiation and engraftment into the vasculature of the lung and secretion of factors that mobilize endothelial and progenitor cells and represent a promising source for pulmonary vascular regeneration [55]. Amniotic fluid stem cells (AFSCs), multipotent fetal-associated cells that can be easily and ethically obtained from amniocentesis specimens, have been shown to improve lung density and function in models of diaphragmatic hernia [56]. AFSCs have also been shown to integrate into areas of distal lung epitheleial injury with expedited repair and expression of NKX2-1 and SFPTC (markers of alveolar type 2 cell diffrernitation) [24, 49]. Human amnion epithelial cells (hAECs), found in the lining of the placenta, display low immunogenicity and also appear to possess regenerative and anti-inflammatory properties [57–60].

7.5 Endogenous Stem Cells

Recent work has called into question the existence of stem cells in slowly renewing tissues like the lung [44, 45]. The epithelium lining pulmonary airways turns over slowly during the normal process of tissue maintenance and is replaced far more slowly than specialized post-mitotic cell types of the gut or the epidermis, a property that is reflected in the functional characteristics of airway progenitor cells in their resting versus proliferative states. Therefore, it is not surprising that progenitor cell hierarchies of the lung and other slowly renewing tissues do not fit the classical stem cell hierarchies described in tissues of rapid turnover [42]. In fact, Hu and colleagues, through use of in vivo injury models, have recently described the existence of a stem cell capable of renewing the endocrine pancreas, a tissue that until recently was thought to be maintained

solely through self-duplication of differentiated β cells [61]. This, and previous work in lung to be discussed in more detail below, illustrate a deviation from the classical stem cell hierarchy marked by lack of an obligate transit-amplifying progenitor cell in the steady state. Rather, slowly repairing tissues such as lung are maintained at steady state by an abundant facultative transit-amplifying progenitor that fulfils characteristics of a differentiated cell type in the quiescent state, yet retains proliferative capacity and the ability to generate daughter cells capable of generating other specialized lineages [61]. Therefore, the endogenous stem cell at steady state likely remains quiescent. However, studies utilizing in vivo injury models have revealed stem cells that can be functionally distinguished from facultative progenitors, based upon their resistance to environmental stimuli and spatial localization in the conducting airway.

7.6 Clara Cells

Within the normal lung, Clara cell proliferation maintains the facultative progenitor cell pool (self-renewal) and restores terminally differentiated cells of the conducting airway epithelium (ciliated cells). This vast reparative reservoir distinguishes lung epithelia from tissues such as the intestine that are maintained through proliferation and differentiation of tissue-specific stem cells. The unique features of lung epithelial maintenance and repair suggest that chronic lung disease could be treated through interventions that stabilize the Clara cell pool or by cell replacement strategies that restore this abundant cell type [62].

- Clara cells are non-ciliated secretory cells in the small airways and trachea. Their morphology and biochemical composition display amazing heterogeneity within the airway epithelium of a single species, among different species, and in response to injury.
- Clara cells have several lung protective functions. They detoxify xenobiotics and oxidant gasses, control the extent of inflammation, participate in mucociliary clearance of environmental agents, and proliferate/differentiate to maintain the ciliated cell population.
- Clara cells are secretory and the source of Clara cell secretory protein (CCSP) and contribute surfactant apoproteins A, B, and D, proteases, antimicrobial peptides, several cytokines and chemokines, and mucins to the extracellular fluid lining the airspaces.
- In humans, many forms of lung cancer may originate from Clara cells, including adenocarcinoma, the most frequently diagnosed form of lung cancer. Whether Clara cells have a similar etiologic function in mouse models of adenocarcinoma is more controversial [62].

7.7 Mesenchymal Stem Cells

MSCs, first desccibed by Friedenstein et al. in 1968, are a class of multipotent stem cells with self-proliferative and differentiation potential [40, 42]. MSCs may be isolated from bone marrow as well as other tissues including gingival, adipose, umbilical cord, and placenta. Isolation of MSCs requires that cells (1) exhibit fibroblastic morphology, clonogenicty, and plastic adherence when cultured in standard tissue culture conditions; (2) differentiate into adipocytes, osteoblasts, and chondrocytes in vitro; and (3) express certain cell surface markers such as CD44, Sca-1, CD29, and CD90 but not CD45, CD34, CD14, and CD11b [43–45].

In addition to their capacity for multipotent differentiation and their ease of isolation, MSCs are characterized by a number of features that make them attractive subjects for research in regenerative medicine (Fig. 7.2). MSCs lack immunogenicity, home to areas of injured tissue, and have anti-inflammatory and immunomodulatory effects [26, 61]. Because MSCs have linited expression of MHC class I and II molecules, both autologous and allogeneic administration are easily achieved (Fig. 7.2) [40, 63]. In addition, MSCs can be genetically modified using viral vectors to enhance their therapeutic potential [64–66]. Because of these favorable characterstics, the therapeutic potential of MSCs has

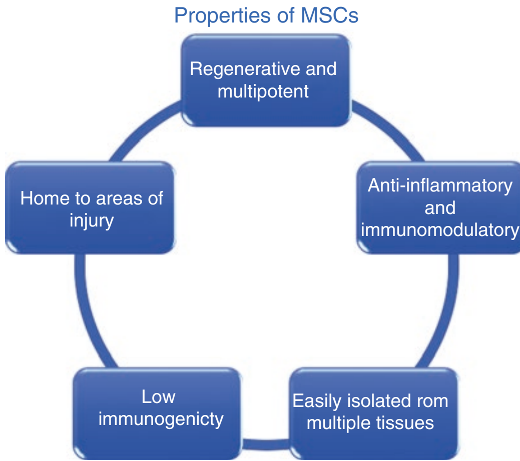

Fig. 7.2 Properties of MSCs

been investigated not only for lung diseases [61] but also for a wide variety of other conditions, including hepatic failure [55], myocardial infarction [67], diabetes [68], sepsis [56], and acute renal failure [24].

7.8 MSCs in the Treatment of IPF

A number of preclinical studies have examined the therapeutic potential of stem and progenitor cell populations in animal models of pulmonary fibrosis and MSCs have been studied in human phase I clinical trials [2]. In addition to those discussed above, a number of additional features suggest that MSCs may be beneficial in the treatment of IPF. MSCs inhibit cytotoxic T-cells and natural killer cells, are known to secrete growth factors including KGF, HGF, VEGF, and Ang-1, and play an important role in the repair of alveolar epithelium through mitochondrial transfer [51, 69].

Because epithelial injury underlies the pathogenesis of pulmonary fibrosis, the delivery of exogenous stem or progenitor cells capable of participating in alveolar reepithelialization may have therapeutic potential to break the cycle of aberrant epithelial–mesenchymal crosstalk and halt disease progression. It is possible that, due to changes in the lung microenvironment and the continued presence of injurious stimuli, exogenous progenitor cells, like endogenous cells, may simply participate in the characteristic pathological repair process. On the other hand, intrinsic factors are thought to play a role in alveolar epithelial cell injury, and preclinical studies suggest that stem cells can exert profound effects through the secretion of soluble mediators [31, 53, 54].

Systematic reviews reveal numerous preclinical studies of MSCs in the treatment of BLM-induced lung injury [5]. To date, these studies suggest that MSCs are effective in improving histopathology, Ashcroft scores of lung fibrosis, lung collagen deposition, and survival in animal models of BLM-induced lung injury. However, most of these studies used young animals and examined the initial inflammatory phase rather than the chronic fibrotic phase. As previously discussed, spontaneous reversal of BLM-induced lung fibrosis may occur spontaneously in young mice [70], but does not occur in aged mouse models [70, 71]. A more recent study utilizing an aged mouse model of BLM-induced lung fibrosis found that treatment with adipose-derived MSCs may promote a systemic acute repair phenotype to prevent fibrosis in multiple organs and enhance wound healing by modulating pro-fibotic factors such as miR-199 and its downstream target, CAV1 [1].

Preclinical studies have shown MSCs to be efficacious in the treatment and prevention of lung fibrosis [53, 72]. Nonetheless, concerns remain regarding the activity of MSCs within a pro-fibrotic microenvironment [73–76]. While some preclinical studies suggest that MSCs might promote fibrosis, to date, no human studies have found a similar pro-fibrotic effect [37, 43, 63, 70, 74, 75, 77–85].

7.9 Clinical Trials of MSCs for the Treatment of IPF

Twenty years ago, Lazarus conducted the first clinical trial using bone marrow cell injection in patients with hematologic malignancies [52]. Since then, numerous clinical trials have been conducted to test the feasibility and efficacy of MSC-based therapy, and more than 2000 patients have recieved allogeneic or autologous MSCs for the treatment of various diseases [52].

Early clinical studies of MSCs in patients with IPF have shown promising safety profiles [30, 78, 86]. A phase Ib study of endobronchially administered autologous adipose-derived MSCs showed not only acceptable safety outcomes but also improvements in quality of life parameters [78]. Longitudinal outcomes of this study also demonstrated an acceptable safety profile with a 100% survival rate at 2 years after the first administration of MSCs and a median overall progression-free survival of 26 months [87].

Studies of intravenously administered placental-derived MSCs [79, 83] have found that administration of up to 2×10^6 cells per kilogram is safe in subjects with moderately severe IPF [83]. Importantly, only minor and transient alterations in peri-infusion hemodynamics and gas exchange were reported, ameliorating concerns regarding the potential embolization of stem cells to an already compromised pulmonary vasculature. At 6 months, there were no observable declines in forced vital capacity (FVC), diffusing lung capacity for carbon monoxide (DLCO), six-minute walk test (6MWT), or CT fibrosis score [88].

The AETHER trial also showed favorable safety outcomes for the single-dose intravenous delivery of up to 2×10^8 allogeneic MSCs in patients with IPF [79]. Although this study was underpowered for the detection of significant changes in functional indices, the mean decline in % predicted FVC and DLCO were below the thresholds for disease progression [1, 46].

At this time, the only study actively recruiting IPF patients for treatment with stem cells is a Phase 1/2 clinical trial (NCT02745184) taking place at two sites in China. Researchers intend to isolate autologous lung stem cells, MSCs from the patient's own bronchi, expand them in the laboratory, and then deliver the expanded cell population via a single injection directly into an area affected by IPF. Safety parameters will be monitored for 1 year and efficacy will be measured by changes in lung function and exercise ability tests.

Looking ahead, ReCell, an FDA-approved phase 1b trial, is planned but has not yet begun enrollment. In this multidose, randomized, double-blind trial, 10×10^6 MSCs will be delivered intravenously to patients with IPF.

A team of scientists from the UNC School of Medicine and North Carolina State University demonstrated that they could harvest lung stem cells from people using a noninvasive office procedure. They snipped tiny, seed-sized samples of airway tissue using a bronchoscope, this method involves far less risk to the patient than does a standard, chest-penetrating surgical biopsy of lung tissue. Cheng and his colleagues cultured lung spheroid cells from these tiny tissue samples until they multiplied to the thousands and were enough to be therapeutically injected.

Once the stem cells were harvested they were able to multiply these lung cells in the lab. The results yielded enough cells sufficient for human therapy. In 2017 these researchers were working with the FDA for preparation of clinical trials in patients with IPF. Cheng, Lobo, and their teams are now planning an initial study of therapeutic lung spheroid cells in a small group of IPF patients.

Currently this study is still undergoing data collection.

7.10 Moving Forward

Stem cells have been used in medicine since the 1950s when bone marrow transplants were first used to treat leukemia. Congressional involvement in stem cell policy started as early as 1974. The first major amendment related to the use of federal funds for research involving embryonic stem (ES) cells occurred in 1996. In 2016 President Obama signed into effect, the 21st Century Cures Act, which includes provisions intended to assure timely regulatory review of regenerative therapies, including cell therapies enabled by stem cell therapy.

While preclinical trials suggest that MSCs may be effective in the treatment of IPF, and early clinical trials support their safety, currently the data to support their efficacy for the treatment of IPF is insufficient. Despite this lack of evidence, cell-based therapies are being aggressively marketed to vulnerable patient populations. A review carried out in November 2018 of the FDA web-

site lists over 1000 stem cell-related businesses registered. These clinics offer unproven, experimental treatments for a wide variety of conditions [89, 90].

In the case of IPF, desperate patients and their physicians continue to succumb to an onslaught of marketing and branding of as yet unproven "stem cell" treatments. Unfortunately, these businesses are also almost wholly unregulated [91]. A review of unapproved stem cell interventions by Turner and Knoepfler and the harm arising from the misuse of unproven treatments support increased government oversight in the interest of patient safety [92]. A sense of urgency exists to establish solid evidence regarding the efficacy of stem cell therapies for the treatment of chronic lung disease.

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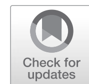

8 Paving the Road for Mesenchymal Stem Cell-Derived Exosome Therapy in Bronchopulmonary Dysplasia and Pulmonary Hypertension

Vincent Yeung, Gareth R. Willis, Elizabeth Taglauer, S. Alex Mitsialis, and Stella Kourembanas

8.1 Introduction

Infants born with extremely low birth weight (ELBW) have increased risk of developing a number of diseases including periventricular leukomalacia (PVL), retinopathy of prematurity (ROP), hypoxic-ischemic encephalopathy (HIE), bronchopulmonary dysplasia (BPD), and pulmonary hypertension (PH) [1]. With notable advancements in surfactant therapy, postnatal nutrition, the refining of steroid-based approaches and an ongoing improvement in ventilation strategies, the current standard of care for preterm infants not only supports their survival but also strives to minimize further organ injury and promote functional long-term recovery [2, 3]. Whereas recent studies established that the frequency of several morbidities associated with prematurity is decreasing [4], the prevalence of BPD is rising, perhaps as a consequence of our own successes and this persists in the era of less-invasive mechanical ventilation [5].

BPD occurs almost exclusively in preterm infants that have required oxygen therapy and mechanical ventilation [6]. Although an accurate definition of BPD has historically lacked uniformity, it is characterized by delayed or disordered lung development, emphysema-like features of lung disease (alveolar simplification), decreased vascular surface area, and abnormal long-term pulmonary function [7, 8]. Notably, secondary PH has been linked to moderate to severe cases of BPD and is characterized by remodeling of pulmonary arterioles, elevated pulmonary vascular resistance, right ventricular hypertrophy that may progress to failure and overall increased infant mortality [9, 10]. Arguably, current pharmacological interventions and past medical advances appear to have only a subtle impact on the severity and long-term consequences of the disease. Thus, there is an imperative need to expand our therapeutic arsenal by developing novel and safe interventions to effectively treat the primary damage and reduce the risks of further complications associated with extreme preterm birth [11]. Herein, we will highlight the application of cell-based therapies, focusing on mesenchymal stem cells (MSCs), and commenting on their immunomodulatory role in experimental models of

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neonatal lung injury and PH, as well as the first clinical trials on MSC therapy on neonates. Furthermore, we will expand on the paracrine attributes of MSC therapeutic properties, presenting current progress towards the development of cell-free therapeutics based on MSC-derived exosomes/extracellular vesicles (MEx).

8.2 Stem Cell-Based Therapies

Stem cells have crucial roles in normal development and in maintaining homeostasis by aiding organ repair and regeneration throughout life, as they can undergo self-renewal and can differentiate into multiple cell types. They can be broadly divided into two categories: pluripotent stem cells (PSCs) and adult (somatic) stem cells, MSCs belonging to the latter category. The defining characteristic of these two stem cell types is that PSCs, either of embryonic origin (ESCs) or induced (iPSCs), can activate an extensive repertoire of differentiation pathways and are able to become almost all specialized cell types. Adult stem cells can only differentiate into a limited number of cell types closely related to their tissue of origin, or, in the case of MSCs, into cell types of mesenchymal origin. A property most relevant to the field of regenerative medicine is that PSCs have essentially unlimited renewing capability, and thus they can be readily expanded in tissue culture without losing their differentiation capabilities. That ability represents a major advantage over cultures of adult stem cells, which can be expanded for only limited passages and eventually lose their "stemness." The PSC advantage is also the basis of the great disadvantage in terms of live cell therapeutic treatments, as they have been associated with teratoma formation in vivo. For a recent concise review please see Kolios & Moodley [12].

The immature organs of preterm infants are vulnerable to the detrimental effects of oxidative stress, infection, and associated insults resulting in tissue damage and perturbations of developmental pathways that, in the case of the immature lung, can lead to BPD and PH [6, 7]. It has been a valid assumption that depletion or dysregulation of endogenous stem/progenitor cell populations in the developing lung could arguably underlie tissue simplification and disease progression [13]. Therefore, an approach to restore homeostasis could be based on harnessing the well-established regenerative and immunomodulatory properties of stem cells. Indeed, over the past decade, a large number of studies, both in preclinical models of disease as well as in clinical trials, explored the promise of the therapeutic potential of interventions based on stem cell transplantation in a number of diverse diseases, albeit the original high expectations have yet to be fulfilled.

8.2.1 Pluripotent Stem Cells (PSCs)

PSCs are comprised of embryonic stem cells (ESCs) [14] and induced pluripotent stem cells (iPSCs) [15]. ESCs have extensive self-renewal capacity and can differentiate into specific germ layers. The identification of four key transcription factors (KLF4, OCT4, SOX2, and c-MYC) that can reprogram fully differentiated somatic cells into PSCs [16] has been a pivotal paradigm shift in the field. The potential to reprogram fully differentiated somatic cells is enormous, as the expansion could occur in vitro, but also, this could overcome immune-mediated rejection by providing as an autologous source of stem cells for transplantation.

There is a scarcity of studies addressing the potential of PSCs for in vivo lung repair. Notably, it has been reported that ESCs, differentiated into alveolar epithelial type II cells (ATII) can populate the mouse lung parenchyma upon endotracheal injection [17]. More recently, innovative approaches using in vitro differentiation regimens and reimplantation or organoid models have demonstrated the capacity of iPSCs to recapitulate the lung architecture and environment by generating distal alveolar epithelial cells and conducting airway epithelial cells [18, 19]. Further, the airway delivery of human iPSCs-derived ATII to a hyperoxia-induced lung injury in vivo model improved lung function and structure [20], but the actual use of PSC transplantation in clinical trials on lung

disease may be far in the future, as ethical constraints surround the generation of ESC lines and the pluripotent nature of both iPSCs and ESCs does raise grave concerns for potential teratogenic risks, as reviewed in [21–23]. In the lung field, the foreseeable current application for iPSCs will be in basic research, as they can be used to generate complex 3D lung organoids [24], useful in studies to understand lung development using in vitro tools. In the near future, iPSCs could be instrumental in whole organ tissue engineering to achieve the repopulation of decellularized xenogeneic lung matrix scaffolds with human cells. When proper topological distribution, cell-type differentiation, and long-term viability is achieved, these efforts could pave the road for unlimited availability of "humanized," transplantation-competent lungs in the not-too-distant future [25–27].

8.2.2 Adult Stem Cells

Originally, the best-described adult stem cells were hematopoietic stem cells, with their potential to reconstitute the entire hematopoiesis program of the bone marrow (BM). Since then, progress in stem cell biology has identified numerous stem/progenitor cells, including endothelial progenitor cells (EPCs), human amnion epithelial cells (hAECs), and MSCs. Transplantation of different adult stem cells types in preclinical models of neonatal disease, including neonatal brain injury [28], hypoxia-ischemia, and cerebral palsy [29], as well as stroke, BPD, and PH [13, 30, 31], have produced very promising results.

EPCs were first identified and isolated from adult peripheral human blood [32], and shown to retain the capacity to differentiate into functional endothelial cells and sustain vasculogenesis. It was hypothesized that EPCs administration would benefit infants at risk with BPD. Indeed, administration of conditioned media (CM) derived from endothelial colony-forming cells (ECFCs) promoted angiogenesis in vitro and ameliorated parameters of PH subsequent to experimental BPD [33]. Intravenous (IV) infusion of human umbilical cord blood (UCB)-derived ECFCs into immuno-deficient rodents exposed to hyperoxia promoted lung vascular growth and attenuated alveolar injury [34]. A disconcerting observation in these rodent animal models was the appearance of aberrant tissue growth in the lungs upon transplantation of long-term cultured EPC-like lines [35]. Although such growth was not observed using fresh EPC cultures, safety concerns may be raised in using such cells in human clinical trials. In contrast, hAECs may represent a promising perinatal tissue-derived product for BPD treatment, as they exhibit mostly anti-inflammatory properties and are isolated from the amniotic membrane at birth [36]. Studies have highlighted that hAECs attenuate the fetal pulmonary inflammatory response and promote the lung-protective effects in neonatal mouse and sheep models [37, 38]. Administration of hAECs in neonatal models of BPD by IV or intratracheal (IT) routes effectively ameliorated alveolar injury and reduced fibrosis with some evidence of transdifferentiation events and cell engraftment [39]. As it has been firmly established for MSCs (see below), it has been suggested that the main mechanism of protection by hAECs against acute and longer-term injury is predominantly paracrine [40, 41]. The donor cells exert their beneficial effects by modulating the local inflammatory response, rather than through tissue repair and engraftment in the recipient lung, as had been postulated in earlier studies [37, 39].

8.3 Mesenchymal Stem Cell-Based Therapies in Preclinical Models of Lung Injury

Mesenchymal stem cells were originally isolated from the bone marrow [42], and subsequently from several other adult tissues such as adipose [43], dental pulp [44], and Wharton's jelly [45]. Early work stressed the multipotent attributes of MSCs, as witnessed by their ability to differentiate into mesoderm-derived lineages. The International Society for Cellular Therapy (ISCT) proposed minimal requirements to define human

MSCs, which entail: (1) adhesion to plastic; (2) expression of the cell surface markers CD73, CD90, and CD105; and (3) lack of CD11b or CD14, CD34, CD45, CD19 or CD79α, and HLA-DR surface expression; (4) ability to differentiate into adipocytes, chondrocytes, and osteoblasts in vitro [46]. The exact definition of MSCs is still evolving, as methodologies used in isolating, expanding, and characterizing MSCs differ considerably between research groups. It has been realized that MSC isolates can exhibit significant inter-culture and intra-culture variation both in surface markers and differentiation potential [47, 48]. It is therefore important to carefully consider the source, lineage, and age of MSC cultures to be used for studies involving therapeutic applications and be aware of possible significant variations in their transcriptome and proteome [43, 49–51]. Indeed, development of novel screening technologies is critical to fully characterize large panels of surface markers and the MSC populations they represent [52, 53]. Driven by these considerations the current consensus is that mesenchymal *stromal* (rather than *stem*) cells should be the more appropriate designation for MSCs, a moniker that reflects observed heterogeneities in functional properties and differentiation potential.

The ease of isolation, expansion, and in vitro differentiation potential of MSCs made them a favorite reagent in preventive and regenerative studies on a wide spectrum of animal models of disease, and MSC transplantation was shown to be very effective in ameliorating and even reversing critical parameters associated with lung diseases, as reviewed in [54–59]. In order to model interstitial lung diseases, bleomycin-induced lung injury is commonly used, inducing pulmonary fibrosis as well as pulmonary hypertension through proinflammatory and fibrotic reactions. The mouse bleomycin model allows for mechanistic studies in these two pathological processes that also exist in BPD—fibrosis to a milder degree and PH seen in moderate to severe cases of BPD [9]. Early studies using the bleomycin mouse model of pulmonary fibrosis [21, 60–63] reviewed in [64] demonstrated that MSC treatment was very effective if administered during the initial phase of inflammation, improving collagen deposition and the Ashcroft score for fibrosis. Animal survival was also improved, and inflammatory markers were reduced in the bronchopulmonary lavage. Nevertheless, MSC treatment was less effective in reversing the fibrotic phase of the disease. Similarly, studies on a rat monocrotaline (MCT) model of pulmonary arterial hypertension demonstrated that either IV or IT administration of BM-derived endothelial progenitors [65] or BM-derived MSCs [66–69] ameliorated arteriolar narrowing, alveolar septum thickening and right ventricular hypertrophy (RVH), and improved RV function and pulmonary vascular resistance, whilst improving endothelium-dependent responses.

Using the mouse model of hypoxia-induced pulmonary hypertension (HPH), Liang et al. demonstrated that the therapeutic effect of mouse BM-MSC treatment was associated with the suppression of the lung inflammatory response induced in the early stages of hypoxic exposure [70]. In this work, a careful assessment of a number of male donor MSCs residing in the recipient female lung demonstrated that the dramatic physiologic effect was associated with insignificant donor cell engraftment. In studies on the hyperoxia-induced neonatal rodent model of BPD, BM-MSC treatment, either by the IV or IT routes, was shown to be effective in protecting against arrested lung vascular and alveolar development [71, 72]. Significantly, and congruently with the low donor cell engraftment observed in the HPH studies, media conditioned by MSCs conferred a similar, or even more robust, protective effect in vivo [71], buttressing the concept that MSC therapeutic action in the lung is predominately paracrine (see below). Subsequent confirmatory studies showed that MSCs isolated from human umbilical cord blood (UCB) can be effective in attenuating hyperoxia-induced lung injury in neonatal rats, exerting both short-term and long-term (6 months) therapeutic benefits, with persistent improvement in exercise capacity and lung structure and without adverse lung effects [73, 74]. Significantly, in the hyperoxia-induced BPD model, UCB-derived MSCs showed significant protection only in the early,

but not the late phase of lung inflammation [73]. This parallels the studies demonstrating that suppression of the early inflammatory peak induced by hypoxia in the mouse model of HPH, by either BM-MSCs [70] or ectopic expression of the cytoprotective enzyme Hmox1 [75], confers long-term protective benefits. This strongly suggests that MSC treatment for lung disease may prove to be more efficacious in preventing the establishment of pathology, arguably by restoring homeostasis at the onset of injury, rather than reversing established injury.

8.3.1 The Therapeutic Action of MSCs in the Lung Is Predominantly Through Paracrine Mechanisms

The original promise of MSCs was that their plasticity and multipotent differentiation capacity would permit the development of therapies where the donor cells would participate in extensive repair and regeneration of injured or diseased lung tissue. Indeed, some early reports, fueled by enthusiasm for the new regenerative paradigm, claimed wide-spread engraftment of donor MSCs into the recipient lung, and massive in situ differentiation of donor cells into pneumocytes residing in the recipient lung [60, 61, 76–78]. Subsequent studies, however, clarified that the impressive improvement in lung physiology observed by MSC transplantation therapy could not be accounted for by donor cell engraftment and trans-differentiation as originally reported. Indeed, careful studies transplanting male mouse BM-MSCs in hypoxic females revealed that donor MSCs survive only a few days in the lung, and although engraftment and transdifferentiation into pneumocytes, in particular alveolar type II epithelium, was observable, the actual number of engrafted donor cells was miniscule [70]. In parallel studies, using a hyperoxia-induced neonatal mouse model of BPD, Aslam et al. reported that media conditioned by mouse BM-MSC (MSC-CM) were more beneficial than IV injections of MSCs in reducing fibrosis and improving alveolar simplification [71]. In a follow-up study, when neonatal mice were exposed to hyperoxia (75% O_2) for 2 weeks to initiate lung injury, it was demonstrated that a single dose of mouse MSC-CM reversed the hyperoxia-induced parenchymal fibrosis and peripheral devascularization (pruning), ameliorated PH and RVH, improved lung alveolar development, and normalized lung function (dynamic lung compliance and airway resistance) [79].

The realization that paracrine factors play a major, if not the sole role in the mechanism of MSC therapeutic action [79–81] has by now been confirmed by many independent studies [82–84]. More recently, a meta-analysis evaluated the therapeutic potential of utilizing MSCs and MSC-CM in experimental BPD [85]. This analysis of 25 controlled studies that met the inclusion criteria used lung alveolarization as the primary outcome and the authors reported that specifically, the administration of MSC-CM significantly improved alveolarization, ameliorated lung inflammation and fibrosis, without comprising safety and efficacy.

Results from these preclinical models strongly suggested that the beneficial effects of MSC paracrine activity are associated with modulation of the host immune system, congruent with the known attribute of MSCs as potent immunomodulators [86–88]. Nevertheless, the exact molecular mechanism(s) remain under active investigation and certain hypotheses have been advanced on the nature of the MSC immunomodulatory paracrine activity and on the host target cell(s) that respond to these MSC signals. Initial efforts in identifying the active therapeutic moieties in the MSC secretome had predominantly focused on growth factors, chemokines and cytokines [89–91], but it became evident that not one single molecule could possibly account for all the observed diverse effects, and not one single molecule could recapitulate all the therapeutic function when administered in isolation. As discussed below, it was soon realized that the vector of MSC therapeutic function was represented by a higher order of complexity, to wit MSC-derived extracellular vesicles.

8.4 Extracellular Vesicles: The Therapeutic Vector of the MSC Secretome

Extracellular vesicles (EVs) are a heterogeneous class of lipid bilayer-enclosed microparticles, harboring diverse plasma membrane and cytoplasmic components. Such submicron structures are produced by all cells (including prokaryotes), and they were originally characterized as a mechanism through which the cell may jettison unwanted molecules, akin to a cellular "garbage shuttle." Although components of the mechanism have been coopted in evolution to also serve other purposes, notably the generation of retroviral particles, the great majority of EVs produced by a normal, healthy cell is, arguably, garbage. Nevertheless, it appears that certain cell types may have evolved a mechanism to produce a subclass of EVs designed for broadcasting signals to their environment, to sustain surveillance of, and, in turn, to affect other cells. That active EV subpopulation, which we can term the "signalosomes," plays a significant role in health and disease, and this realization has opened new research themes for future EV therapeutic application across multiple disciplines. We will discuss here basic tenets of EV biology and the recent and exciting realization that MEx represent the therapeutic vector of the MSC secretome.

8.4.1 EV Diversity and Nomenclature

The size and molecular characteristics of EVs are diverse, and full definition of EV subclasses and their biogenesis remains an active field of research [92]. As described in more detail in Chap. 4, EVs can generally be divided into three main types: (1) exosomes, defined as vesicles of approximately 30–150 nm in diameter and of endosomal origin, appear as intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) and they are released as EVs upon fusion of the MVB with the plasma membrane; (2) microvesicles (MVs), defined as vesicles of approximately 150–1000 nm in diameter that bud directly from the plasma membrane; (3) apoptotic bodies or blebs (ABs), defined as vesicles greater than 1 μm in diameter and released from cells undergoing apoptosis [93]. Of these three major classes, the exosomes are the products of a most intricately controlled biogenesis pathway, with a finely tuned selection of cargo. It is therefore safe to hypothesize that the *signalosomes*, the putative therapeutic vector of MSCs, represent an exosomal subclass [94]. The International Society of Extracellular Vesicles (ISEV) has defined minimal framework guidelines to characterize EVs and their function [94–96]. The term "EVs" is suggested to denote all extracellular vesicles where the purity of a particular preparation cannot be ascertained and ISEV requirements include biophysical properties, subcellular origin, and protein markers, useful as standards to identify the heterogeneous nature of EVs. This incomplete understanding of EV diversity is mostly due to technological limitations in efficiently separating EV subpopulations based upon their molecular profile. Novel approaches, separating EVs based upon biophysical properties, such as asymmetric flow field-flow fractionation (AF4) are expected to facilitate EV subclass definitions [97], and, when applied to MEx preparations, will permit the enrichment of a more homogeneous *signalosome* population that will further our understanding of the relevant cargo conferring the action of MEx on recipient cells.

8.4.2 Exosome Biogenesis and Secretion

The formation of MVBs consists of highly dynamic endosomal membrane compartments involved in the internalization of extracellular protein, ligands, or cellular components, their recycling to the plasma membrane, and/or their degradation [98]. Early endosomes mature into late endosomes and are denoted as MVBs due to their morphological features. During this process, they accrue ILVs in their lumen, through invagination of the defining lipid bilayer, and ILVs are considered to be the precursors to the "true exosomes" (Fig. 8.1). ILVs are formed by

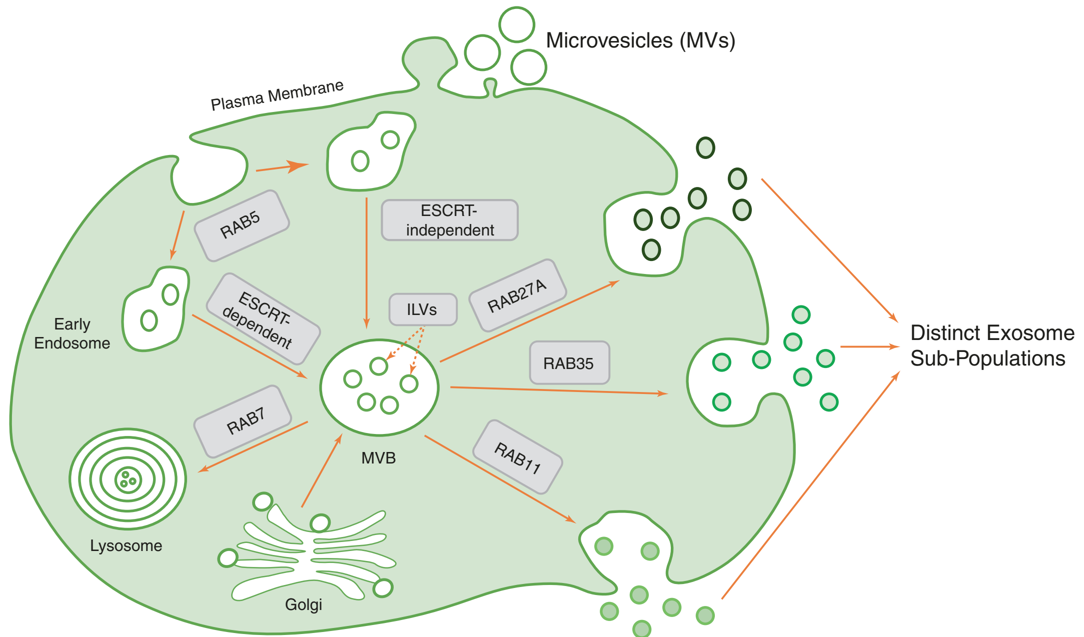

Fig. 8.1 Schematic representation of EVs from different intracellular origin and the molecular machineries of exosome/EV biogenesis and secretion. Microvesicles (MVs) can be released from direct budding from the plasma membrane. Multiple machineries are involved in the biogenesis of intraluminal vesicles of multivesicular bodies (MVB) via endosomal sorting complex required for transport (ESCRT)-dependent or independent mechanisms. It remains unknown whether they act simultaneously on the same MVB or on different MVBs. The Ras-protein (RAB) family has roles in modulating endosomal trafficking; with RAB7 trafficking MVBs for lysosomal degradation; RAB11, RAB27A, and RAB35 have been shown to promote exosome secretion by trafficking MVBs to the plasma membrane. It is proposed that secretion of Distinct Exosome Sub-Populations is governed by the RAB protein family as reported [124, 147, 148]

inward budding of the early endosomal membrane, sequestering proteins and lipids that are specifically sorted. The formation of MVBs and ILVs are processed by the endosomal sorting complex required for transport (ESCRT) complex, containing approximately thirty proteins. This has four distinct ESCRT complexes (ESCRT-0, -I, -II, and -III), with associated proteins such as programmed cell death 6-interacting protein (ALIX), vacuolar protein sorting-associated protein 4 (VPS4), and vacuolar protein sorting-associated protein (VTA1). These complexes and proteins have distinct tasks including: interaction with ubiquitinated membrane proteins to membrane deformation and abscission [99]. Upon MVB maturation, trafficking of MVBs between organelles and the extracellular space is governed by small Ras-associated (RAB) GTPase proteins that are essential for regulating transport between different endosomal compartments [100]. The RAB GTPase superfamily, composed of at least 60 proteins, can play vital roles in controlling membrane identification and MVB budding, motility, uncoating, and fusion [101]. The final step of exosome release, involves fusion with the acceptor membrane that depends on the protein family, soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) [102]. Members of this family are categorized as vesicular SNAREs (v-SNARE) located on the vesicle's membrane and target SNAREs (t-SNARE) located on the membrane of acceptor compartments [103, 104]. This process is proposed to allow the SNARE proteins to form complexes between the MVB and plasma membrane to mediate fusion; thus, allowing the release of ILVs, termed as exosomes, often represented by a heterogeneous population that differs in their molecular composition.

8.4.3 Composition of Extracellular Vesicles

The molecular composition of EVs remains variable and dependent upon the cellular origin, stimuli, and their biogenesis. During biogenesis, EVs incorporate an array of bioactive cargo from their parent cells. Some of the cargo can be reported to include genetic information in the form of DNA, mRNA, noncoding RNAs (microRNA: miRNA), free fatty acids, and proteins. Extracellular vesicle characterization has been aided by the development of comprehensive databases such as ExoCarta [105], Vesiclepedia [106], EVpedia [107], and exoRBase [108] that assembled EV findings from different studies with the goal of aiding investigators in finding molecular signatures specific to cell/tissue type. As a result, certain proteins were identified as being exosome-associated, including flotillin-1 (FLOT1), ESCRT-related proteins, ALIX and tumor susceptibility gene 101 (TSG101), the tetraspanins, CD9, CD63, and CD81, RABs, SNAREs, and others (Fig. 8.2). EV membranes are also enriched in cholesterol, sphingomyelin, ceramide, and lipid raft proteins [95, 109]. Furthermore, EVs express integrins which may contribute to delivering their bioactive cargo to specific tissues and organs [110], but the cargo's fate remains unclear and appears dependent on the route of uptake (caveolin, clathrin, pinocytosis, micropinocytosis, and others) [111]. The bioactive cargo can reflect the stimulus that triggered EV formation, suggesting the packaging of a "signal" can be exported from the parent cell; thus, EVs may act instrumentally for cell–cell communication [112–116]. The specific mechanism(s) by which EVs deliver their cargo and elicit functional responses with target cells remains poorly understood, but EV–target cell interaction is likely to be cell- and EV specific. Some different uptake/endocytic-related pathways may be involved to direct membrane fusion, depending on the cell type.

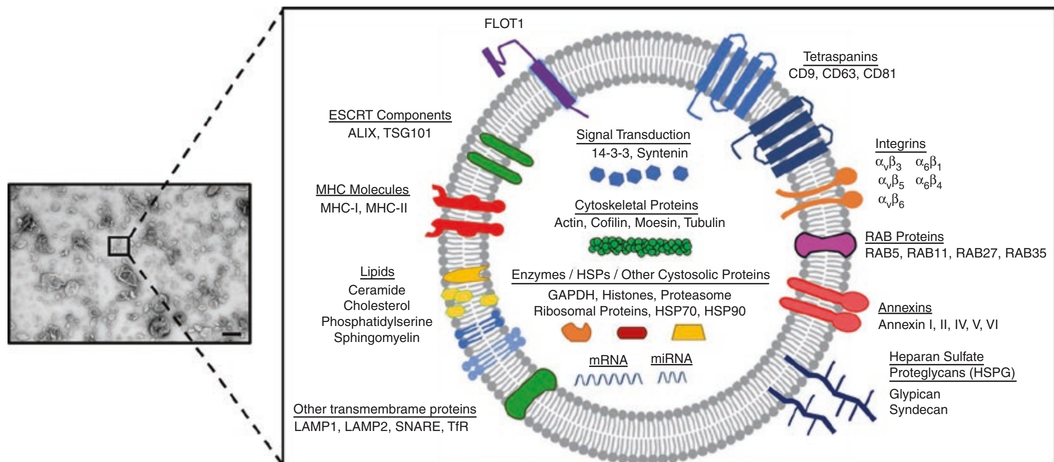

Fig. 8.2 Mesenchymal stem cell exosomes (MEx). *Left panel*: Transmission electron microscopy of MEx derived from human umbilical cord Wharton's Jelly. Exosomes are heterogeneous by nature but typically contain a diameter between 30 and 150 nm. Scale bar: 200 nm. *Right panel*: Typical structure and molecular composition of exosomes. Exosomes are surrounded by a phospholipid bilayer, enriched in several lipids such as ceramide, cholesterol, phosphatidylserine, sphingomyelin. They contain proteins such as flotilin-1 (FLOT1), programmed cell death 6-interacting protein (ALIX), tumor susceptibility gene 101 (TSG101), major histocompatibility complex-I and -II (MHC-I and -II), lysosomal associated membrane protein-1 and -2 (LAMP-1 and -2), transferrin receptor (TfR), tetraspanins, integrins, small GTPase Ras-related (RAB) proteins, annexins, and heparan sulfate proteoglycans (HSPGs). Exosomes can also contain signal transduction molecules, cytoskeletal proteins, enzymes, heat shock proteins (HSPs) and can contain RNA or small noncoding RNAs. The exosomal cargo serves to mediate intracellular communication between different cell types within the body, thus functioning differently in either normal homeostasis or pathological conditions. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; ESCRT: Endosomal sorting complex required for transport; SNARE: soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor

The wide repertoire of miRNAs in MEx could conceivably provide a miRNA-based mechanism for therapeutic function of MSC secretion. It was first reported that RNA was transferred from mouse mast cells to human mast cells via exosomes [117] and this was similarly described in Epstein-Barr virus-infected cells [118] and COS-7 cells [119]. One report found that miRNAs in MSC-derived EVs, more specifically miR-125b-5p (known to target p53) levels were increased in neonatal mouse cardiomyocytes, when cultured with MEx [120], suggesting exosomal transfer of miRNA. By using an anti-miR-125b-5p oligonucleotide, the authors demonstrated MSC-derived EVs effects, may be entirely dependent on miRNA transfer and this was supported in similar studies [121, 122]. Although, the mechanism of action still needs to be addressed, with the broad application of MSC-derived EVs or MEx-based therapeutic application, a single mechanism may not be definitive to address all related diseases. A collective effort remains in elucidating the mechanism(s) of action of EV function and the specific biological moiety responsible for their functional output. With the heterogeneous population of EVs, studies have demonstrated a range of effects in vitro and in vivo disease models, based on density [123], protein [124] or size [97] of EVs. It still represents a prominent challenge in unravelling the complexity behind EV biogenesis and secretion, as identifying the vital bioactive moieties responsible for their biological effect remains unknown. We understand that different EV subpopulations harbor diverse protein and transcriptional cargo; it is fair to speculate they are likely to mediate different effects on target cells. Thus, improved separation techniques between the "non-active" and enrichment of the "bioactive" EV subpopulation will allow us to understand the key mechanistic function potentially utilized in neonatal disease models such as BPD and PH.

8.5 MEx-Based Therapies for Lung Disease

As already discussed, the current dogma is that MSCs act predominately via paracrine mechanisms to confer protection in animal models of lung disease [71, 79], and that the main vector of this paracrine mechanism is comprised by MSC-EVs (MEx) [125, 126] as reviewed in [30, 127–129]. The first report, to our knowledge, that EV preparations isolated from MSC-CM albeit not extensively purified or thoroughly characterized, could substitute for MSC treatment in vivo*,* was that of Bruno et al., who used an acute kidney injury animal model [130]. Subsequently, Lai et al. reported that EVs secreted by human MSC cultures mediated the cardioprotective effects of MSCs in a myocardial ischemia/reperfusion injury model [131]. The report of Lee et al. [125] was the first demonstration that MEx treatment represented a therapeutic alternative for lung pathologies. Using a mouse model of HPH, we reported that mouse BM-MSC MEx, isolated and characterized as per ISEV requirements and further purified through size exclusion chromatography, could protect the animals against the development of lung vascular remodeling, elevated artery pressures, and RVH after 3 weeks of hypoxic exposure at 8.5% O_2. A single dose of MEx suppressed the early hypoxic inflammatory response, characterized by the pulmonary influx of macrophages, attenuated the levels of hypoxia-induced inflammatory cytokines in the lung and protected against the development of vascular remodeling and pulmonary hypertension. Significantly, EVs produced by fibroblasts were inactive, and MSC-CM depleted of exosomes by size exclusion chromatography had no effect, demonstrating that the therapeutic activity was clearly associated with the vesicular fraction of the MSC secretome. Extending these studies to the hyperoxia-induced BPD model, we demonstrated that treatment with MEx derived from human MSCs was effective in radically improving lung morphology and pulmonary development, decreasing lung fibrosis, rescuing pulmonary vasculature loss, and ameliorating vascular remodeling in the mouse lung [126]. Significantly, EVs produced by either human BM MSCs or Wharton's Jelly MSCs were shown to be equally efficacious. This observation should facilitate future large-scale production of MEx, as umbilical cords represent a more abundant source of MSCs than bone marrow, and no invasive procedures are involved in collecting this material.

Arguably even more important is the fact that the young MSC cultures from the cord stroma are pristine, as they do not carry the mutational or epigenetic imprints of the adult donor's health record. This should generate greater reproducibility in MSC clone characteristics, and more uniformity in MEx preparations. Interestingly, the drastic effects we have observed in the murine models of lung disease have been achieved by a single bolus of MEx delivered IV. This strongly suggests that MEx treatment may result in a reprogramming of the recipient's immune system, bestowing long-term protection, despite the continuous environmental insult (hyperoxia or hypoxia) [129]. The hypothesis had originally been advanced that modulation of lung macrophage polarization may represent a protective mechanism against lung injury [75]. Indeed, the immunomodulatory action of MEx on lung macrophages was readily evidenced by assessing the expression of markers associated with either the "M1-like" (classical; proinflammatory) or the "M2-like" (alternate; nonclassical) states of polarization [126]. It is important to realize that macrophage polarization represents a continuous spectrum, and the simple M1/M2 dipolar classification is no longer able to describe the diverse phenotypes. Therefore, the detailed definition of the protective state into which lung macrophages are reprogrammed by MEx treatment is currently an active area of study.

Subsequent studies in the field confirmed and expanded the above original reports, and the bulk of the more recent reports have established the effectiveness of rodent MEx, but also, most significantly, of MEx of human origin in diverse preclinical models of lung disease. Notably, MEx of human origin, systemically delivered, were shown to be even more effective than mouse MEx in mitigating Th2/Th17-mediated allergic airway inflammation in a mouse of asthma induced by Aspergillus hyphal extract [132]. This model is of relevance to neonatal lung pathologies, since there is high prevalence of reactive airway disease at 8 years of age in former preterm infants with BPD [5]. In the rat model of monocrotaline (MCT)-induced PH, MEx isolated from rat BM were reduced right ventricular hypertrophy (RVH) and pulmonary remodeling [133]. In the same model, MEx-containing EV preparations prevented parameters of PH when given at the time of MCT administration and ameliorated these pathologic features when given after the establishment of disease [134]. The mechanism of therapeutic/protective action of MEx treatment for lung pathologies is based on immunomodulation, and more specifically on reprogramming the macrophage phenotype [30, 129]. Studies in the BPD model, using MEx derived from preterm cords, also suggested the importance of TSG-6 protein (TNF alpha-induced protein 6; product of the TNFAIP6 gene) as a MEx cargo component crucial for their therapeutic action [135], but the mechanistic details at the molecular level remain to be defined. The IT route of administering MEx was reported to be effective in a rat model of BPD, where both MEx and MSCs were shown to be effective against hyperoxia-induced damage, but MEx were observed to be more efficacious in terms of alveolarization and lung vascularization parameters [136]. The aggregate promising results on MSC-based and MEx-based therapies on preclinical models of lung disease have therefore created the stage for the first clinical trials.

8.6 MSC-Based Clinical Trials on Neonates

Ongoing clinical trials using MSCs to treat lung diseases, most notably Acute Respiratory Distress Syndrome (ARDS), Chronic Obstructive Pulmonary Disease (COPD), and Idiopathic Pulmonary Fibrosis (IPF) have established the safety (at least in the short- and intermediate term) of MSC treatment and have yielded invaluable information for crafting the strategies necessary to translate procedures from bench to bedside and improve the efficiency of live cell MSC-based therapies [137–140]. Analyzing the lessons from these pilot studies is beyond the scope of this discussion, and herein we will focus on reviewing ongoing clinical trials of MSC treatment for BPD and highlighting the considerations for MSC-based or MEx-based treatment of the neonate.

In 2014, the first Phase I clinical trial of MSC-cell therapy for BPD prevention was reported [141]. Using a single-arm, dose-escalation design, the study involved 9 preterm infants born at

25.3 ± 0.9 weeks gestational age. These patients had a mean birthweight of 793 ± 127 g, with no known severe congenital anomalies and no clinical evidence of septic shock or severe (≧Grade 3) intraventricular hemorrhage at the time of the study. At a postnatal age ranging from 5 to 14 days, the patients were determined at the "highest risk" for BPD based on the clinical criteria of continuous ventilatory support and an inability to tolerate weaning of ventilatory support in the 24 h prior to recruitment. The 9 recruited infants were administered a single, IT dose of the allogenic human UCB-MSCs at an average timeframe of 10.4 ± 2.6 postnatal days. The first three patients were administered a dose of 1×10^6 cells (low dose). With no onset of short-term adverse events noted in these series of patients, the subsequent 6 patients were administered 2×10^6 cells IT (high dose). The infants were closely monitored for signs of cardiorespiratory compromise, anaphylaxis, infection and other serious adverse events (SAE) as defined by the Consolidated Standards of Reporting Trials [142]. The reportable SAE within the cohort included patent ductus arteriosus (PDA) ligations (4/9 patients). Apart from these events, IT administration of MSCs appears well tolerated within these patients with no significant clinical compromise noted in the short-term period.

Levels of lung inflammatory markers were investigated in the tracheal aspirates that included IL-6, IL-8, and TNF-α which have roles that may augment an inflammatory cascade and exacerbate lung injury by mediating macrophage "classical" activation, neutrophil infiltration, and maintaining proinflammatory cytokine expression inhibiting lung development. A reduction in these markers was observed at 7 days post transplantation compared to pretransplant levels. However, lacking a parallel control group during the study invites further questioning, as it remains unclear whether this change was related to the natural infant's lung disease progression or MSC administration. However, due to the timeframe of this study (up to 36 weeks corrected gestational age), 3/9 patients went on to develop moderate BPD and no formal conclusions can be made regarding the clinical efficacy of MSCs in treating BPD.

This study demonstrated the feasibility and short-term safety of IT administration of MSCs, laying the groundwork for future clinical trials. In their follow-up study, patients were continued to be monitored at regular intervals for 2 years following MSC administration and reported the clinical outcomes of this longer-term evaluation [143]. From the 9 patients that received IT MSC therapy, 8 survived long term, with 1 patient's death occurring due to *Enterobacter cloacae* sepsis/enteritis following discharge from the NICU. The 8 surviving infants were rehospitalized an average of 1.4 times during the 2-year post-NICU-discharge evaluation period, with approximately 50% of these admissions due to respiratory viral infections (rhinovirus, parainfluenza, and RSV). Furthermore, the infants did not show any signs/symptoms of teratoma formation during this follow-up period, and no visible mass lesions were detected on chest radiographs taken on their third interval visit. This was an important follow-up parameter to consider as administration of some stem cells (PSCs or ESCs) into immunocompromised, premature patients carries a potential risk for teratoma formation. Therefore, MSC administration based upon the follow-up study appears to be safe with no SAE occurring in the neonatal population.

A similarly designed Phase I trial was reported on 12 ELBW infants that demonstrated feasibility and tolerance of MSC administration [144]. Further conclusions regarding efficacy of MSC therapy in the neonatal population will require larger patient cohorts with the concomitant evaluation of appropriate control populations. With accumulating preclinical evidence on MSC's ability to also reverse BPD-associated PH, future MSC clinical trials for BPD should continue concomitant evaluation of cardiac function, in both short-term and long-term outcome evaluations. In the Phase I BPD MSC study [141], serial echocardiograms were performed pre and post MSC therapy showing no alteration in cardiac function and specifically no PH. However, a larger proportion of the neonatal "at-risk" BPD population with more long-term evaluations of cardiac function will be significantly valuable in determining the potential of MSC therapy in prevention of BPD-associated PH. The pertinent clinical data from these studies and current active trials are summarized in Table 8.1.

Table 8.1 Update on ongoing and completed clinical trials using mesenchymal stem cells for bronchopulmonary dysplasia (BPD)

Study type	Age	Weight (Birth)	*n*	Dose	MSC source	Frequency	Delivery	Follow up	Outcome	Status	Ref/NCT identifier
Phase I	23–29 weeks	500–1250 g	9	10×10^6/kg ($n = 3$ patients) 20×10^6/kg ($n = 6$ patients)	Umbilical Cord Blood	Single dose	IT	36 weeks corrected gestational age	No short-term adverse outcomes	Completed	Chang et al. 2014 (Won-Sung Park, Korea)
Phase I Follow up	2 years	500–1250 g	8	10×10^6/kg ($n = 3$ patients) 20×10^6/kg ($n = 6$ patients)	Umbilical Cord Blood	Single dose	IT	2 years chronological age	No long-term adverse outcomes	Completed	Ahn et al. 2017 (won-Sung Park, Korea)
Phase I Follow up	5 years	Unknown	8	10×10^6/kg ($n = 3$ patients) 20×10^6/kg ($n = 6$ patients)	Umbilical Cord Blood	Single dose	IT	5 years chronological age	N/A	Active, not recruiting	NCT02023788 (Won-Sung Park, Korea)
Phase II	7–14 days	Unknown	70	10×10^6/kg	Umbilical Cord Blood	Single dose	IT	36 weeks corrected gestational age	N/A	Unknown	NCT01828957 (Won-Sung Park, Korea)
Phase I/II	7–14 days	Unknown	12	10×10^6/kg or 20×10^6/kg	Umbilical Cord Blood	Single dose	IT	36 weeks corrected gestational age/20 months	N/A	Completed	NCT02381366 (Steven Powell, USA)
Phase I	Infant (1–6 months)	Unknown	10	3×10^6/kg	Umbilical Cord	Single dose	IT	1 year	N/A	Unknown	NCT01207869 (Huiying Liu, China)
Phase I	>14 days	Unknown	10	5×10^6/kg	Umbilical Cord Blood	Single dose	IT	2 years	N/A	Not yet recruiting	NCT02443961 (Maria Jesus del Cerro, Spain)

IT Intratracheal, *MSC* Mesenchymal stem cell, *NCT* National clinical trial

Interestingly, the safety profile of stem cell-based therapy for infant cardiac disease has been explored for several years prior to the initiation MSC cell-based therapy for BPD [145]. However, the clinical trials to date have primarily focused on autologous cardiac progenitors or EPCs administered to infants with hypoplastic left heart syndrome or idiopathic PH. Overall, this demonstrates safety profiles for stem cell transplantation for neonatal/infant cardiovascular conditions with some potential promising outcomes for improvement particularly in right ventricular function. While cardiac progenitor cells may align with the physiologic needs of congenital cardiac disease, the PH associated with BPD is multifactorial in origin and will likely benefit from the broader therapeutic capabilities of MSC-based therapies.

8.7 Considerations and Challenges for MSC-Based Therapies in the NICU

The initial results from MSC-based clinical trials for BPD are highly promising. While BPD is treated as a "multisystemic" disease given its established causal relationship with PH, the pre- and postnatal events leading to BPD development (such as growth restriction, premature birth, hyperoxia) are also implicated in other organ pathologies in the multisystemic syndrome of prematurity. For example, apart from PH, retinopathy of prematurity (ROP) and intraventricular hemorrhage are among the highest comorbidities associated with severe BPD phenotypes. While these diseases can be attributed to separate organ-specific pathologies, they are combined under the concept of a developing preterm infant fetus that is having to further develop in an "extrauterine" environment. This environment may provide basic life-sustaining support, but still lacks the benefit of a healthy gestational molecular network within the developing womb. This is a potential reason why Wharton's Jelly MSCs should be considered as a "first-line" treatment modality for neonatal disease such as BPD. These MSCs growing in the intrauterine environment exist within and likely contribute to the same "homeostatic" complex network of molecular signals as the developing infant [51]. In this context the promising reports from the ongoing clinical trials give us hope that future refinement of methodology and careful selection of the MSC cultures will lead to high therapeutic efficacy and reproducible outcomes. It is safe to assume that, if in the future live cell-based therapy for the neonate becomes commonplace, MSCs from the umbilical cord will be used. They have been demonstrated to be as efficacious as BM-derived MSCs in preclinical models, and they represent a young, pristine cell type, that does not carry the possible burden of a lifetime history to pathogens and environmental insults that adult BM MSCs have been exposed to.

Based on the positive therapeutic findings from preclinical studies from a range of different diseases [114], there is good reason to be highly optimistic for the outcomes of clinical trials on MEx-based therapeutics for BPD. The advantages of a reagent that encapsulates the therapeutic action of MSCs but has not the multitude of drawbacks, in both safety and logistics, associated with live cells, are evident and paramount. As further discussed in Willis et al [116], the efficacy of MEx treatment has been reproducibly and robustly established in the laboratory, using diverse preclinical models of disease, but, for the industrial-scale production of GMP-grade pharmaceuticals based on MEx to be used in clinical trials a number of technological and mechanistic issues must be resolved. These include the definition of a widely acceptable Potency Unit for MEx preparations, the standardization of the conditions for MSC culture and the protocols for MEx harvesting and storage. In addition, safety considerations also need to be addressed, although it may be expected that such concerns will be arguably milder than those relevant to live cell MSC treatment, as mutagenicity and oncogenicity concerns will be null.

The transition of MEx to the clinic will require the safety and production to be certified to a good manufacturing practice (GMP) quality. There is no current "gold" standard to isolate and purify exosomes in this still budding field. Commonly used methods such as ultracentrifugation often coprecipitate exosome preparations with non-exosomal-associated proteins/molecules, promot-

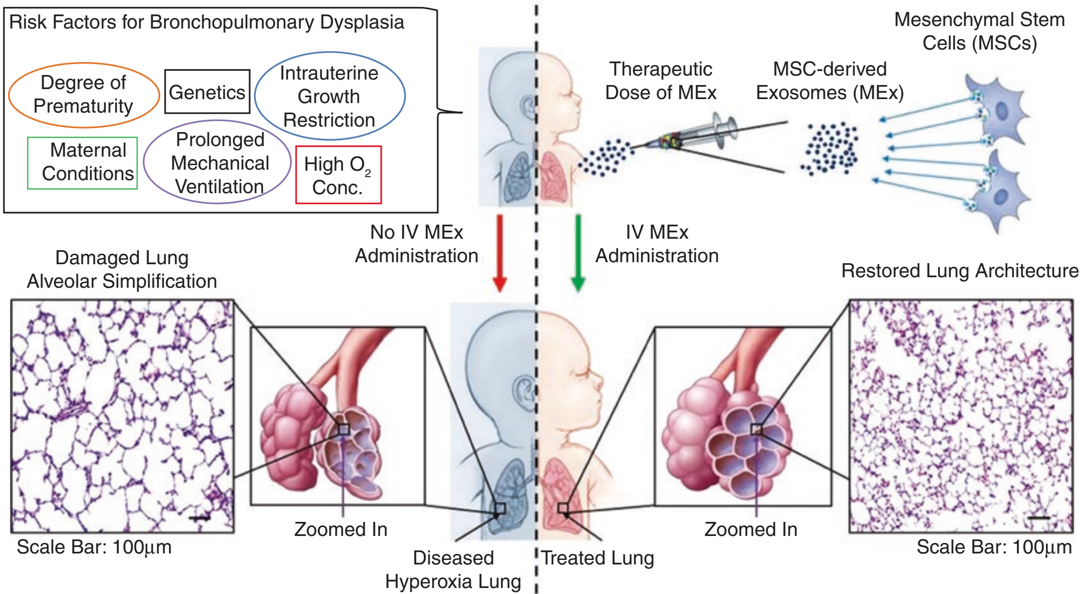

Fig. 8.3 Schematic summary for using mesenchymal stem cell-derived exosomes (MEx) for treating neonatal infants with bronchopulmonary dysplasia (BPD). Preterm infants with BPD have respiratory difficulties, characterized by lung growth arrest and emphysema-like features of lung disease (alveloar simplification), that can lead to pulmonary hypertension (PH). Accumulating preclinical studies have demonstrated that the functional therapeutic vector present in the mesenchymal stem cells (MSCs) secretome are extracellular vesicles, including exosomes (MEx), and MSC's therapeutic efficacy can be recapitulated by a cell-free MEx-based treatment. Here, we propose that intravenous delivery of MEx into preterm infants with BPD can restore lung architecutre and restore homeostasis in neonatal disease

ing exosome aggregation which may alter its in vivo distribution and function [123, 146]. For application to our preclinical models of lung disease we have isolated MEx from MSC CM by differential centrifugation and concentrated the product by tangential flow filtration (TFF) before MEx purification either by size-exclusion chromatography (SEC) [125] or through density flotation in an Iodixanol-based cushion gradient (Optiprep™) [126]. Using the latter method, the MEx-containing fraction has a biochemical density of 1.18 g/ml and contains a characteristically low protein to particle ratio, signifying efficient separation from nonvesicular components of the secretome. This methodology results in a product of high purity, but it is not suitable for manufacturing. MEx-based clinical trials will probably use products purified by alternative methods, amenable to large-scale production and GMP standards, most probably SEC. Without belittling the formidable challenges, we will face in our efforts to translate MEx-based therapy from the laboratory bench to the NICU (Fig. 8.3), we are confident that we will witness the first clinical trials using MEx to treat BPD in the very near future.

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CRISPR/Cas9 Editing in Induced Pluripotent Stem Cells: A Way Forward for Treating Cystic Fibrosis?

9

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9.1 Introduction

Cystic fibrosis (CF) is a genetic disorder characterized by dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) protein, an ion channel found in a variety of epithelial tissues. CF is caused by mutations in the CFTR resulting in an imbalance in cellular chloride, sodium and bicarbonate ion concentrations. This leads to the generation of thick mucus plugs forming biofilms colonized by numerous bacteria. Patients have chronic lung infections and these progressively lead to a fatal decline in lung function [1]. Improvements in diagnosis and treatments of CF have had a substantial impact on life expectancy in patients in recent years raising the average to over 37 years. To date the most promising treatments, small molecule modulators and potentiators of CFTR, are a result of significant investments in CF research and utilization of high-throughput drug screening in the 2000s [2, 3]. With recent advancements in cellular reprogramming and genetic manipulation techniques, there is another possibility for CF breakthroughs utilizing state-of-the-art technologies.

The Darwinian theory explains evolution as a product of natural selection, a process of genetic recoding through acquisition of advantageous mutations occurring over millennia to adapt for survival. Through the years following Charles Darwin's epochal discoveries, scientists have proposed leveraging the tools of evolution to intentionally edit the human genome as a therapeutic approach for genetic diseases like CF. Deliberate genetic engineering is a field that continues to evolve decades after its first discovery, holding so much promise yet, to date, has delivered little success in the clinic. Some have considered it one of the greatest challenges to modern human medicine. After a trial in the early 2000s, when gene therapy was used to successfully correct the gene causing severe combined immunodeficiency, but led to leukemia several years later due to off-target oncogene activation, enthusiasm for and public perception of the field became somewhat stagnant [4]. However, the recent advent of induced pluripotent stem cells (iPSC) has provided an unlimited source of patient-specific stem cells for gene editing and subsequent differentiation, an advance that will work in tandem with recent gene editing breakthroughs to propel the field forward and lead to autologous cell therapy. A new wave of research has begun to develop novel approaches to generate healthy autologous cells for gene editing and clini-

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cal translation, but there are still a multitude of challenges to overcome before this concept becomes reality.

Patient-specific disease models represent the ideal approach to personalized medicine; however, using patient-derived iPSC alone is likely inadequate, as many models require optimized differentiation protocols and isogenic controls. The differentiation potential of iPSC to each of the three germ layers is highly variable among iPSC cell lines from different donors and is likely due to genetic variation [5–9]. The genetically inherent variability in differentiation efficiency of iPSC lines highlights the need for efficient and adaptable differentiation protocols, and the importance of pairing mutant lines and nonmutant lines on otherwise identical genetic backgrounds [10–12]. Coupled with state-of-the-art advances in gene-editing technology, most notably clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR/Cas9), patient-derived and commercially available iPSC lines can be used to model disease phenotypes across genetic backgrounds. By coupling CRISPR/Cas9-mediated gene editing with iPSC, clonogenic mutant cell lines can be easily derived or corrected and paired with isogenic controls. These gene-edited iPSCs can then be differentiated into any adult cell type to model disease where primary cells are difficult to isolate and genetically manipulate. CF is one such disease in which difficulties procuring and culturing patient cells are compounded by the thousands of genetic mutations that would need representation in screens aimed at providing new treatments to as many patients as possible. This chapter will summarize the evolution of iPSC and gene editing techniques and discuss the potential for their future utilization in the clinic in the treatment of CF.

9.2 Cystic Fibrosis

9.2.1 Prevalence and Incidence of CF

CF is the most common lethal autosomal recessive disorder affecting at least 70,000 people worldwide [13]. Although multiple organ functions are affected due to mutations in the cystic fibrosis trans-membrane conductance regulator *CFTR*, lung failure due to chronic bacterial infections and inflammation is responsible for most of the morbidity and mortality associated with the disease [14]. Advances in the therapeutic options for CF patients have substantially improved both the quality of life and lifespan, with current life expectancy for those born from 2013 to 2017 at 44 years, an increase of 70% over two decades based on data obtained from the 2017 CF patient registry (https://www.cff.org/Research/Researcher-Resources/Patient-Registry/). CF, however, remains without a cure. Severe pulmonary disease is the primary cause of mortality in almost 80% of CF-related early deaths [15–17]. The only effective therapy currently available for CF end-stage lung disease is lung transplantation.

9.2.2 Mutation Classes Identified in CF

To date there are over 2000 *CFTR* gene variants in the CFTR mutation database, with more than 300 mutations confirmed to be causative of CF (www.cftr2.org). The heterogeneity of causative CF mutations results in several types of transcriptional, translational, or posttranslational malfunctions depending on the type and location of the CFTR mutation. These mutations are divided into six classifications depending on their effect on the CFTR protein [18] and are summarized in Fig. 9.1.

9.2.3 Current Therapeutic Options for CF Patients

The increased average life expectancy of CF patients is a result of advances in therapeutic options. CF is highly heterogenic and the severity of disease is variable even among patients with identical CFTR mutations, as such, most treatment options are symptom based. Treatments can comprise of a combination of preventive measures, therapies, medications, and surgical proce-

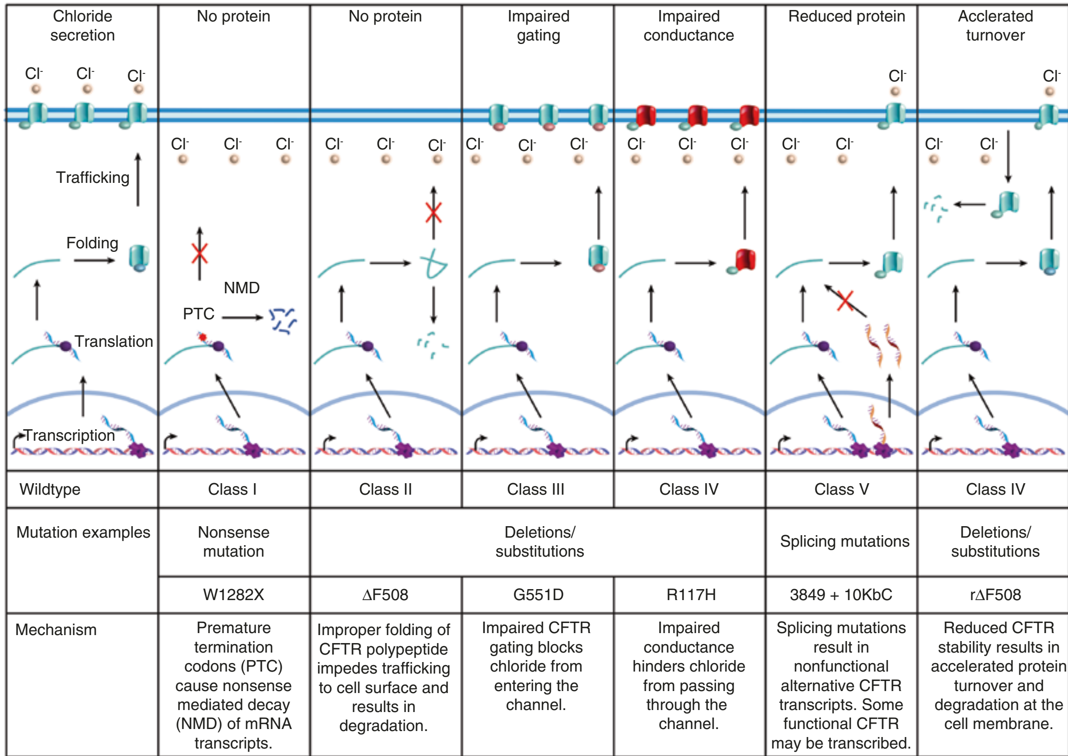

Fig. 9.1 Schematic of the functional changes caused by the different CFTR mutation classes. Normally, CFTR is transcribed, translated, folded into the correct conformation and trafficked to the cell membrane where it functions as an ATP-gated chloride channel. Frameshift and nonsense mutations that introduce a premature termination codon (PTC) can result in Class I mutations, producing nonfunctional truncated proteins as well accelerated turnover of transcripts by nonsense-mediated decay (NMD). Deletions and substitutions are common causes of Class II, III and IV mutations. Class II mutations, such as the most prevalent CFTR mutation delF508, result in misfolded protein that does not traffic to cell membrane. Class III mutations caused impaired gating, keeping the CFTR channel constitutively closed. Class IV mutations are characterized by impaired conductance, reducing the amount of chloride passed through the channel. Class V mutations result in reduced amounts of functional protein trafficked to the membrane. This can be caused by splicing mutations resulting in increased production of nonfunctional alternative transcripts for CFTR. Finally class VI mutations decrease the stability of CFTR at the membrane, resulting in increased endocytosis of the protein

dures. Ultimately, lung transplantation is commonly performed for patients with end-stage lung disease from CF [19–22]. CF is the most common indication for lung transplant in the pediatric population and the third most common indication in the adult population [23, 24]. Unfortunately, long-term survival following lung transplantation is still poor compared to that for other solid organ transplants. Within 5 years of receiving a lung transplant, nearly 50% of patients develop bronchiolitis obliterans syndrome (BOS) the most common cause of morbidity and mortality beyond 1 year following lung transplantation [23]. BOS is thought to be a manifestation of chronic graft rejection and/or Obliterative bronchiolitis (OB), an inflammatory and fibro-proliferative disorder that affects the distal airways in the transplanted lung and results in a progressive decline of lung function [25].

Recent advancements in medications have focused on the development of drugs known as CFTR modulators that aim to treat CF at the level of CFTR protein. The types of CFTR modulators and their applications have recently been reviewed [26, 27]. Briefly, some Class I mutations caused by premature termination codons

(PTC) can be treated by aminoglycosides that allow translational read-through of the PTC [28]. CFTR correctors can treat Class II mutations by correcting protein mis-folding, allowing variant CFTR to traffic to the cellular membrane. Both class III and class IV mutations may be treated by CFTR potentiators, restoring channel gating or conductance respectively. Stabilizers may treat class VI mutations reducing turnover of unstable CFTR at the plasma membrane [26, 29]. Finally, CFTR amplifiers may be used in combination therapies with modulators of all classes of mutations to increase the total amount of functional CFTR at the cells apical surface. Combination therapy is an important aspect of CFTR modulator treatments. Currently there are three drugs approved for use with specific mutations: Tezacaftor, Lumacaftor, and Ivacaftor. Lumacaftor and Tezacaftor are both CFTR correctors for treatment of the deltaF508 mutation; however, there is still impaired conductance of the rescued variant protein. Combined treatment with Ivacaftor, a potentiator of the G551D mutation, can restore some function of the variant deltaF508 CFTR protein at the membrane. These drugs target some of the most common mutations, but with patient-to-patient variability and inadequate efficacy for all genotypes new therapeutics are necessary.

9.3 Editing the Genome

9.3.1 Manipulating the Genome

Gene therapy involves directly manipulating a host genome by inserting new DNA that results in altered biological functions. Genetic engineering, on the other hand, harnesses the cells own genetic repair pathway of homologous recombination to correct a defective endogenous gene, restoring status quo in affected tissue. The concept of genetic engineering is one met with both great scientific intrigue and excitement, but also a level of public reservation; as such the field is plagued by controversy. Despite this, one has to acknowledge that the manipulation of the genetic make-up of an organism, or indeed just a cell, has the potential for enormous scientific opportunity for discoveries in medicine, agriculture, and industry.

Progress in gene therapy is dictated by the generation of delivery systems that have the capacity to efficiently transfer functional genes into a variety of cells or tissues with no pathogenicity. In the 1960s/early 1970s the notion that exogenous DNA could be permanently introduced into a mammalian cell line, enabling functional gene expression through generations of cell division, started to become reality [30]. Synthetic and viral approaches were subsequently developed and the first clinical trials in humans extended from viral vector testing in animal models using retro, adeno, and adeno-associated viruses. Utilizing viruses as gene delivery systems requires “stripping down” the viral genome by replacing the replicating structural and regulatory proteins with the gene of interest and leaving alone the cis-acting elements essential to the packaging and integration of the genetic information into a host genome. The vectors are derived from viruses with RNA or DNA genomes, which are capable of infecting cells and directing gene expression. Viruses either integrate into the host genome producing a lifelong expression of the delivered gene or are non-integrating proffering a more transient gene expression. The choice of viral vectors adapted for gene delivery to human cells to provide either permanent or transient transgene expression depends upon a number of factors; efficiency of infection, expression level required, and ease of production, in addition to safety, toxicity, and stability. Virus options include adenoviruses, γ-retroviruses, lentiviruses, poxviruses, adeno-associated viruses, baculoviruses, and herpes simplex viruses.

By 1996, over 100 clinical trials had been initiated in the United States; however, evidence for success was in short supply [31, 32]. Enthusiasm for the trials diminished as therapeutic promise was overshadowed by the challenges of efficient delivery to the target cells, sustaining gene expression, and minimizing off-target effects and adverse immune reactions [33]. CF was an obvious disease candidate for treatment by gene ther-

apy and the first clinical trials targeted the lungs by aerosol delivery of DNA. The success of these trials was hampered by the efficiency of delivery to the target cells. The correct CFTR DNA would need to be expressed at the apical surface of epithelial cells lining the lung in patients suffering from CF. Herein lies the problem: CF patients have a damaged epithelial layer covered in biofilms of thick sticky bacteria-ridden mucus making delivery by aerosol to the target cells virtually impossible [34]. Gene therapy has made substantial progress since its inception, but it has been far slower than initially predicted and hindered by adverse clinical effects [35]. Insertional mutagenesis, efficiency of delivery, access to the cell target for delivery, immunological responses, and sustained and sufficient expression are just a few of the confounding factors that needed to be resolved for gene therapy to fulfill its potential. There is still hope. As a new era of gene editing has begun with the advent of regenerative medicine and newer, more precise gene editing methods. It is these new tools that become the focus of this chapter.

9.3.2 Gene Editing with Endonucleases

Unlike viral approaches that introduce pieces of exogenous DNA, these newer technologies target the DNA sequence within the endogenous nuclear genome. Genes can now be modified or knocked out in situ leaving no exogenous genomic trace. The technology of this field has rapidly evolved from a very inefficient traditional homologous recombination (HR) approach to highly precise and efficient methods involving designer nucleases. This technology has already transformed basic biological research around the world and progressed to the clinical with the three types of designer nucleases currently active, or slated to begin, clinical trials in 2019 based on the National Institute of Health's clinical trials database (https://clinicaltrials.gov/).

Propagation of genetic material is essential to the continuation of life, yet the process is flawed, and thousands of genetic lesions are created in each cell every single day. Many of these DNA lesions are relatively benign; however, some, such as a double-stranded break (DSB), can be considerably toxic leading to genomic instability and carcinogenesis. In fact, the genetic information on an entire chromosome arm can be lost due to a DSB. If DSB are not rapidly repaired, mutations and chromosome rearrangements can eventually cause cell death. Evolution has led to cunning ways to exploit the generation of a two-sided (frank) DSB to control biological processes. For example, programmed DSBs occur to initiate class switch recombination during maturation of immunoglobulin genes [36], they occur during meiosis [37, 38] and during transposition [39]. Cells have also developed a number of competing repair pathways which include homologous recombination (HR), nonhomologous end joining (NHEJ), and single-strand annealing (SSA). HR is a DNA metabolic process critical for genomic preservation through supporting DNA replication and telomere maintenance. It consists of a number of subpathways that culminate in high-fidelity repair of DNA damage using DNA strand invasion and template-directed DNA synthesis. Such pathways include the classical DSB repair [40, 41], Holliday Junction-mediated HR (HJHR) [42–44], break-induced replication (BIR) [45–48], and synthesis-dependent strand annealing (SDSA) [49, 50].

Gene editing essentially hijacks this endogenous pathway, utilizing the creation of double-strand breaks (DSB) in the vicinity of an extra-chromosomal fragment of donor DNA to insert an exogenous sequence into the DNA repair locus. In the absence of HR, error-prone NHEJ can result in indels that may be useful to knock out genes by targeting their genomic sequences [51]. Spontaneous HR with extrachromosomal DNA is exceptionally inefficient, but the ability to create a DSB at a desired genomic locus significantly stimulates the efficiency of HR-mediated recombination [52–54]. Scientists realized this and began to develop strategies for creating DSB at specific genomic loci to target specific genes. Capecchi and colleagues were among the first to develop and utilize this technology, which they used to create the first genetic knockout mice [55–58]. Since

this seminal discovery, the field has continued to evolve and new technologies have been developed that specifically target a single desired genetic loci. Such technologies include customized engineered endonucleases including zinc finger nucleases (ZFN) [59, 60], transcription activator-like effector nuclease (TALENS) [61–64] and, most recently, CRISPR [65, 66].

9.3.2.1 Zinc Finger Nucleases (ZFNs)

Over a decade ago, the laboratory of Chandrasegaran was among the first to design a ZFN to target specific DNA sequences, first in *Xenopus laevis* oocyte nuclei in 2001 followed shortly by human cells in 2003 [67, 68]. These consisted of several three-finger zinc finger proteins (ZFP) linked to the nonspecific cleavage domain of the FokI Type IIS restriction endonuclease, creating customized nucleases [59, 69]. The targeting of specific DNA sequences is thus possible by manipulating the modular assembly of the ZF domains [70, 71]. The ability to create domains for sequence-specific recognition enabled relatively efficient cleavage specific to the corresponding ZFN and subsequent HR at those targeted sites [67, 68]. ZFNs, like the FokI endonuclease, must dimerize to be able to cleave and generate a DSB. The number of ZFPs can vary from a minimum of three fingers up to six fingers. Increasing the number of fingers boosts specificity but limits the possibility of finding a suitable target site. While the modular assembly is a desirable feature of ZFNs, it is also their limitation—target sequences are limited to the ZFP triplets of base pairs available. Additionally, there are some issues in assembling ZF domains; certain combinations significantly alter the DNA binding properties in ways that are unpredictable. While the use of ZFNs represented a significant breakthrough in gene editing, the limitations in their construction did not make the technology easily accessible for all.

9.3.2.2 Transcription Activator-Like Effector Nuclease (TALENS)

Several of the limitations of ZFNs were overcome when TALENs were discovered in 2009. Site-specific DNA binding is achieved by *proteins* known as TAL effectors, which are derived from the plant pathogen *Xanthomonas* sp. that uses TAL effector proteins to weaken its host's defense. TALs are assembled in a modular fashion, similar to that of ZFN, and can be linked to the catalytic domain of the FokI endonuclease domain to form TALENS capable of generating site-specific DSB [72–74]. They differ from ZFNs in that each TAL domain is able to recognize a single base. TALENs therefore offer greater specificity and versatility in targeting desired locations in the genome. Each TAL domain consists of a unit of 33–35 amino acids arranged in tandem, which contains a central repeat domain specifying a single base preference; this is determined by two critical amino acids adjacent to each other known as the "repeat variable di-residue" or RVD. Back-to-back papers published in Science at the end of 2009 enlightened us on exactly how TAL effector specificity is determined [72, 73]. While the increased specificity and reduced production costs made them a promising alternative to ZNF, TALENs were quickly overshadowed by CRISPR/Cas9.

9.3.3 The CRISPR/Cas9 Revolution

A discovery by Dr. Jennifer Doudna's laboratory has made specific gene editing accessible to all. The CRISPR/Cas9 system is the most recent advance in the gene-editing field. This technology is less labor-intensive, cheaper, and surmounts the necessity to rely on protein-based recognition of target DNA sequences. Research into the function of uncharacterized repetitive DNA sequences in a variety of prokaryotes led to the fortuitous discovery of a bacterial adaptive immune system comprised of a RNA-guided DNA-cleaving enzyme (Cas9) and CRISPR transcripts that target foreign DNA for degradation. CRISPR-Cas9 has already been reviewed extensively and we refer you to a selection of these reviews for more detail [75–81]. Briefly, while there are three types of CRISPR-Cas9 systems that have been identified, it is the type II system that has evolved to be what we now refer to when discussing gene editing. Different organisms have different CRISPR systems, but the CRISPR type II sys-

tem in *Streptococcus pyogenes* is the simplest. Cas9 is the feature protein of this system critical for CRISPR RNA (crRNA) maturation, but is only triggered to cleave DNA in the presence of transactivating crRNA (tracrRNA). In fact, the tracrRNA, a small noncoding RNA, has two essential functions: to initiate pre-crRNA processing by RNase III enzymes and to activate crRNA-guided DNA cleavage by Cas9. Two single-stranded RNA oligonucleotides mimicking the naturally occurring dual tracrRNA:crRNA complex can be used for gene editing. For simplicity single guide RNAs (gRNA) maintain two key features: the 20/21 nucleotide guide sequence for DNA binding specificity and the hairpin that will recruit the Cas9 protein. The 20/21 nucleotide sequence can be designed to target any stretch of DNA adjacent to a protospacer motif (PAM), which is an NGG sequence, at its 3′ end. This directs the DSB to a specific site in the genomic DNA for subsequent repair by HR or NHEJ (Fig. 9.2). CRISPR/Cas9 represents a technology that can be applied to an enormous variety of scientific research because its limiting factor is gRNA design and production, which is much faster and cheaper than TALEN and ZNF protein With CRISPR, it is possible to either generate mutations in specific genes [82, 83], correct mutations at the endogenous locus [84–91], or tag endogenous genes with reporters to study specific signaling pathways and trace cells during differentiation [92–96].

Since the first experiments in *E. coli*, the application of CRISPR to scientific research has grown exponentially [83, 97]. Experiments have shown that a modified Cas9 protein lacking endonucleolytic activity (dCas9) co-expressed with a specific 20/21 nucleotide single guide RNA (sgRNA) could specifically silence the gene it is complementary to by blocking transcriptional machinery access to DNA. With dCAS9 fusion proteins, transcriptional co-regulators or epigenetic modifiers can be targeted to specific sequences, allowing for construction CRISPR libraries for repressing and activating genes [98–107] or manipulation of the epigenome through targeted methylation or chromosome looping [108–113].

The CRISPR gene editing technology has exploded at such speed that there are still questions poised over potential safety and ethical issues. As with TALENs and ZFNs, there is concern over the potential for off-target effects that remain to be fully evaluated [114–121]. It has been suggested that the potential for off-target effects can be reduced by truncating the gRNA to <20 bp [122] with sgRNA sequences of 14–15 bp still guiding Cas9 but inhibiting enzymatic activity [123], while mutant Cas9 nickases (nCas9) have also been developed that make single strand nicks instead of DSBs [124]. Using nCas9 with two multiplexed sgRNAs complementary to offset sequences on opposite strands around a target site will cause a DSB. Because single nicks are naturally repaired by the cell's high-fidelity base excision repair, and two sgRNA-nCas9 are required for a DSB; this method reduces off-target activity by 50–1500 fold. In addition, four multiplexed sgRNAs designed to target nCas9 simultaneously to donor and genomic DNA have been shown to produce accurate HDR at rates comparable to standard DSBs [125]. Recent work with DNA- and RNA-based editors fused with dCas9 or another variant dCas13 has enabled editing of the genome without making any cuts, or bypassing the genome altogether and editing transcripts directly [126, 127]. Nonetheless, gene editing is widely accessible to the entire research community and Fig. 9.3 and Table 9.1 provides a summary of the current applications of CRISPR.

9.4 Pluripotent Stem Cells

9.4.1 Defining and Inducing Pluripotency

Embryonic stem cells (ESC) and especially iPSC are often considered to have revolutionized our access to human biology due to their limitless self-renewal and capacity for differentiation. Human ESC were first cultured by Thomson and colleagues back in 1998 [171]. Derived from the inner cell mass of a blastocyst, a pre-implantation-

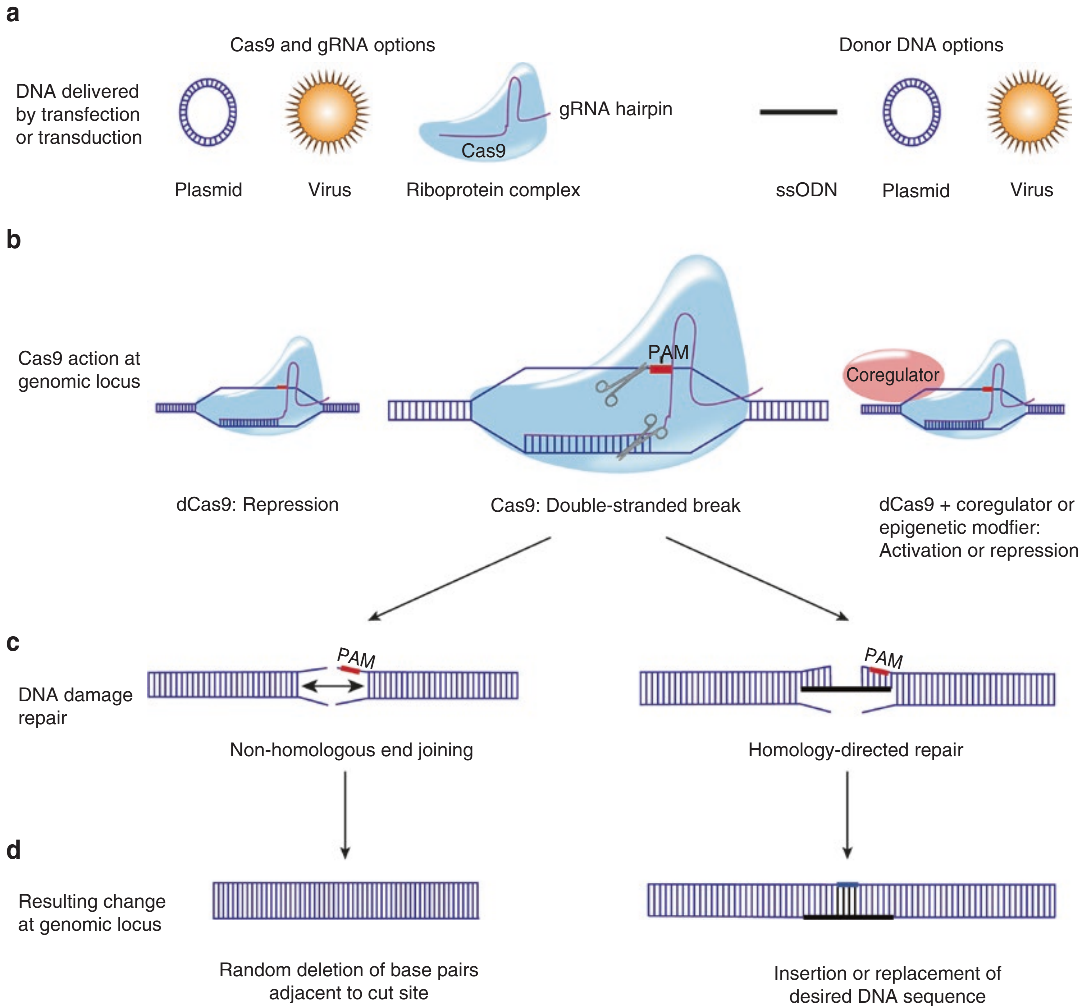

Fig. 9.2 CRISPR/Cas9 Gene Manipulation. (**a**) Delivery methods for the Cas9, guide RNA, donor DNA or pre-formed riboprotein complex. Plasmid and viral delivery requires exogenous expression and formation of the Cas9/gRNA riboprotein complex in the cell. Donor DNA can be delivered as single-stranded oligodeoxynucleotide or as double-stranded DNA usually as plasmid or packaged in virus. (**b**) Once the riboprotein complex has bound the correct sequence adjacent to the protospacer adjacent motif (PAM) site, Cas9 nuclease activity creates a double-stranded break (DSB) approximately 3 bp upstream of the PAM. Enzymatically inactive Cas9 (dCas9) can regulate gene expression by steric hindrance, through attached co-regulators or epigenetic modifiers such as VP16. (**c**) In the absence of homologous donor DNA DSB are repaired by nonhomologous end joining (NHEJ) which often resects several base pairs before joining the DNA strands. In the presence of donor DNA homology arms invade the free strands, loose DNA is excised, and gaps filled in and ligated by the cells endogenous enzymes. (**d**) The end result of NHEJ is the deletion of base pairs near the cut site, often leading to nonsense mutations when targeted to gene exons. As a consequence of homology directed repair, homology arms are integrated into genomic sequence along with any base pair changes or insertions accompanying them

stage embryo, these cells are able to differentiate into all derivatives of the three primary germ layers: the endoderm, ectoderm, and mesoderm. The use of ESC in scientific research has been plagued by ethical and religious concerns surrounding the issue of “what constitutes life,” resulting in vast differences in regulations pertaining to their use worldwide. Unlike ESC, iPSC can be generated, or “induced” from almost any somatic cell type in the human body. This circumvents any of the ethical concerns raised from obtaining human ESC. Induced pluripotency became a reality in

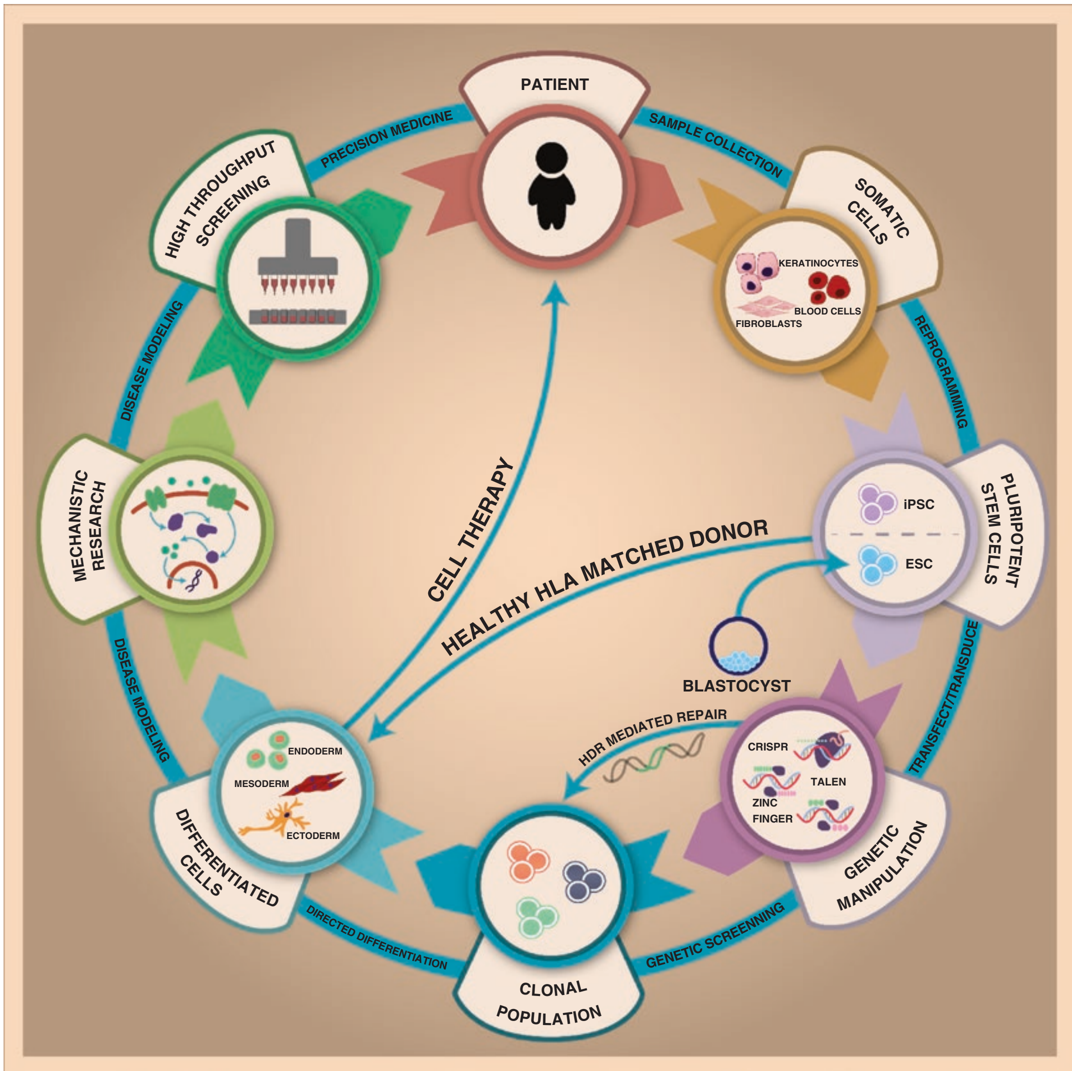

Fig. 9.3 Overview of Pluripotent Stem Cells and Genetic Engineering for Autologous Cell Therapy. Somatic cells taken from a patient or donor are reprogrammed into induced pluripotent stem cells. Human Leukocyte antigen (HLA) match donor iPSC or embryonic stem cells (ESC) can be differentiated to a specific cell type and used for cell therapy, while patient derived iPSC will need to be mutation corrected, clonally expanded and genetically screened. Differentiated patient iPSC can also be used to model diseases in vitro for study of molecular mechanisms or for high-thoughput drug screening on patient-specific genetic background to be used for precision medicine

2006 when Yamanaka and colleagues discovered a combination of genes and culture conditions that were able to reprogram fully differentiated mouse fibroblasts to cells that resembled mouse ESC [128]. They followed this study in 2007, demonstrating the same reprogramming capacity in human skin fibroblasts generating the first human iPSC [131, 132]. Thomson's laboratory published a similar protocol concurrently in Science, solidifying the generation of iPSC from somatic cell sources [136]. iPSC are able to undergo regular passaging in vitro without loss of their pluripotent state and generate cells representing all three primary germ layers. iPSC may not replace the necessity for studying primary human tissues but they provide the ability to gen-

Table 9.1 Current applications of CRISPR-Cas9

Application	Variations of CRISPR	Examples
Disease Modeling	Cas9 alone for disruption or with HDR template for correction	Knocking in an exon of dystrophin corrects DMD in patient-derived iPSCs and differentiated skeletal muscle cells [175] Correction of CFTR restores gene function in airway epithelial cells derived from cystic fibrosis patient-derived iPSCs [141] Inserting common mutations of colorectal cancer into intestinal organoids with a Cas9-sgRNA plasmid and HDR ssDNA template caused them to develop tumors and metastasize into the spleen, modeling colorectal cancer [176]
Gene repression (CRISPRi)	dCas9 alone or fused to gene repressor	dCas9 alone sterically hinders binding of RNA polymerase to target gene [177] Multiple dCas9-gRNAs targeting the same gene stack repression [83] Fusion of dCas9 to repressor effector protein (KRAB domain of Kox1) silences GFP expression [178]
Gene activation (CRISPRa)	dCas9 fused to gene activator	dCas9 fused to RNA polymerase ω subunit binds to promoter to activate gene expression in bacteria [177] dCas9 fused to herpes simplex activation domain tetramer VP64 activates genes in human cells [179]
Transcriptional programming (CRISPRi + CRISPRa)	dCas9 fused to scRNA	Lentiviral delivery of dCas9 and scaffold RNAs (scRNAs) that encode both target site and protein-binding site allows for simultaneous gene activation and repression at multiple genes in human HEK293 cells [180]
Creation of transgenic animals	Cas9 alone, Cas9 with HDR donor, or Cas9 nickase	Microinjection of Cas9 D10A nickase and ssDNA HDR template into mouse zygotes for analysis of SNPs in mice [181] Multiplexed repression and directed point mutation of multiple genes in mouse ESCs with single injection into zygotes [182] Cas9 and two gRNAs make large 10 kb deletions in mouse zygotes that pass on to offspring [183] Cas9n nickase with two gRNAs creates specific gene knock-in or knock-out mice [184] Correction of dystrophin gene in mouse germline prevents muscular dystrophy [88] Somatic cell nuclear transfer from gene-edited fibroblasts to zygotes induces biallelic knockout mutations in goats [185] and pigs [186] for agricultural research Creation of DMD disease model in rhesus monkey [187] Creation of transgenic African turquoise killifish, the shortest-lived captive vertebrate at 4–6 months' lifespan, for study of age-related diseases [188] Transfection of plasmid with Cas9, sgRNA, and antibiotic resistance to chicken germ cells and creation of homozygous egg white gene knockout rooster offspring [189]
Gene editing in adult animals	Cas9 alone, Cas9 with HDR donor, or Cas9 nickase in viral or nonviral delivery vector	Hydrodynamic injection of Cas9-gRNA plasmid and ssDNA donor to mice tail vein corrects *Fah* gene and rescues weight loss phenotype in tyrosinemia model mice [190] Delivery of Cas9 mRNA in lipid nanoparticles and sgRNA/HDR donor in AAVs corrects *Fah* in mouse hepatocytes and corrects weight loss phenotypes [191] Lentiviral delivery of CRISPR-Cas9 causes chromosomal rearrangement of EML4 and Alk genes to generate tumors [192] Adenoviral delivery of Cas9 nickase and 2 gRNAs to excise DMD exon 23 in DMD model mouse muscle restores regenerative capacity of stem cells [193]
Whole genome functional screening	Library of sgRNAs combined with normal Cas9 or dCas9 fused to activators or repressors	Activation of 70,000 genes to find ones that provide resistance to BRAF inhibitor in patient-derived samples [101] Identification of genes required for cell transmission or cell free entry of hepatitis C virus [194] Cas9-EGFP lentivirus with lentiviral sgRNA library represses genes in nonmetastatic tumor cells to identify tumor suppressors preventing metastasis in mice [100]

Table 9.1 (continued)

Application	Variations of CRISPR	Examples
Inducible regulation	iCRISPR or dCas9 fused to N terminal fragment of CIB1	Doxycycline-inducible CRISPR (iCRISPR) for controllable knockout and knockin of genes in human iPSCs during specific points in differentiation [195] Light-activated CRISPR (LACE) for gene activation in specific tissue by blue light-mediated activation of cytochrome 2p to interact with CIB1 [196]
Antiviral therapy	Cas9/gRNA plasmid	Targeting surface antigen of hepatitis B virus to remove it from mouse liver tissue [197] Disrupting and preventing against HIV infection in iPSCs [198]
Cell therapy	Cas9 alone, Cas9 with HDR donor, or Cas9 nickase	Correction of β-globin mutation in human HSCs for transplantation into mice and rescue of sickle cell anemia phenotype [199] Reversion of chromosomal inversion in coagulation factor VII in human iPSCs to rescue hemophilia A phenotype after transplantation to disease model mice [86]
DNA labeling	dCas9 fused to fluorescent protein	Visualization of regions of DNA to study spatiotemporal organization i.e. labeling telomeres to study elongation and disruption [200] Multicolor CRISPR labeling by fusion of different fluorescent proteins to dCas9-sgRNAs to find distance between chromosomal loci and degree of compaction [201]
DNA isolation	dCas9 fused to epitope	Attaching a tag to a genomic region to purify with immunopreciptation and identify local DNA-binding proteins with mass spectrometry (enChIP) [202]
Epigenetic regulation	dCas9 fused to an epigenetic effector or repressor	dCas9 fused to p300 acetyltransferase acetylates H3K27 to activate gene promoters and enhancers in human cells [203] dCas9 fused to demethylase represses enhancer activity in mouse ESCs [204]
Enzymatic DNA base editing	dCas9 fused to cytidine deaminase	High efficiency enzymatic conversion of C to T in window 4 to 8 bases from PAM without inducing DSBs [205]
RNA detection diagnostics	CRISPR-C2c2 of (type VI CRISPR)	CRISPR-C2c2 cleaves all RNA when target mRNA is present, causing fluorophore-labeled RNA to signal when human β-actin mRNA is present [206]
Gene drives	Plasmid with Cas9-sgRNA flanked by 2 homology arms (MCR)	Propagation of edited homozygous loss of function mutation to offspring by insertion of DNA sequence for CRISPR-sgRNA into disrupted gene in single parent i.e. for disease gene editing in mosquitos [207]
Editing human germline	Cas9 mRNA, gRNA, GFP, and ssDNA template	Insertion of 6 silent mutations to β-globin gene in human tripronuclear (3PN) zygotes with Cas9 and HDR donor resulted in low HDR efficiency, mutations caused by delta-globin being used as a template, off-target effects, and mosaic embryos [208]

DMD Duchene muscular dystrophy, *iPSCs* induced pluripotent stem cells, *CFTR* Cystic fibrosis transmembrane regulator, *GFP* green fluorescent protein, *SNP* single nucleotide polymorphism, *ESC* embryonic stem cell, *AAV* adeno-associated virus, *EGFP* enhanced green fluorescent protein, *PD-1* programmed cell death protein 1, *enChIP* engineered DNA-binding molecule-mediated chromatin immunoprecipitation, *PAM* protospacer adjacent motif

erate many cell types and tissues that remain inaccessible, such as neurons from the brains of Parkinson's patients or tissue-specific cells from those with rare hereditary diseases. Thinking ahead to a future of personalized, precision medicine and cell-based therapeutic approaches, iPSC allow for treatment with one's own cells thus guaranteeing HLA haplotype match and negating the necessity for immunosuppression. Multiple groups worldwide, including the California Institute for Regenerative Medicine (CIRM), the Center for iPSC Cell Research and Application (CiRA), and the European Bank of Induced Pluripotent Stem Cells (EBiSC), have already begun amassing cell banks containing iPSC from healthy donors with homozygous HLA to develop a stock for cell therapy that is immunocompatible with a continually growing proportion of the population. The generation and use of pluripotent stem cells is summarized in Fig. 9.3.

As stem cell treatments progress toward the clinic, there is an increased focus on the stringent and rigorous regulatory concerns that cell therapies will need to surpass in order to make it into patients. Additionally, it is evident that iPSC face additional scrutiny over ESC. The major concerns over the use of ESC reflect issues with their long-term genetic stability in culture, their potential to be tumorigenic and the purity of the cell product to be used. iPSC add additional trepidations, particularly regarding the methodology for reprogramming influencing the genetic stability, identity and safety of the cells. Initial reprogramming studies used integrating viruses (retrovirus and lentivirus) to express the reprograming genes [131, 132, 136]. This method of reprogramming integrates proto-oncogenic genes into the host genome, leading to potential for insertional mutagenesis and an increased risk of tumorigenesis due to the spontaneous reactivation of the viral transgenes [172–174]. A multitude of options for transgene delivery to somatic cells now exist, enabling reprogramming without integration into the host genome. Table 9.2 provides a comprehensive analysis of the currently available reprogramming methods with examples of tissue and species variety.

Despite significant advances in the methodologies available for generation of iPSC, the efficiency of reprogramming remains notably poor—there is inherent variability and a necessity to optimize reprogramming for each cell source. That said, the generation of iPSC is now a relatively routine procedure in many laboratories. A distinct heterogeneity in iPSC lines, even those generated from the same cell source, leads to characteristic differences in pluripotency and differentiation potential. Labs have now started carrying out larger scale “omics” analyses between ESC and iPSC generated from the same or different donors using multiple reprogramming techniques and media [209]. The data generated from the Progenitor Cell Biology Consortium, published in Stem Cell Reports, can be accessed online via https://synapse.org, a resource that will be continually added to and will provide a valuable analytical reference for the scientific community. Additionally, detailed protocols for generating clinical-grade iPSC and derivatives are now being published [210, 211]. The potential use of iPSC and their derivatives in modeling disease, screening new drugs for novel therapeutic targets and toxicity, and as a source of autologous cell therapy should not be undermined nor forgotten and may be a more pertinent value and application of iPSC.

9.4.2 Specification of Lung Epithelium from iPSC

The application of pluripotent stem cells in clinical trials has already begun for treatment of macular degeneration; using stem cell-derived retinal pigmented epithelium (RPE). RPE is on the forefront of pluripotent-derived cell therapy, because RPE is composed of a terminally differentiated, nonproliferating, long-lived, and engraftable cell type where relatively pure populations can consistently be differentiated from iPSC and ESC [212–214]. To use iPSCs to treat lung diseases such as CF an effective differentiation protocol deriving lung epithelial cell progenitors is necessary.

The main epithelial progenitor cells of the proximal airways are basal cells, characterized by the expression of tumor protein p63 (TP63), cytokeratin 5 (KTR5), integrin α 6 (ITGA6), and nerve growth factor receptor (NGFR) and ultimately their capacity for differentiation to functional cell types in pseudostratified polarized respiratory epithelium in vitro. Chap. 2 provides a comprehensive overview of both airway and alveolar epithelial cells. In particular, this differentiation requires the generation of a specialized postmitotic multiciliated cell (MCC). Several groups have shown iPSC differentiation to MCC and other specialized cell types of the proximal and distal airways yet differences in protocols, cell lines and differentiation efficiencies highlight the need for a standardized differentiation protocol amendable to different cell lines as well as guidelines for characterization of iPSC-derived basal cells [141].

Current lung differentiation protocols share similar strategies attempting to mimic lung development from embryogenesis through fetal development. This is accomplished via combinations of growth factors, cytokines and small molecules added in temporal specific pattern to direct differentiation stepwise from pluripotency towards endoderm, to anterior foregut endoderm (AFE), to

Table 9.2 Currently available methods of reprogramming

Technology	Genes	Species	Comments
Retrovirus ×4	OKSM OSNL OKS ± M	Ms Fb [128–130], Hu Fb [131–134], Hu renal Ep (urine) [135] Hu Fb [136] Ms. Fb [137], Hu Fb [137], Hu Fb [134]	Large footprint *Enhanced by*: Silencing of PUMA and p21 [129], small molecule OAC1 [130], SSEA3+ fibroblasts [133],miR-19a/b [134]
Lentivirus ×4	OKSM	Hu Fibroblasts [138]	Large footprint *Features*: Feeder Free+ ROCK Inhibitor [138]
Polycistronic Lentivirus + CRE/LOXP	OKSM OKSMNL	Hu Kt [139], Hu Fb [140] Hu Fb [141], Hu BM [141], Hu PBCD34+ [142]	Small footprint *Features*: Inclusion of shRNAp53 [141], STEMCCA-loxP lentivector [142]
miRNA	*miR302/367*	Ms Fb [143], Hu Fb [143]	No footprint *Features*: Viral [15], polyketal nanoparticle-mediated [144]
Sendai virus	OKSM OKS	Hu Fb [145–147], Hu Skeletal Myo [148], Hu NEC [149], Hu WJ-MSC [150], Hu PBCD34+ [142] Chimp PBC [151], Hu PBC [151]	No footprint *Features*: Replication-defective and persistent Sendai virus [146], Xeno-Free [147], feeder-free [148], TS12KOS [151], *Enhanced by*: Inhibitors of histone deacetylase (sodium butyrate) and SB431542 [148]
Episomal	OKSMNL OKSM OS OKSL+ L-MYC	Hu Fb [152], Hu CBMNC [153] Hu PBMNC [154] Hu PBCD34+ [155] Hu PBCD34+ [156], Hu CBCD34+ [156]	No footprint *Features*: 6-in-1 oriP/EBNA1-based vectors [152, 153, 156] *Enhanced by*: inclusion of BCL-XL [154], inclusion of Wpre [155], shRNA for TP53 [156], EBNA1 [156]
mRNA	OKSML+Rarg and Lrh-1 OKSM OKSML	Hu Fb [157] Hu Fb [158] Hu Fb [158]	No footprint *Features*: Xeno and Feeder free [157] *Enhanced by*: Oct4 incorporating an N-terminal MyoD transactivation domain [157]
piggyBac transposon	OKSM	Ms Fb [159, 160], Rat Fb [161], Bo Fb [162]	No footprint *Features*: Polycistronic [160] *Enhanced by*: inclusion of EOS [160], inclusion of shRNA TP53 [161]
Sleeping beauty transposon	OKSM	Hu Fb [163], Bo Fb [162]	No footprint
Minicircles	OSNL	Hu ASC [164], Hu Fb [165], Ch Fb [166]	No footprint *Features*: 4-in-1 codon-optimized minicircle (CoMiC) [165]
Protein	OKSM	Ms Hp [167]	No footprint *Features*: 9R-fused reprogramming proteins [167]
Adenovirus	OKSM	Rat Fb [168, 169], Rat BM MSC [169]	No footprint *Features*: Polycistronic [169]
Mini-intronic plasmid (MIP)	OKSM OKSM±L	Ms Fb [170] Hu Fb [170], Hu PBMNC [170]	No footprint *Features*: 4-in-1 plus shRNAp53 [170]

OKSM OCT3/4, KLF4, SOX2, c-Myc, *OSNL* OCT3/4, SOX2, Nanog, LIN28, *OKSMNL* OCT3/4, KLF4, SOX2, c-Myc, Nanog, LIN28, *Hu* human, *Ms* Mouse, *Fb* Fibroblast, *Ep* Epithelial, *CBMNC* Cord Blood Mononuclear Cell, *PBMNC* Peripheral Blood Mononuclear Cells, *Kt* Keratinocytes, *BM* Bone Marrow, *Hp* Hepatocytes, *ASC* Adipose Stem Cell, *MIP* Mini intronic plasmid, *Ch* Chicken, *SB431542* TGF-beta signaling inhibitor, *NEC* Nasal Epithelial Cell, *PBCD34+* Peripheral blood non mobilized CD34+ cells, *PBC* Peripheral Blood Cells, *WJ-MSC* Wharton's Jelly Umbilical Cord Mesenchymal Stem Cells, *Wpre* Woodchuck posttranscriptional regulatory element, *EOS* early transposon promoter and Oct3/4 and Sox2 enhancers, *Bo* Bovine

specified lung progenitor and finally to mature cell types. Of the three germ layers formed during embryogenesis, the endoderm lineage eventually gives rise to the gut, pancreas, liver, lung, and thyroid. As such, directed endoderm differentiation was of early interest in the ESC field. The transforming growth factor beta (TGF-β) signaling pathway had been shown to be critical for endoderm formation in mice, and in 2005 D'Amour and colleagues used activin A, a TGF-β signaling molecule, to generate ESC-derived endoderm cells [215]. Most early research into ESC-derived endoderm lineage, focused on liver, intestine, and pancreas as AFE derivatives proved more difficult to produce. In 2011, efficient AFE differentiation from endoderm was established by Green and colleagues who modulated TGF-β and bone morphogenic protein (BMP) signaling through use of the TGF-β signaling inhibitor SB-431542 and the BMP antagonist Noggin [216].

Reports on iPSC-derived lung epithelium soon followed, with several groups using combinations of BMP and fibroblast growth factor (FGF) signaling molecules to derive cells expressing NKX2.1, a transcription factor identified as earliest marker of lung lineage specification during development. In order to select for these cells, Hawkins and colleagues used an Nkx2.1-GFP fluorescent reporter iPSC cell line to isolate prospective lung progenitor cells from their differentiation and perform single cell RNA-seq analysis. From their experiments, they identified a cell surface marker expression profile of CD26low/CD47 high that could be used to isolate NKX2.1 positive cells in the absence of a fluorescent reporter [217]. Current research focuses on isolation and expansion of these Nkx2.1 cells in conditions that facilitate the expression of same markers found in basal cell progenitors isolated from proximal airways in hopes of producing bona fide iPSC-derived basal (iBasal) cells. While iPSC-derived lung epithelial cells are already being used to model lung disease in vitro, future research will need in-depth characterization of iBasal cells before they can be applied for stem cell therapy.

9.5 Gene-Edited iPSC Progeny: A Therapeutic Option for CF?

The purpose of gene therapy is to treat disease-causing mutations by delivery of the correct or therapeutic gene to the nucleus, enabling physiologically functional gene expression. Direct in vivo gene therapy has been limited by a number of factors discussed throughout this review. A combination of two of the most groundbreaking technological advances this decade, iPSC and gene editing with engineered endonucleases, bestows a novel therapeutic approach for autologous gene therapy.

It did not take long for Yamanaka and colleagues to adapt the iPSC technology from mouse embryonic fibroblasts (2006) to human fibroblasts (2007); therefore it should come as no surprise that iPSC have now been generated from a multitude of species [161, 162, 218–226] and tissues [85, 132, 142, 149, 227–230]. By utilizing a patient's own cells, the necessity for immunosuppression is eliminated and an unlimited supply of autologous cells is theoretically plausible. Any identified disease-causing mutation can be specifically targeted in these cells and corrected by the combination of a nonmutant donor DNA template with one of the technologies generating a DSB, be it ZFN, TALEN or CRISPR. These gene-corrected autologous cells could then be used in cell therapy either to repair damaged tissues or as a delivery vehicle for small molecules to treat disease. While their value in vivo is still yet to be realized, in vitro they are proving to be an invaluable source for modeling human tissue, organs and disease development. Correction of a disease-causing gene in iPSC provides the perfect experimental control for in vivo studies, whether it be for high-throughput drug screens or for investigation of poorly defined cellular mechanisms contributing to disease pathogenesis. As the cell lines are isogenic, the only difference between the gene corrected cells and the mutant cells would be the single mutation of interest. This rapidly emerging field of stem cell gene therapy has already witnessed many "proof-of-principle" studies where patient-derived iPSC have been gene-corrected to restore normal cell function.

9.5.1 iPSC Models of CF

Understanding the complexity of cellular phenotype and function in the adult human lung is hindered by lack of reproducible and patient-specific in vitro models in which to study the molecular mechanisms and transcriptional programs that govern cell specification and function. Several animal models have been generated including the CF mouse, rat, ferret, rabbit and pig [231–236]. While these models have provided critical information into how CFTR defects lead to the development of the CF phenotype they have often fallen short in emulating the severity of lung disease in a CF patient. While the larger animal models are proving to be close representations of the human CF phenotype, they are less accessible to the research community. Rodent models, on the other hand, are more accessible but not perfect due to inherent differences between mouse and human lung structure, physiology, and immunology, so findings do not often translate well into the human setting. In addition, there is evidence that genetic lesions in mouse and human can lead to different phenotypes as seen in the analysis of CFTR [235]. This poses one of the biggest limitations for studying cellular heterogeneity and progenitor cell niche in healthy and diseased lungs. When considering the more proximal airways, mouse lungs, for example, have far fewer submucosal glands and differences in the distribution of cell types across the trachea and more proximal airways. Species differences highlight the importance of evaluating cell phenotype and function in a human model system. Primary cells isolated from patient donated lung tissues are the current gold standard for studying the human lung epithelium.

Isolation and culture of human airway epithelial cells (HAECs) from both healthy and diseased patients has been well established [237]. However, availability of primary lung tissue is limited, with the demand continuously increasing, most healthy uninjured lungs are allocated for transplantation. Diseased lung tissue is usually isolated postmortem or posttransplantation: at the end stage of lung disease after years of infection and damage. Alternatively, for living patients a small number of cells can be isolated by nasal brushing, bronchial brushing, or biopsy. Utilizing recent improvements in cell culture techniques, most notably conditional reprogrammed cell (CRC) culture, as small number of cells can be expanded by five orders of magnitude [238]. This allows for implementation of genetic manipulation and expansion for downstream applications and functional analysis [239]. Despite advances in culture techniques for expansion of primary airway cells, they still encompass a poorly defined heterogeneous population of lung cells that have a restricted capacity for in vitro expansion while maintaining phenotype [238, 240]. Additionally, the CRC method relies on co-culture with the 3T3-J2 cell line derived from mouse fibroblasts and the clinical applications of primary cells grown in these xenogeneic conditions may be restricted.

The ease of genetic manipulation in iPSC and their capacity for indefinite passaging allows for a potentially unlimited source of cells that can be derived and maintained in xenofree conditions making them suitable for both high-throughput screening and autologous cell therapy. iPSC can model airway in vitro through either air–liquid interface (ALI) cultures or three-dimensional spheroid and organoid cultures [141, 241–243]. ALI cultures rely on specialized porous inserts that allow for a layer of cells to be suspended in a culture dish in an isolated apical chamber. Once cells have formed a monolayer with tight junctions, differentiation media can be added to the basolateral side of the cells while media can be removed from the apical. This forms the air–liquid interface that facilitates pseudo stratification and differentiation of lung basal cells. While there are limitations when trying to recapitulate complex diseases in vitro, such as loss context-specific phenotypes, primary ALI cultures remain the gold standard for the study of CF [244, 245]. Specialized ALI inserts can be placed in an Ussing chamber to measure changes in membrane potential, a readout of CFTR function. In 3D cell culture, basal cells can be suspended in extracellular matrix proteins such as Matrigel™ and then allowed to grow into organoids. Addition of forskolin or cAMP analogues/cAMP elevating agents to organoid cultures stimulates CFTR chloride conductance into the lumen of the organoid. Increased luminal chloride concentration is followed by osmotic swelling resulting in a visible enlargement of organoid as a readout of CFTR function [246].

Shortly after the establishment of human iPSC, scientists began reprogramming and banking patient cells for use in future studies. The first CF patient iPSC were derived before the development of lung differentiation protocols and CRISPR/Cas9 technology, making establishment of transgene free cell lines capable of endoderm differentiation the focus [140]. CF patient iPSC lines were differentiated into CFTR expressing lung progenitors, and mature iPSC derived lung epithelium displayed responsiveness to small molecule that acts as a CFTR "corrector." The CFTR corrector enhanced CFTR trafficking to the cell membrane in deltaF508 cell lines serving as a proof of concept for the use of differentiated iPSC in small molecule screens [247]. Next, efficient CFTR gene editing in iPSC was achieved using CRISPR/Cas9-mediated HDR. Donor DNA containing an excisable double selection cassette flanked by donor homology arms with wild-type CTFR sequence generated footprint-free transgene-free corrected iPSC clones with almost 90% efficiency. Subsequent differentiation of CFTR corrected iPSC demonstrated restoration of CFTR function, serving as a proof of concept for CFTR gene editing in iPSC [248]. CRISPR/Cas9-mediated editing of CFTR in iPSC is not only a promising tool for functional studies but also gives hope for the application of the differentiated cells for autologous cell therapy.

9.5.2 Autologous Cell Therapy: A Therapeutic Reality?

Autologous cell therapy (ACT) involves the isolation and ex vivo manipulation of a patient's cells, usually by culture and expansion, before reintroduction to the patient. ACT holds advantages over traditional donor cell therapy in that cellular source precludes the need for a donor search and immunosuppressants. In the late 1950s the first successful human bone marrow transplants were only possible because they were analogous to autologous cell transplantation, using genetically identical donor cells transplanted from a healthy identical twin [58]. Development of HLA matching, graft versus host disease treatments and patient care increased viability of allogenic bone marrow transplants for treatment of hematological disease [74]. Autologous bone marrow transplants followed the success of allogenic transplants, eliminating the need for HLA donor matching, but are still limited to certain disease context [83]. Of the >100,000 reported hematopoietic cell transplants (HCT) in the United States from 2012 to 2016, 58% were autologous: data is available in the donor registry of the C.W. Bill Young Cell Transplantation Program (https://bloodcell.transplant.hrsa.gov/research/citation_guidelines/index.html).

ACT is already a therapeutic reality for many hematological diseases, but for broader clinical applications such as the treatment of CF, it may not be so straightforward. Most routine HCT fall under the category of "minimal manipulation" and do not require FDA drug regulations. Matrix-induced autologous chondrocyte implantation (MACI®) and autologous cultured fibroblast (LAVIV®) are currently the only two FDA approved ACT not sourced from hematological cell, but neither utilize stem cells or genetic modification (https://www.fda.gov/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/default.htm). For iPSC-based ACT major hurdles to overcome are efficient derivation of the specific mature cell population relevant to the disease and delivery of the cells to the target tissue in quantities sufficient for functional disease correction. Perhaps mature cells would not be most suitable to transplant, as a committed progenitor may be more adaptable in this situation. Several recent studies have shown engraftment and differentiation ex vivo expanded primary airway progenitor or iPSC-derived lung progenitor cells in mice, but long-term persistence and functional gene correction in a disease model has yet to be established [249–251]. Furthermore, immune rejection remains a possibility due to the ex vivo manipulation of autologous cells. This issue should be address in both preclinical and clinical trials when they commence.

Autologous cell therapy offers many advantages over allogeneic cell therapy. However, patient-specific production of clinical and GMP grade genetically modified cells will be costly,

requiring highly specialized procedures and operators to meet the scalability and regulatory demands of cell therapy. Providing a cell product that is efficacious at an acceptable cost is going to present a demanding task. It is critical to remember that there is still much to achieve to ensure that quality gene corrected autologous cell therapy can become a safe therapeutic approach for genetic diseases in the future.

9.5.3 Direct In Vivo Gene Editing

While autologous cell therapy combined with gene editing allows maximum manipulation of cellular product prior to transplantation, direct delivery of gene editing materials to the patient presents a quicker and possibly safer means to achieve the same goal. Both methods result in the patient's own cells being intentionally edited to correct pathogenic elements in the genome, and rely on these cells to cooperate with the rest of the body to produce functional therapeutic effects. Direct in vivo gene editing eliminates the issue of tumorigenicity stemming from iPSC reprogramming or incomplete differentiation, but the possibilities of oncogene activation or other unintentional off-target effects remain. Currently, the primary obstacle to overcome for successful in vivo gene editing is delivery of genetic material to the cells of interest, which needs to be extremely specific to prevent the wrong types of cells in the body being edited. Work is underway to develop safe, specific, and efficient delivery methods for in vivo gene editing.

Some early studies have focused on gene delivery by hydrodynamic injection, which increases membrane permeability by coaxing hepatocytes into forming endocytic vesicles that can deliver genetic material [252]. One study at the Massachusetts Institute of Technology focused on delivering CRISPR-Cas9 plasmids and a ssDNA donor into the mouse liver by injection into the tail vein [190]. The purpose was to correct a splicing mutation in the *Fah* gene that is causative for human hereditary tyrosinemia (HTI) in a mouse model for this disease. The gene was repaired in only 0.4% of hepatocytes, but mice that received the treatment showed functional rescue of the HTI weight loss phenotype and reduced liver damage. Hydrodynamic injection was also demonstrated to be safe, with wild-type mice showing no weight loss or hyperplasia after Cas9:sgRNA injection and extremely low Cas9 expression after 3 months.

Hydrodynamic gene therapy has been attempted only in one human clinical trial, where cirrhotic patients with thrombocytopenia had a human thrombopoietin plasmid injected into their middle hepatic vein [253]. Patients tolerated the treatment without side effects and experienced an increased platelet count 7 days after injection, but platelets declined back to normal levels after 4 weeks due to the transient nature of this treatment. With permanent gene editing like CRISPR-Cas9, therapeutic effects may have lasted much longer. Just 2 years after the above study, the same group corrected the *Fah* mutation in HTI model mice with CRISPR-Cas9 again, but used a combination of viral and nonviral delivery to achieve >6% gene correction in hepatocytes—fifteen times more than in the previous study [191]. Cas9 mRNA was delivered using lipid nanoparticles a week after sgRNA and the donor template were delivered using adeno-associated virus (AAV). Delivering Cas9 by lipid nanoparticles is preferable because Cas9 is too large to fit in an AAV vector with the other components and needs to be removed from the body soon after gene editing to prevent DNA damage, unlike the sgRNA and donor. Only a week after Cas9 nanoparticle delivery, the treatment effectively cured the mice of HTI, rescuing the weight loss and liver damage phenotype. Less than 0.3% indels were detected at the top three predicted off-target sites, which was comparable to indel levels in mice not injected with Cas9 mRNA. This was an incredible improvement over just 2 years, highlighting the astounding rate of progress of CRISPR-Cas9 and gene delivery technologies.

Another recent study used a pair of AAVs to deliver CRISPR-Cas9 and gRNAs to mouse skeletal muscle to splice out exon 23 of the *DMD* gene, which is mutated in Duchene muscular dystrophy (DMD) [193]. The goal was to restore in-frame

transcription of truncated but functional dystrophin to prevent muscle deterioration resulting from this disease. The group used a smaller Cas9 orthologue from *Staphylococcus aureus* (*SaCas9*) that can fit in an AAV vector, with paired gRNAs that reduce off-target effects. For delivery, they used two serotype 9 AAVs, which are specific for mouse skeletal and cardiac muscle, with Cas9 going in one vector and the dual gRNAs going in the other. The AAVs were injected into tibialis interior (TA) muscles of mice, and 4 weeks after treatment, they showed exon 23 excision at a rate of 39% in mRNA in addition to restored dystrophin protein expression. The muscle cells treated with CRISPR even had increased specific force and attenuated force drop after damage compared to contralateral untreated muscles. This treatment was also given systemically when mice at postnatal day 3 were intraperitoneal injected with the CRISPR AAVs, resulting in exon 23 excision in multiple skeletal muscles at a rate of 3–18%. The group even harvested endogenous satellite cells and differentiated them to myotubes that retained gene correction and dystrophin protein expression, demonstrating that muscle stem cells could be transfected with CRISPR and regenerate muscle damaged by DMD pathology. This study exhibits the remarkable efficacy and simplicity of CRISPR treatments and potential of AAVs for specific delivery.

Although AAVs are highly specific, it may be more desirable to deliver the entire Cas9:sgRNA ribonucleoprotein complex to cells to enable more transient protein activity. Bio reducible lipid nanoparticles are an effective method to deliver protein cargo across the cell membrane. One group has developed a combinatorial method to synthesize bioreducible nanoparticles that effectively deliver Cas9:gRNA complexes to cells [254]. The cationic nanoparticles readily bind to anionic Cas9:gRNA complexes, and degrade in response to the reductive intracellular environment to enable endosomal escape by protein cargo. These CRISPR-carrying nanoparticles enabled genome editing at a surprisingly high rate of 70% in cultured human cells. When the nanoparticles were bound to negatively supercharged Cre recombinase and delivered into the mouse brain, gene editing was confined to the injection site with minimal diffusion, implying the potential for this delivery method in targeting small regions of the brain with specific neural populations.

In vivo gene editing is especially effective for diseases that involve defective cellular function without excessive cell death, and in tissue that is easily accessible or targetable. Before it becomes a reality in the clinic, researchers need to develop a diverse arsenal of safe delivery methods with individual strengths and weaknesses depending on the nature of disease and target tissue. In vivo gene editing and autologous cell therapy should work in tandem to treat human genetic disease by harnessing the potential of intentional genetic manipulation.

9.6 Future Perspectives

The scientific community has embraced the recent and rapidly advancing fields of induced pluripotency and gene correction, opening the door for vast development of new cell-based approaches for treating, and potentially curing, diseases. Although there are still significant scientific challenges to overcome before these treatments become reality, substantial hope remains for future clinical application. It is now over 40 years since those first bacteria were genetically modified; so much progress has been made in this field that we have now reached a critical point that may have a profound impact on the future of gene therapy. In 2016, it was announced that a request from a team of scientists in London to edit the genomes of human embryos using the latest state-of-the art gene editing technology, CRISPR-Cas9 was approved the UK Human Fertilization and Embryology Authority (HFEA). This represented a world's first for such an endorsement by a national regulatory authority. From this study we gained fundamental insights into early human development, and this lead to the world's first approval for research of this kind [255]. The United States followed suit in 2017 after the National Academy of Science and National Academy of Medicine joint committee established strict guidelines for embryonic gene manipulation, which was quickly followed by a group at Oregon Health and Sciences University correcting a patho-

genic mutation in human embryos [256]. Due to different regulation standards, Chinese researchers have already progressed in manipulating germline mutations [257]. Indeed, the alleged birth of gene-edited human twins was recently reported and has been highly condemned across the international scientific community: the implantation of gene-edited embryos violates the current code of conduct around the world. This report has affected both public and scientific opinion regarding human genome editing and will surely impact regulations moving forward [258]. CRISPR Therapeutics and Vertex Pharmaceuticals have submitted a joint clinical trial application in Europe, with studies planned to start next year. They also plan to submit to the FDA to being trials in the United States. Undoubtedly, this will pose an enormous challenge for bioethicists and regulatory authorities, but its progress will be interesting to follow in the coming years. The field of gene editing is exploding, and it is truly an exciting time in the field of genetics.

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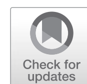

10 Clinical Application of Stem/ Stromal Cells in Cystic Fibrosis

Steven T. Leung, Timothy S. Leach, Anthony Atala, and Sean V. Murphy

10.1 Introduction

Cystic fibrosis (CF) is a progressive genetic disease associated with an increased morbidity and mortality caused mainly by respiratory impairment and pancreatic deficiencies. CF is an autosomal recessive disease caused by a mutation in the Cystic Fibrosis Transmembrane Conductance Regular (CFTR) gene located on chromosome 7 [1, 2]. CFTR is a chloride channel that is primarily expressed in the apical membrane of epithelial cells, and serves to regulate transepithelial fluid homeostasis [3]. By regulation of chloride secretion and subsequently sodium reabsorption, CFTR plays a pivotal role in regulation of the hydration in the airway surface liquid and mucus layer. As such, loss of function of the CFTR channel leads to deficient cAMP-dependent chloride secretion into the airways, leading to airway surface dehydration and tethering of mucins to the bronchial apical surfaces and decrease in airway surface fluid pH [4]. In addition, recent research indicates that the decreased pH induces significant defects in host antibacterial defenses [5–8]. CFTR is also expressed in inflammatory cells, and a lack thereof in CF immune cells has been shown to be associated with functional defects and dysregulation in neutrophils [9–16], macrophages [17–23], and T cells [24–26]. Consequently, chronic infections with pathogens such as *Pseudomonas aeruginosa* occurs, which is associated with lower childhood FEV_1 [27], faster decline in FEV_1 in spite of optimal respiratory management [28, 29], higher mortality rates [30], and shorter median survival [31]. Chronic infections lead to a chronic inflammatory response, which results in tissue destruction and respiratory insufficiency. There is thought to be an imbalance between protease and antiproteases in the lung, with serine proteases and matrix metalloproteases overwhelming the antiproteases. This leads to a proinflammatory state with resulting degradation of the extracellular matrix components, such as elastin, and cleaving of immune receptors such as T-cell receptors, complement receptors, and CXCR1 [22, 32, 33].

While CF affects multiple organs of the body such as the lungs, liver, pancreas, sinuses, and reproductive tract, approximately 90% of CF

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patients will ultimate succumb due to respiratory failure. As such, most treatments target the respiratory system, which is highly susceptible to increased inflammation and infection. Repeated bacterial infection and chronic inflammation for CF patients results in the characteristic phenotype of the disease: detrimental lung scarring, bronchiectasis, and airflow limitations [34]. Since there is not yet an effective cure, current treatments instead focus on management of the disease in order to prolong life and improve quality of life [35, 36]. Due to the singular genetic mutation of the disease, gene therapy has believed to be the most obvious treatment moving forward. Recently, a new class of drugs termed CFTR modulators have been developed. These drugs aim to improve function of the mutant CFTR protein, either by increasing the time that the CFTR channel remains open or acting as a chaperone protein to enable proper folding of the CFTR channel. Still, the lack of clinical success and permanent gene correction along with possible mutagenesis has resulted in investigation of other avenues for treatment [37].

In the past couple of decades, cell therapy has become a widely investigated treatment option for diseases, such as CF, due to their regenerative, anti-inflammatory, and immunomodulatory capabilities [38]. Other lung diseases with similar chronic inflammation phenotypes have already been treated with cell therapies with various levels of success and include chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and acute respiratory distress syndrome (ARDS) [39–42]. COPD is hallmarked by chronic inflammation in the lungs most commonly as a result of prolonged tobacco inhalation, which can result in any combination of emphysema, severe airflow obstruction, mucus hypersecretion, and small airways fibrosis [43]. IPF is a chronic pulmonary disease marked by unknown progressive fibrosis of the lower respiratory tract that has been associated with numerous proinflammatory cytokines and mediators, including CCL-2, CCL-3, CCL-7, and IL-8, that play a role in the lung function's rapid deterioration [44, 45]. Lastly, ARDS is a severe form of acute lung injury frequently caused by bacterial or viral infection that is characterized by dysregulation of the inflammatory response, uncontrolled and sustained activation of the coagulation pathways, and hyper-permeability of the alveolar endothelial and epithelial layers [46–49]. Treatment with stem cell therapy is believed to target various aspects of these diseases and could provide similar beneficial results in CF due to analogous chronic inflammation and location of the diseased area. For instance, heterogeneous or gene-corrected bone marrow- or amniotic fluid-derived mesenchymal stem cells could potentially graft into the host and differentiate into a functioning airway epithelium [37, 50–52]. Furthermore, the capabilities of stem cells to reduce inflammation and inhibit bacterial growth have been well analyzed [53–55]. CF along with other diseases, such as COPD, have shown to have dysregulation and senescence of the resident lung stem cells during injury furthering the pathologies [56–59]. Thus, the delivery of stem cells, both endogenous and exogenous, to the CF lung has become an interesting therapy route. Delivery of endogenous lung stem cells though has been less studied due to the difficulty of derivation and lack of understanding. Instead many studies have investigated regulating the resident endogenous stem cells through significant signaling pathways that involve molecules such as Wnt, Notch, Histone deacetylases (HDACs), and noncoding mRNAs [60–63]. With regard to delivery of stem cells as a therapeutic approach, many of the therapies that have been investigated involve non-lung stem cells, specifically adult mesenchymal stem/stromal cells and perinatal stem cells. In the following sections, the role of stem cells, their limitations, and potential applications for treating CF will be discussed.

10.2 Stem Cells Used for Therapy

Initially, stem cell research was focused on using stem cells as a tool to reconstitute the damaged airway epithelial layer. Instead, extensive research in stem cells has shown the diversity of their benefits ranging from antimicrobial properties and anti-inflammatory properties to trans-differentiation into any somatic cell. Each

of these properties can have beneficial effects on the disease profile of CF patients. The four types of stem cells that will be discussed in this chapter and their influence on the treatment of CF are embryonic stem cells, induced pluripotent stem cells, adult mesenchymal stem/stromal cells, and perinatal stem cells.

10.2.1 Embryonic Stem Cells (ESCs)

Embryonic stem cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos [64]. As such, they can differentiate into cells from all three germ layers, and thus contain enormous potential for research. Unfortunately, ESCs are also subject to ethical and religious controversies, as their harvest requires termination of embryos. Currently, funding for ESC research is limited in the US, and directed differentiation into somatic cells remains inefficient [65]. Furthermore, ESC cells may become tumorigenic and immunogenic, which further limits their feasibility in a clinical setting. However, cell lines derived from ESCs may be useful for research and drug screening. CF cell lines can be obtained in two ways: introduction of a specific mutation via homologous recombination into a normal ES cell line; or (two), collecting ESC from an affected embryo [66]. CF embryos can be obtained from carriers of the CFTR mutation undergoing reimplantation diagnosis, which allows for the detection of genetic disorders of an embryo fertilized in vitro prior to implantation into the uterus [65]. In one study, an air–liquid interface model complete with tight junctions and functional apical polarized CFTR protein channel was created using proximal epithelial cells generated from differentiation of ESCs [67].

10.2.2 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells were developed to overcome the ethnical challenges arising from collection of ESCs. These cells are produced by reprogramming adult somatic cells using genes unique to ESCs, such as Oct3/4, Sox2, c-Myc, and Klf4, via viral transfection [68]. Similar to ESCs, iPSCs are now most commonly used for drug discovery and disease modeling to study the pathogenesis of CF lung disease. By using a cocktail comprising of growth and morphogenic factors, differentiation of foregut spheroids into NKX2-1^{+} lung progenitor cells were possible, with formation of airway and alveolar-like architecture [69]. Similarly, McCauley et al. produced functional airway epithelium from human-induced pluripotent stem cells to generate patient-specific cystic fibrosis data [70]. Lung bioengineering using ESCs and iPSCs is also an attractive option. These cells are optimal due to their capability to differentiate into pulmonary progenitor cells following reprogramming that mimics fetal lung development [67, 71]. A scaffold is used to provide a matrix for attachment and growth of cells, and offer the opportunity to incorporate therapeutic agents such as growth factors which can be released to drive differentiation as the cells remodel the scaffold during growth and development [72]. Furthermore, the scaffold can be either biofabricated or biologically derived and seeded with autologous stem cells to prevent an immune response. Biofabricated scaffolds offer the ability to precisely construct components that mimic the extracellular matrix but fail to incorporate the complexity of the surrounding neurovascular and alveolar architecture [68]. Conversely, biologic lung scaffolds can be obtained by decellularization of native lung tissues and subsequent reseeding with autologous cells [73].

10.2.3 Adult Mesenchymal Stem/Stromal Cells (MSCs)

One major source of stem cells that is currently being widely investigated for cell therapy is the adult mesenchymal stem/stromal cell. MSCs are multipotent adult stem cells that were first derived from bone marrow, but are present in many organs and can now be derived from essentially any tissue, such as the lungs [74, 75]. Depending on tissue location, the MSCs can be

derived from a myriad of methods that involve retrieving the tissue, digesting the ECM, and migration of the cells out of the tissue with specific media [76]. According to the International Society for Cellular Therapy, MSCs can be categorized as cells that express the surface markers CD44, CD73, CD90, CD105, CD146 while lacking the following markers: CD11b, CD14, CD34, and CD45 [77]. Additionally, these cells are characterized by their differentiation capabilities into osteoblastic, adipocytic, and chondrocytic lineages in vitro [77, 78]. This type of stem cell is of interest for several cell therapies due to their immunomodulatory and anti-inflammatory properties [79, 80]. Furthermore, as discussed in Chap. 3, other properties that have been expressed by these cells that could be beneficial in cell therapy include antifibrotic and microbicidal effects [81].

10.2.4 Perinatal Stem Cells (PSCs)

Another major type of stem cells for therapeutic use in the lung are perinatal stem cells, which can be either epithelial or mesenchymal in origin. These cells originate from the perinatal tissue which includes the placenta and its membranes, umbilical cord, and amniotic fluid [82]. Human amniotic epithelial cells are derived from simple digestion of amniotic membranes, and do not express mesenchymal or hematopoietic stem cell markers [83–85]. These cells are isolated from the epithelial layer of the amniotic membrane, which is the innermost layer of the amniotic membranes that surrounds the fetus [86]. The source of the amnion originates from embryonic ectoderm before gastrulation, and as such, human amniotic epithelial cells (hAEC) are pluripotent with expression of several embryonic stem cell markers such as OCT-4, Nanog, SSEA-3, SSEA-4, TRA 1-60, and c-kit [87–90]. In addition, they do not express HLA-A, B, C, and DR, or β2-microglobulin antigens on their surfaces, and thus does not elicit an immune response and can be safely injected into an allogenic recipient without rejection [91]. In vitro studies have shown that hAEC can differentiate into cells from all three embryonic germ layers, such as cardiomyocytes, myocytes, osteocytes, adipocytes, pancreatic cells, hepatocytes, as well as neural and astrocytic cells [88, 89, 92].

Human amniotic mesenchymal stem cells (hAMSC), on the other hand, are isolated from the stromal layer deep beneath the amnion epithelium, where they are sparsely dispersed [93]. Similar to hAEC, hAMSC can also differentiate into cells from all three germ layers and do not express HLA antigens on their surfaces [94, 95]. They express mesenchymal cell surface markers such as CD105 and CD90, and negatively express hematopoietic markers, such as CD29, CD34, CD45, and CD105 [96]. A comparison study completed between hAMSC and hAEC showed that while both show excellent secretory profiles for regenerative medicine applications, including angiogenesis, there were key differences in their cell migration and proliferation capabilities [97]. Therefore, the application of the stem cells could dictate the optimal stem cell source for treatment.

Stem cells isolated from umbilical cord blood consist of both hematopoietic and progenitor cells, and contains fewer T cells than bone marrow, which may decrease the incidence of Graft versus Host Disease [98]. In addition, they express the CD34 antigen, thought previously to be expressed exclusively by hematopoietic cells [99]. Compared to similar MSCs derived from bone marrow or peripheral blood, cord blood cells have greater proliferative response to cytokines and are less dependent on stromal cells [99]. In addition, cord blood contains a higher proportion of more primitive hematopoietic cells than bone marrow, characterized by long telomere DNA [99]. These cells are also able to differentiate into cells derived from all three germ layers [100]. Umbilical cord matrix cells are cells isolated from the Wharton's jelly which also exhibit MSC-like properties, and can be easily isolated, frozen or thawed, clonally expanded, engineered to express exogenous proteins, and extensively expanded in culture [101].

Two types of stem cells have also been isolated from amniotic fluid: amniotic fluid MSCs and amniotic fluid stem cells. Amniotic fluid is most commonly obtained via amniocentesis dur-

ing the first and second trimesters of pregnancy. Amniotic fluid MSCs were the first stem cells isolated from amniotic fluid and display mesenchymal features similar to MSCs isolated from other sources such as bone marrow, liver, and blood. These cells express Class I major histocompatibility (MHC) antigens (HLA-ABC), were negative for markers of the hematopoietic lineage (CD45) and of hematopoietic stem cells (CD34, CD133), and stained positively for a number of surface markers characteristic of mesenchymal and/or neural stem cells, but not embryonic stem (ES) cells, including CD29, CD44 (hyaluronan receptor), CD73, CD90, and CD105 (endoglin) [102]. In addition to MSCs, a different subpopulation of proliferating cells was identified in amniotic fluid, expressing the pluripotency marker Oct4. These cells have been termed amniotic fluid stem cells, and were demonstrated to be able to differentiate into cell types of all three germ layers [103].

Overall, perinatal stem cells are convenient sources for future banking and possible therapeutic use due to the disposal of the tissue source after fetal birth. Similar to adult MSCs, PSCs have shown to have multipotent, and sometimes pluripotent, differentiation capabilities, and even exhibit characteristics of embryonic stem cells (ESCs) [102]. For example, they have shown to express markers of both MSCs and ESCs and can differentiate into cells of all three germ layers. They express similar surface markers of adult mesenchymal stem cells, while also increasing expression of other characteristic markers such as CD29 and CD166 expression for placental-derived stem cells [104] and CD9, integrin α6, and integrin β1 expression for amniotic epithelium-derived stem cells [105]. In addition to the increase of specific markers, some PSCs have shown downregulation of MSC-specific markers such as the lack of CD40, CD80, and CD86 for umbilical cord stem cells and the lack of CD44, CD90, and CD105 for amniotic epithelium-derived stem cells [104]. Besides the ease of derivation of PSCs, their importance for cell therapy revolves around their immunomodulatory and anti-inflammatory properties similar to MSCs [106–110].

10.3 Approaches to Stem Cell Therapies for Cystic Fibrosis

Although life expectancy in CF has improved dramatically over the last four decades, the majority of CF patients will succumb to respiratory failure [111, 112]. As such, the mainstay of therapy has been focused on improving lung function through three routes: eradicating chronic infections with pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, reducing chronic inflammation, and promoting mucociliary clearance.

The first study looking at long-term use of systemic corticosteroids was conducted in the 1980s. This trial demonstrated that alternate-day prednisone was associated with improved lung function and weight gain, with fewer hospital admissions [113]. However, subjects also had multiple corticosteroid-associated adverse events, such as persistent growth defects, and loss of improved respiratory function after discontinuation of prednisone [114]. Therefore, systemic corticosteroids are not recommended for maintenance therapy in CF [115]. However, it demonstrated that lung function can be preserved by decreasing the inflammatory response in the lungs. Currently, ibuprofen is the only anti-inflammatory drug recommended for long-term management of CF patients, which has been shown to slow lung function decline, preserve body weight, and decrease hospital admission with no known adverse effects [115, 116]. However, the major concern with implementation of ibuprofen as chronic maintenance therapy in CF is its potential adverse effects, such as abdominal pain, elevated transaminase levels, hemoptysis, hematuria, renal failure, gastrointestinal bleeding, or decreased esophageal motility [117, 118]. Chronic infections in the CF lungs exacerbate inflammation, therefore antibiotics are also pivotal in long-term management of CF patients. Inhaled tobramycin and azithromycin are antibiotics that have been shown to have anti-inflammatory effects and are commonly used in CF management [119, 120]. Still, there has been growing concern over antibiotic-resistant bacteria associated with CF and the inability of current antibiotic to combat this resistance [121, 122]. As

a result, practitioners have to be vigilant in their treatment regimens as this bacterial resistance plays a key role in the morbidity and mortality of most CF patients. Airway clearance can be achieved using chest physiotherapy and mucolytics. Hypertonic saline and mannitol are also currently used in patients with moderate or severe pulmonary disease to rehydrate the airways [115, 123]. Since the abnormal mucociliary clearance is due to airway surface dehydration resulting in an abnormal thick mucus layer, stem cell therapy could improve lung function of CF patients through correction of the defect through stem cell engraftment and differentiation into CFTR expressing epithelial cells. This in turn would lead to promotion of mucociliary clearance and reduction of chronic infection and inflammation.

10.3.1 Reduction of Chronic Infections

CF patients are more susceptible to polymicrobial colonization and infections by various bacteria, especially *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Stem cells, specifically MSCs, have demonstrated antimicrobial properties both in vitro and in vivo. In one study, Sutton and colleagues demonstrated that MSC exhibit efficacy in treatment of both Gram-positive and Gram-negative pathogens through production of the antimicrobial peptide LL-37 in a CF rat model [124]. LL-37 is a member of the human cathelicidin family produced by many cell types including macrophages, natural killer cells, and epithelial cells of the skin, airways, ocular surface and intestines [125]. The positive charge on LL-37 associates with negatively charged phospholipid membranes, allowing membrane penetration, transmembrane pore formation, and subsequent bacterial cell lysis [126–130]. Furthermore, MSCs and their supernatant are also able to slow the growth rates of a variety of bacteria, which if administrated concomitantly with antibiotics can theoretically create a synergistic combination that can improve efficacy and response [124]. One study indicated that when adipose-derived MSCs are cocultured with *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the two of the most common bacteria associated with CF, the bacterial growth is actually significantly inhibited [131]. In a murine Gram-negative pneumonia model, MSC enhanced bacterial clearance, reduced lung injury, and improved survival by upregulating production of lipocalin-2 in response to LPS and inflammatory mediators generated by activated macrophages [132]. Lipocalin-2 is a secretory protein initially isolated from neutrophils that sequesters iron via siderophore, thus inhibiting bacterial growth and expansion [133]. MSCs also upregulate genes involved in phagocytosis and bacterial killing and contributing to enhanced bacterial clearance [54]. Finally, MSCs have also been shown to secrete β-defensin 2, an inducible defensin against Gram-negative bacteria, such as *P. aeruginosa*, that achieves its antimicrobial effect via creation of transmembrane pores, leading to bacterial cell lysis [134, 135].

Studies have also shown that perinatal sources for stem cells have similar antimicrobial effects to adult MSCs. Sung and colleagues intratracheally delivered umbilical cord stem cells to *Escherichia coli* infected mice to reduce bacterial growth and damage associated with acute lung injury and pneumonia [136]. The umbilical cord stem cells were shown to have a crucial paracrine effect by the secretion of the antimicrobial peptide β-defensin 2 similar to adult MSCs. Additionally, comparable to adult MSCs, the umbilical cord stem cells were shown to upregulate and activate receptors associated with the innate immune response such as Toll-like receptors (TLRs) [136, 137]. These TLRs play a significant role in the immune system by recognizing specific pathogen-associated molecular patterns (PAMPs) expressed by foreign microbes in order to initiate the appropriate immune response [138]. The amnion membrane, another perinatal source for stem cells, has been well characterized to have natural antimicrobial effects [139–141]. Specifically, amniotic epithelial and mesenchymal stem cells, when induced with the proinflammatory cytokine IL-1β, will secrete a variety of antimicrobial peptides including LL-37 and β-defensin 2 [142]. The antimicrobial capabilities of MSCs have been well characterized in literature and can be directly translated to the persistent bacterial colonization seen in CF lungs.

10.3.2 Reduction of Chronic Inflammation

Recently, anti-inflammatory properties of stem cells have been well characterized, thus making it an ideal adjuvant therapy for long-term management of CF chronic inflammation. Specifically, CF lungs contain large amounts of proinflammatory factors, such as TNFα, IL-1β, IL-6, and IL-8, in addition to expressing poor regulation of counter-regulatory molecules for inflammation such as IL-10 and nitric oxide [143]. Adult mesenchymal stem cells have shown to alleviate airway inflammation by modulating immune cell activation and function [80]. This may be useful for addressing the dysregulated immune cell functions observed in CF disease. In rat models of mechanically- and bacterially-induced lung injury, human adult MSCs improved lung compliance and survival, reduced alveolar edema, and decreased bacterial load and proinflammatory cytokines such as neutrophil chemoattractant-1 and IL-6 [144, 145]. The authors attributed the improvement in lung function primarily to the immunomodulatory effect of MSCs, as the decrease in infiltration of proinflammatory immune cells resulted in subsequent effects. In another study, Gu and colleagues demonstrated that MSC administration to cigarette smoke-induced rats alleviated airway inflammation and emphysema by downregulating cyclooxygenase-2 (COX-2) and COX-2-mediated prostaglandin E2 (PGE2) production in macrophages through inhibition of mitogen-activated protein kinase (MAPK) signaling of p38 protein kinase and extracellular signal-regulated kinases (ERK) [146]. This in turn leads to decreased production of proinflammatory prostaglandins, mitigating the inflammatory response.

Similar to MSCs, perinatal stem cells have demonstrated anti-inflammatory properties. For instance, amniotic-derived stem cells were shown in vitro to suppress CD4+ and CD8+ T-cell proliferation and decrease production of the associated proinflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-5, IL-9 and IL-22 [106]. Several types of perinatal stem cells such as amniotic epithelial cell, amniotic MSC, and umbilical cord MSC have all been shown to reduce fibrosis in a murine bleomycin-induced lung injury model; hAEC and umbilical cord MSC have been shown to decrease expression of proinflammatory cytokines such as MCP-1, TNF-α, IL-1, IL-6, and the profibrotic cytokine TGF-β [147–149]. In one study, hAEC were demonstrated to remain engrafted in mouse lungs 4 weeks after injection, which resulted in decreased inflammation; in addition, lung collagen content was significantly reduced, and thought to be secondary to increased degradation of MMP-2 coupled with downregulation of inhibitors of MMP-1 and MMP-2 [149]. hAEC also express anti-inflammatory IL-1 receptor antagonist, IL-10, collagen XVIII, thrombospondin-1 and all four isotypes of tissue inhibitors of metalloproteinase (TIMPs) [150]. Furthermore, amniotic mesenchymal stem cells can skew macrophage polarization towards the anti-inflammatory M2 phenotype and induce T-regulatory cell differentiation [80, 106], alleviating the chronic inflammatory response in the lungs and limiting tissue damage and fibrosis. The source of placenta-derived stem cells did not seem to be an important factor, as both allogeneic and xenogeneic stem cells both abrogated bleomycin-induced lung fibrosis [148]. Interestingly, amniotic mesenchymal stem cell supernatant also exhibits similar anti-inflammatory properties with the cells from which they were derived, further suggesting that stem cells secrete and release biologically active factors that have immunomodulatory properties [151–153]. All of these studies further provide evidence of the immunomodulatory ability of stem cells, as they can regulate immune cell proliferation, differentiation, and polarization. Thus, delivery of stem cells could potentially alleviate chronic inflammation, and inhibit the cycling of bacterial infection and inflammation that results in epithelial damage associated with CF.

10.3.3 Lung Epithelium Correction

Another avenue for treatment of CF with stem cell therapy is cellular reprogramming and differentiation of the stem cells into CFTR-corrected epithelial cells. Previous research has shown that even minimal restoration of CFTR function in the

epithelium could drastically improve the condition and quality of life of the individual [154]. Still, this route of treatment is the most difficult to bring to the clinic, as safety and regulation inhibit growth of this field due to the risk of tumor formation. In addition, introduction of the wild-type CFTR gene into airway epithelial cells is challenging due to the viscous mucus layer covering these cells [155].

Nevertheless, the possibility of restoring CFTR function through differentiation of exogenous stem cells has been investigated primarily in vitro. For instance, a protocol has been developed to differentiate embryonic stem cells in vitro into CFTR functional airway epithelial cells in over a third of the cells before isolation [67]. A similar protocol was developed for the same purpose but directed at iPSCs and showed similar CFTR expression to native lung by day 45 [156]. Still, this has only recently been developed since lung epithelial cells have shown to be difficult to differentiate from embryonic stem cells since they originate from the endoderm, the last germ layer to form [157]. While no CF animal models have been investigated with ESCs, Wang and colleagues successfully transplanted human embryonic stem cells differentiated into type I alveolar cells into an acute lung injury mouse model [158]. Their results indicate that engraftment and differentiation of these cells prevented and repaired tissue damage, but the actual mechanisms and effect of the stem cells on the resident lung niche was not investigated. In another study, stem cells were derived from embryonic human fetal tissue and intravenously delivered to an acute naphthalene lung injury mouse model [159]. The treated mice had improved lung tissue compliance and function that was associated with the engrafted patches in the lung, but again secondary mechanisms were not investigated. Most of the work involving iPSCs instead has investigated developing patient-specific stem cells that can be corrected then transplanted back in the host. As discussed in Chap. 9, many groups have examined various methods of CFTR correction, such as TALENS and CRISPR/Cas9, with various success in vitro [160–162]. For example, Firth and colleagues were able to correct the CFTR defect with CRISPR and quantify the correction with chloride conductance and Western blot [160]. Still, this has yet to be translated into any in vivo models of CF.

Furthermore, bone marrow-derived MSCs, when cocultured with airway epithelial cells, have shown to differentiate to the respective epithelial phenotype with functional CFTR expression [163]. The same study demonstrated CFTR restoration of CF-derived MSCs through gene correction that was capable of significantly affecting epithelial chloride secretion. In one of the few in vivo studies completed for CFTR correction, bone marrow-derived MSCs have been intratracheally delivered to CF mouse models resulting in an increase in CFTR expression [164]. Despite the fact complete differentiation of the MSCs was not confirmed, the partial restoration of CFTR function can be attributed to expression of the functional protein from the delivered MSCs. Duchesneau and colleagues were also able to show delayed infection and increased survival associated with the treatment.

Similar to bone marrow-derived MSCs, human amniotic epithelial cells (hAEC) can also be induced to produce functional CFTR channel. In vitro studies by Carbone and colleagues with amniotic stem cells in coculture with CF epithelial cells showed an increase in gap junction tightness and cell communication that resulted in partial correction of the diseased phenotype after differentiation of the cells into an epithelial phenotype [165, 166]. Furthermore, the results indicated that gap junction intracellular communication played a significant role in the differentiation and correction of CFTR dysfunction. hAEC express the lung-specific marker NKX2.1, which is critical for lung lineage differentiation and type 2 alveolar cell formation [150]. In one study, hAEC were cultured in lung differentiation media, which resulted in expression of functional CFTR channel in the apical plasma membrane similar to native lung ciliated cell [167]. Furthermore, the authors reported that induction of CFTR was further enhanced when hAEC were cocultured in direct contact with lung airway cells. However, although there has been preliminary data demonstrating epithelial correction with stem cells, further studies need to be completed to ensure viability and long-term efficacy of lung epithelial correction both in vitro and in vivo.

10.3.4 Promotion of Mucociliary Clearance

One of the characteristic symptoms of CF patients is the build-up of thick mucus along with poor mucosal clearance in the airways due to the dysregulation of fluid homeostasis. While the promotion of mucociliary clearance has been less examined, studies have shown reduced mucus production and mucus-producing cells with stem cell treatment. For example, multiple studies have shown that treatment with either adult and perinatal-derived MSCs in animal models results in decreased mucus production along with reduced numbers of mucus-producing cells [168–170]. Additionally, a study by Li and colleagues confirmed that placental stem cells actually reduced goblet cell hyperplasia in asthmatic rats through Notch signaling resulting in reduced mucus production [171]. Beyond mucus production, Duan and et al. examined changes in mucus clearance time and mucosal edema in a radiation-damaged nasal mucosa guinea pig model after treatment with umbilical cord stem cells [172]. The results indicated that the stem cells migrated to the site of injury and resulted in increased mucosal clearance within the first week and month. More focused studies need to be completed to correlate the exact mechanisms and benefits that stem cells could provide with regards to mucociliary clearance and mucus production. Still, there is preliminary evidence that stem cell therapy could reduce one of the more aggravating symptoms associated with CF that reduce the patient's quality of life.

10.4 Current Clinical State of Stem Cell Therapy

While stem cell therapy has made significant strides in preliminary in vitro and animal in vivo research, it still is in the transitioning state to clinical success. Currently, there are almost 50 clinical trials involving stem cells for lung diseases that are listed by the U.S. National Library of Medicine (http://www.clinicaltrials.gov/), and the majority are phase I trials to primarily ensure safety of delivery of these cells. The actually efficacy of the treatment has yet to be seen or fully examined in humans for CF, as later clinical phases have yet to be reached. Many of the clinical trials for stem cells have been organized based on the anti-inflammatory and immunomodulatory effects of the cells. As discussed in Chap. 6, one of the first clinical trials for lung disease involved four intravenous deliveries of 1×10^8 adult MSCs per infusion to COPD patients over the span of 3 months [173]. While the results showed no detrimental effects or worsening of the disease from the stem cell delivery, there were no indications of improvement or change besides a decrease in circulating C-reactive protein. After this initial clinical trial, there have been similar trials for COPD, IPF, bronchopulmonary dysplasia (BPD), ARDS, and emphysema that have all warranted further trials as the treatment appeared to be feasible and safe [174–177]. A majority of the treatments involve adult mesenchymal stem cells, but there are clinical trials available that utilize several types of PSCs such as umbilical cord-derived stem cells.

With regards to CF, there are currently only two clinical trials listed by the U.S. National Library of Medicine: one currently recruiting and one not yet recruiting. The primary objective of both studies is to demonstrate the safety of intravenous MSC administration in CF patients. In the first study, the investigators are completing a dose escalation, intervention study on the safety of infusions of human MSCs and tolerability of the infusion process. The study is investigating a dosage of 5×10^6 cells/kg. To quantify the results, infusion-related toxicities will be measured 24 h after infusion, while five subsequent visits will be completed to perform a physical examination, spirometry, blood work, and measure physical exacerbations. In addition to the main goal of the study, baseline levels and posttreatment levels of common CF inflammatory biomarkers such as IL-1β, IL-6, IL-8, TNF-a, and active proteases in blood and sputum to explore possible efficacy of the treatment will be measured. The trial is expected to be completed in late 2020.

Similar to the first study, the primary objective of the second study is the safety of intravenous delivery of MSCs, while a secondary objective is to examine a change in CF symptoms after

treatment. This study is more extensive with a recruiting timeline starting in 2019 and completion date around 2028. The intervention model includes two phases: a safety and a randomization phase. Both phases will examine two separate doses of MSCs, 20×10^6 and 100×10^6 cells, with the second phase including a placebo. The first safety phase will only examine treatment-emergent serious adverse events within the first 30 days of infusions. The second phase will continue to monitor for adverse events while also examining changes in body mass index, the pulmonary function test, pulmonary exacerbations, and changes in local and systemic inflammation a year after the single infusion for its efficacy on CF symptoms. Clinical investigation of stem cell therapy for CF is still in its infancy. There is still significant room to grow in preliminary and clinical research for CF and other similar lung diseases. Besides determining the optimal source of the stem cells, further research needs to be completed in route of administration, dosage, timing, and other factors before clinical success.

10.5 Current Hurdles in Stem Cell Therapy

10.5.1 Animal Model Translation

Currently, there are several main challenges limiting the use of stem cells in clinical applications. First, there are differences between human disease and animal models, thus limiting the applicability of findings based on animal studies. For example, CFTR-knockout mice have been used previously to study therapies for CF. However these mice do not develop the phenotype of CF lung disease, and therefore have been limited in determining the efficacy of these therapies in alleviating symptoms and in measuring endpoints [178]. In order to overcome this limitation, homozygote CF pigs and ferrets were developed via somatic cell nuclear transfer of targeted fetal fibroblasts, with disruption of the CFTR locus through homologous recombination with an adenovirus vector [179, 180]. Since pigs and ferrets are more similar anatomically and physiologically to humans than mice, these new models will improve testing of stem cell-based treatments [68]. Furthermore, there are species-specific differences in cytokine stimulation and gene expression. In one study, murine MSC failed to express the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase, which inhibits bacterial growth [181]. In addition, only murine but not human MSC express inducible nitric oxide synthase after stimulation, thus challenging the validity of murine in vivo models for the preclinical evaluation of human MSC cell therapies [181]. Lung bioprinting, as mentioned previously, is another attractive alternative to animal models. Fabricated three-dimensional scaffolds can mimic components of the extracellular matrix which can be further seeded with autologous stem cells [182, 183]. Alternatively, decellularized animal lungs can serve as a scaffold for which MSCs can repopulate into a pulmonary tissue with some degree of functionality [184]. However, a major hurdle is that 3-D constructs are unable to replicate the complex architecture of the human lung, which encompasses an intertwining network of blood vessels, nerves, and functional lung tissue [185].

10.5.2 Isolating Pure Stem Cell Populations

Second, it is technically challenging to obtain and maintain a pure population of the desired stem cells. Adult stem cells have limited growth and differentiation potential, making it difficult to maintain a large population of cells needed for treatment. Contamination with other cell types or nonhuman molecules can have detrimental effects. In addition, clinical-grade stem cells should be manufactured according to current Good Manufacturing Practices (cGMP) guidelines. One group of researchers reported that several stem cell lines currently approved in the US are probably contaminated with a nonhuman molecule called Neu5Gc, with encodes one type of sialic acid not normally produced in humans [186]. Many people have developed antibodies to Neu5Gc due to exposure in red

meat; thus, injection of current stem cell lines in humans could produce an immune response and result in rejection. Furthermore, recent studies have demonstrated that MSC can undergo spontaneous transformation [187–189]. There have been mixed results in studies regarding immunomodulation with mesenchymal stem/stromal cells. A study completed by Duijvestein et al. reported that of six Crohn's disease patients who were treated with intravenous MSCs, three of the patients actually had worsened conditions [190]. Furthermore, many studies have shown differing effects of stem cells based on their origin of derivation and age [191–194].

10.5.3 Engraftment and Trans-differentiation

For successful cell correction and replacement therapy to occur, an important aspect to consider is cell homing and engraftment to the site of injury or disease followed by trans-differentiation. MSCs, including PSCs, have shown excellent homing capabilities to the site of injury or disease regardless of the mode of administration due to the chemokines present with lung injury [78, 195, 196]. However, the actual engraftment of the stem cells in the lung injury niche has been less effective resulting in many of the poor clinical results [65, 197, 198]. In vitro studies estimate that restoration of *CFTR* in only 6–20% of airway epithelial cells is sufficient to restore *CFTR*-mediated Cl^- secretory function; however, studies also suggest that nearly all cells in the way must express *CFTR* to restore ENaC-mediated Na^+ absorption [199–202]. As such, there is a dire need to improve engraftment and trans-differentiation rates in order to apply this in the clinical setting.

Currently, a small number of studies demonstrated that less than 5% of the total population of cells administered engraft within 24 h [203–205]. In two murine studies, bone marrow-derived stromal cells expressing wild-type CFTR were administered into transgenic CFTR-knockout mice. In the first study, engraftment rate of donor-derived airway epithelial cells was approximately 0.025%, while the total number of chimeric lung epithelial cells exhibiting CFTR expression was only 0.01% [206]. Similarly, the second study also observed very low levels of engraftment (0.01–0.1%) in the intestines [207, 208]. To complicate matters further, hematopoietic stem cells have been demonstrated to fuse with and transfer genetic material with host cells, thus giving a false impression of trans-differentiation [209, 210]. One group investigated the frequency of fusion events in which male mice lacking the lung-specific protein Sp-C were transplanted with female wild-type bone marrow. The authors concluded that the frequency of Sp-C+ cells containing the Y chromosome was 65%, indicating that these cells originated from the host [211]. Airway secretions have even shown to interact with gene vectors and act as inhibitory factors, while the viscosity of the air-surface-liquid interface in CF lungs can act as a physical barrier that impedes trans-differentiation [212, 213].

In addition, there is insufficient data regarding the fate of cells after administration. Poor cell survival and engraftment leads to the limited and inconsistent clinical benefits. In a murine ischemic heart disease model, administration of MSCs resulted in death of 99% of the stem cell population in less than 4 days [214]. It is currently unknown the mechanism underlying stem cell rejection, but it is postulated that the complement system may be involved in the rapid clearance of systematic stem cells after injection [215]. Stem cells may affect the host immune system by either directly inducing an immune response, or indirectly through a modulatory effect. MSCs have a low immunogenic potential, requiring little to no immune suppression during allogenic administration [216–218]. However, upon differentiation these cells may become immunogenic due to upregulation of MHC molecules [219]. Studies in animal models and clinical trials have suggested that antibody formation and T-cell responses can limit transgene expression, as well as the therapeutic value of repeat viral administrations [220, 221]. Subsequently, graft rejection may lead to loss of function of the injected cells. Previous studies have supplemented stem cells with immunosuppressants and pro-survival agents, which

decreased graft rejection and cell death [222, 223]. However, these agents are not without side effects, and long-term data regarding adverse effects and its use concomitantly with stem cells are lacking. As such, use of autologous stem cells whenever possible is ideal as it mitigates this complication. MSC administration has also been demonstrated to induce T-cell anergy [224]. It is currently unknown how this affects the function of the adaptive immune response and if this predisposes to infections. Furthermore, injection of MSC may suppress the immune antitumor response, predisposing the host to tumor formation and malignancy [225].

In addition, optimization of several parameters such as cell number, timing of delivery, route of delivery, and conditioning protocol has not yet been determined. In the lungs, it seems that extensive tissue damage appears to be a prerequisite for stem cell engraftment, which may not be applicable in the clinical setting [226]. Cells can be labeled safely using a contrast agent, which then allows noninvasive longitudinal tracking using high-resolution MRI (Fig. 10.1).

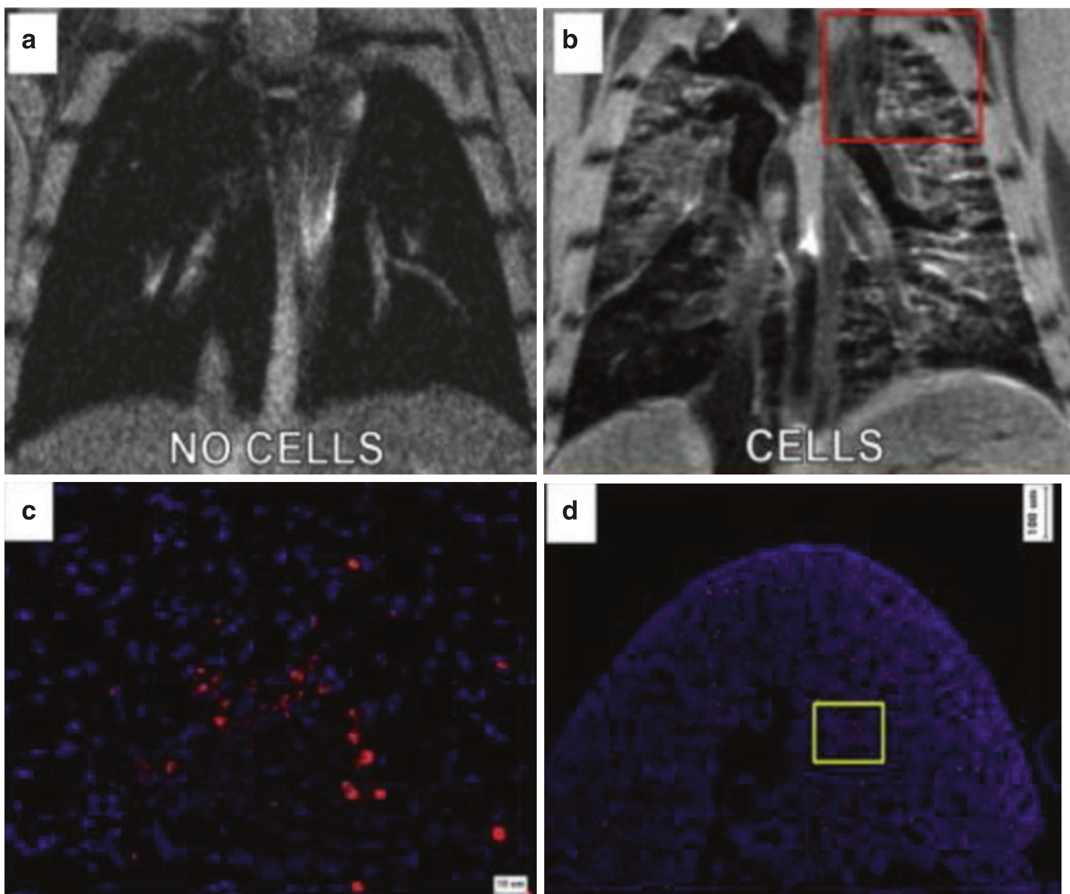

Fig. 10.1 Cell tracking using MRI. Labeled cells were delivered using intra-tracheal administration. (**a**) Animals receiving saline vehicle only demonstrated weakly positive signals surrounding the pulmonary vasculature, secondary to pulmonary blood flow. In (**b**), cells were labeled using the contrast agent Trimetasphere®, which demonstrated areas of hyperintensity throughout the upper lung lobes, consistent with the intra-tracheal route of administration. (**c**) Immunohistochemical analysis of cell localization at low magnification, and (**d**) High magnification of outlined region in (**c**). (Reproduced with permission from Murphy, S.V., et al., *Use of trimetasphere metallofullerene MRI contrast agent for the non-invasive longitudinal tracking of stem cells in the lung*. Methods, 2016. **99**: p. 99–111)

Using this technique, Murphy et al. were able to demonstrate that amniotic fluid stem cells home predominately to injured tissues [227]. Although stem cells typically home to specific tissues especially those that are damaged or under pathologic conditions, it is unclear what percentage of cells migrate to distant nontarget sites. Studies seem to suggest a positive correlation between the number of stem cells homing to the target of interest and improved clinical outcome. In one study of non-ischemic cardiomyopathy, Vrtovec et al. was able to demonstrate that greater myocardial homing led to better improvement in left ventricular ejection fraction [228, 229]. Thus, cell tracking and imaging is vital in determining optimal dosage and predicting clinical response. Unfortunately, current clinical imaging systems have inferior sensitivity compared to animal imaging systems, which only has a lower detection limit of approximately 1×10^4 cells [230]. In addition, radioactive probes used to label cells have a relatively short half-life of less than 3 days [231]. It is also unclear the number of stem cells needed to achieve a therapeutic response in vivo, and the associated risks involved with using such a high number of stem cells with multipotent potential. It has been observed that high number of cells may clump and form aggregates, which may cause thromboembolic events such as pulmonary embolisms or ischemic strokes [232]. Clumping may be associated with the storage duration in suspension, and is correlated with the number of apoptotic cells and may be related to the nucleic acids released by apoptotic or dead cells [233]. As such, cells in solution should be used immediately to ensure cell viability and to reduce clumping. In an ex-vivo extracorporeal membrane oxygenation model, large quantities of MSC adhered to the membrane oxygenator fibers, which resulted in an increase in the trans-oxygenator pressure gradient and a reduction in flow through the circuit [234].

10.6 Conclusions and Future Studies

CF continues to not only shorten the lifespan of those infected but also reduce their quality of life with no cure in the near future. The main characterization of CF has been the recurrent bacterial infections and susceptibility paired with chronic hyperinflammation. Though there are current treatments that have shown to alleviate some of this inflammation and other symptoms, there has been no complete solution [115]. New advancement and understanding of the anti-inflammatory and antimicrobial properties of stem cells has expanded the use of the cells from primarily engraftment and differentiation. Stem cell therapy could play a significant role in the development of new therapies to attenuate the chronic inflammation seen in CF to decrease the developing lung injury, thus improving lung function. Moreover, it could be used in synergy with current therapies to provide a more well-rounded treatment. While preclinical and clinical data demonstrate that there may be a role for stem cell therapy in the treatment of CF, further studies, in vitro and in vivo, need to be completed to elucidate mechanisms, benefits, and any potential side effects. First, more in vitro data needs to be collected to understand the actual mechanisms behind the immunomodulation and anti-inflammatory properties. Specifically, studies involving healthy and CF patient-derived cells and their interactions with stem cells could be highly beneficial. Additional studies will need to be performed to determine the effect of stem cells on the immune response and if this will predispose patients to infections and autoimmune phenomenon. Although there has been increasing evidence in the benefits of stem cells, there are potential risks that need to be addressed and further studied to avoid unnecessary complications. Furthermore, characterization of stem cell niches and dosage stem cell for use as therapy still needs to be elucidated. Significant advancements have been made in the past decade with regards to air–liquid interface and 3D culture of lung epithelial cells can help further disease modeling and drug development. Once the mechanisms are well understood, in vivo and clinical experiments could proceed to optimize procedural aspects such as cell derivation, culture, and dose along with administration location and time. iPSCs are the focus for development and creation of new organs that can hopefully be transplanted into patients. Stem cell

therapy has the potential to be an effective anti-inflammatory and immunomodulatory treatment for CF patients to reduce symptoms and progression of the disease.

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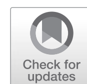

11 Preclinical Evidence for the Role of Stem/Stromal Cells in Targeting ARDS

Tamara Cruz and Mauricio Rojas

11.1 Acute Respiratory Distress Syndrome

Acute respiratory distress syndrome (ARDS) is a high-mortality syndrome that develops following an infection or trauma, leading to a dysregulated inflammatory response in the lung that can cause tissue remodeling, pulmonary dysfunction, and death [1]. ARDS is characterized by acute respiratory failure caused by an increase of fluids in the alveolar space. The breakdown of the immune response increases the permeability of the epithelial–endothelial barrier. This results in an increased filtration of protein-rich fluid from the vascular system into the alveolar spaces, with a subsequent lung edema and a decrease in the ability of gas exchange [2].

ARDS develops as a localized lung response that could have multiple origins including pneumonia, systemic sepsis, major surgery, or multiple trauma. Patients with ARDS have an acute onset of symptoms like severe chest pain and a decrease in their pulmonary function associated to pulmonary infiltrates in the X-ray, indicative of pulmonary edema. These symptoms usually appear after the first week of the injury [3].

ARDS accounts for more than 10% of intensive care unit (ICU) admissions worldwide and has a mortality rate of about 40% [4]. ARDS results in a diminished quality of life and lung function; and survivors often have long-term neuromuscular, cognitive, and psychological symptoms. Additionally, long hospitalization, ICU, and increased use of health care services after hospital discharge have enormous socioeconomical cost [5, 6].

Despite several decades of research, there is no disease-modifying therapy for ARDS. Because the mechanisms driving lung injury are complex and diverse, pharmacological treatments often fail, suggesting that targeting a single mediator or pathway is not enough to achieve therapeutic effects. As an alternative, after their use in several preclinical models of ARDS, cell-based therapies

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with mesenchymal stem/stromal cells (MSCs) are promising, as they can target multiple cellular and extracellular impairments associated with ARDS simultaneously [7].

11.2 Pathology of ARDS

There are multiple initiation agents for ARDS, from microorganism invasion (bacteria or viral) to mechanical stressors, that result from mechanical ventilation [8]. Beyond the initial trigger, aging, like in any other lung diseases, there is an impaired capacity of the lung to recover. In several animal models, when we compared aged with young, there was an increase in the morbidity and mortality as a consequence of an altered inflammatory response [9–11].

Immune activation results in the release of pro-inflammatory cytokines and chemokines with a primary influx of neutrophils into the alveolar space, leading to the release of metalloproteinases (MMP), myeloperoxidases (MPO), collagenase, and the generation of reactive oxygen species (ROS) [12]. This activates and attracts macrophages and lymphocytes to the site of injury with a sequent release of inflammatory cytokines including IL-6, IL-8, IL-1β, and TNFα [13].

In physiological situations, anti-inflammatory mediators act to limit the inflammatory cascade and control the tissue damage. ROS serves multiple functions such as killing phagocytosed microorganism, or the removal of cell debris and signaling. However, high and persistent levels of ROS, MMPs, and MPOs cause tissue necrosis, injury, and destruction [14]. The controlling feedback mechanism seems to be impaired during the onset of sepsis, which leads to a persistent inflammatory response after the resolution of the initial insult [15].

Along with the production of proinflammatory cytokines, other factors are secreted such as endothelin-1 (EDN1), angiotensin-2 (AGT-II), and phospholipase A2 (PLA2), which increase vascular permeability [16, 17]. The disruption of epithelial–endothelial barriers leads to a disruption of the alveolar clearance and production of surfactant [18]. Edema accumulates in the alveoli though the increase permeability of epithelial and endothelial barriers and the decrease in alveolar fluid clearance. Measurements of the protein content in BAL fluid provide an estimate of the alveolar changes and could be used as an indicator of the prognosis of the patient [19]. There is an initial phase of fluid accumulation followed by a proliferation phase, which is characterized by the increase of type II alveolar cells (AEC-II), fibroblast, myofibroblast, and matrix deposition. If the inflammation persists, then there is a disorganized repair that could lead to fibrosis [20, 21].

11.3 MSCs in the Treatment of ARDS

MSCs constitute a great option for the treatment of ARDS because of their ability to regulate the immune response, enhance the phagocytic clearance of bacteria and secrete factors that regulate the capillary–alveolar barrier. Additionally, MSCs appeared immune-privileged with low levels of type I HLA antigens in their surface, which allows them to escape from the patient's immune response [22, 23]. This represents an important advantage that allows the therapeutic use of allogenic MSC [24].

MSC could exert their effect through cell contact-dependent mechanisms and by the release of soluble factors. This chapter summarizes all the processes by which MSCs could be beneficial in the treatment of ARDS (Fig. 11.1) and the preclinical animal models of lung injury in which these have been tested.

11.4 Mechanism of Action of MSC

Tracking MSC engraftment has been possible using MSC expressing GFP or labeled with PKH allowing the localization of the cells by fluorescent imaging [25–27]. There is considerable variety in the number of cells homing into the lungs. We have demonstrated an early retention in the lung of MSCs in large animal models of acute lung injury independent of the way cells were delivered [28]. However, it is well accepted that

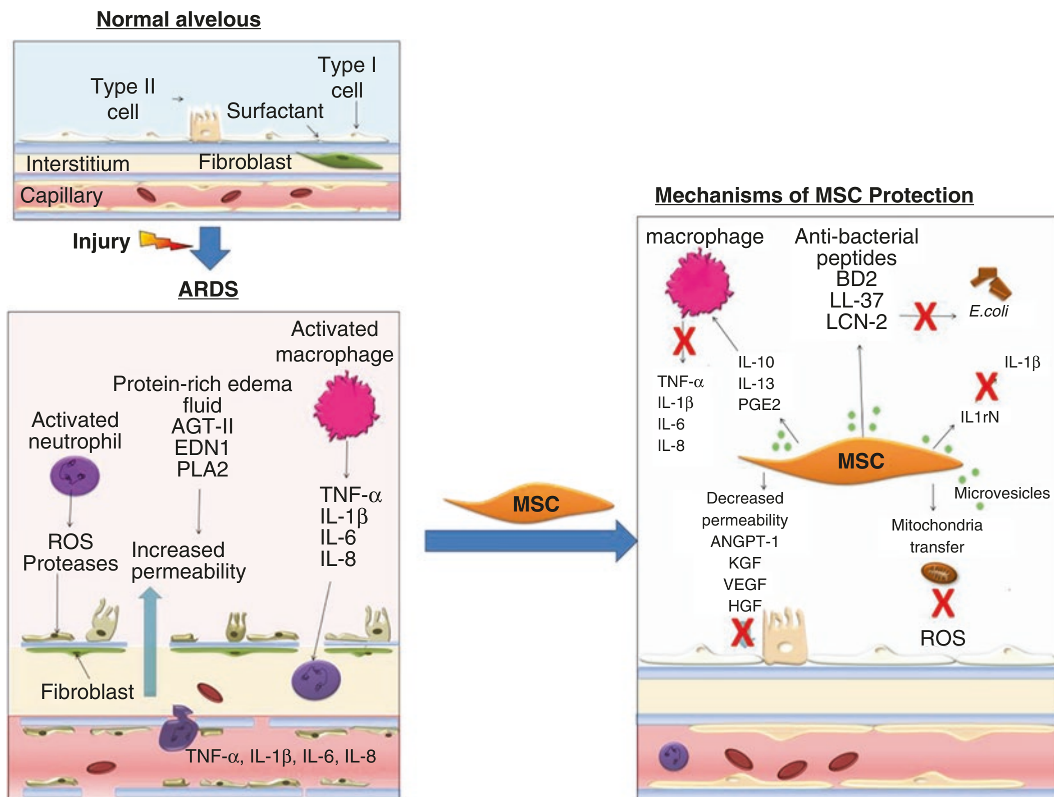

Fig. 11.1 Schematic representation of the mechanisms driving alveolar damage during ARDS and the different pathways targeted by soluble factors produced by MSCs and the consequences on lung repair

by using colocalization of surface markers, infused MSCs that localize in the lung are not differentiating into any other cell type, including alveolar or airway epithelial cells, fibroblasts, or endothelial cells. The actual knowledge suggest that most of the presence of MSCs observed in the LPS model of lung injury reflects a transient process [29] and that the protective effect seen with MSC therapy does not require MSC differentiation into any cell type [14]. Engineered MSC to overexpress important genes for the epithelial lineage like angiopoietin [30], ROR2 [31] or β-catenin [32] showed an enhanced differentiation capacity in vitro, but there was no engraftment and differentiation in in vivo models of LPS-induced acute lung injury.

Despite the initial interest in the multipotent properties of the MSCs, engraftment and differentiation in the lung [14, 33], their beneficial effect more likely derives from their capacity to be recruited by the sites of injury, interact with the host cells, and secrete soluble factors known as the secretome [34]. The MSC secretome is dynamic and could vary depending on the MSC source or the type of lung injury. It includes an extended array of bioactive molecules comprising cytokines, chemokines, growth factors, angiogenic factors, and microvesicles [2]. Table 11.1 summarizes the described molecules of the secretome and their main functions.

11.4.1 MSCs Reduce Endothelial and Epithelial Permeability

The integrity of the microvascular endothelium is essential to avoid the influx of protein-rich fluid from the circulation to the alveolar space. In addition to the generated edema, this permeabilization comes with inflammatory cytokines and

Table 11.1 Secretome of the MSCs and their therapeutic actions against ARDS

Molecule	Mechanism of action
Reduce endothelial–epithelial permeability	
Angiopoietin-1 (Ang-1) [35, 36]	Promotes endothelial survival, reduces permeability, and inhibits leukocyte interactions
Keratinocyte growth factor (KGF) [37–41]	Promotes epithelial proliferation, may be attribute for the promotion of AEC-II and the production of surfactant
Hepatocyte growth factor (HGF) [42–46]	Preserve integrity of the endothelium
Sphingosine-1-phosphate (S1P) [47, 48]	Enhances the resistance of the endothelial barrier and reduces the levels of TNFα
Vascular endothelial growth factor (VEGF) [45, 49–51]	Reduces lung permeability, protects endothelium from apoptosis, controls inflammation, and facilitates VE-cadherin recovery
Alveolar fluid clearance	
KGF [37–41]	Increases trafficking of sodium transport proteins to the cell surface, reduces aquaporin 5 expression, and increases epithelial repair
Fibroblast growth factor 7 (FGF7) [52]	Increases expression of sodium channels
Epithelial growth factor (EGF) [53, 54]	Stimulates the proliferation of epithelial cells, increases sodium channels and Na-K-ATPase function
TGF-β [55, 56]	Increases activity of the sodium channels
Immune response regulation	
IL-4 [57, 58]	Anti-inflammatory cytokine inhibiting type I responses
IL-10 [59, 60]	Anti-inflammatory cytokine inhibiting neutrophil recruitment and activation
IL-13 [61]	Anti-inflammatory cytokine
IL-1rN [62]	Competes with IL-1β receptor binding, inhibiting its effects
Prostaglandin E2 [63, 64]	Stimulates macrophages to produce IL-10
KGF [37, 41]	Induces the secretion of granulocyte–macrophage colony-stimulating factor that increases alveolar macrophage phagocytosis and inhibits macrophage apoptosis
TNF-stimulated gene protein 6 (KGF6) [65, 66]	Anti-inflammatory cytokine
Bacterial clearance	
β-Defensin 2 (BD2) [67]	Inhibits bacterial growth in vitro
LL-37 [68, 69]	Antibacterial, antifungal, and antiviral properties
Lipocalin-2 [70–72]	Regulates chemokines such as CXCL9 to reduce inflammation in front bacterial infections

cells which may further aggravate the ability of the endothelium to reduce edema [33]. Several MSC-secreted factors have the capacity of regulate the alveolar microvasculature reducing its permeability.

Angiopoietin-1 (Ang-1) is a ligand for endothelial Tie2 receptor activating the NF-κB pathway to prevent the formation, on AEC-II, of actin stress fibers and preserve the localization of claudin-18 [73]. Ang-1 has been shown to promote endothelial survival, reduce endothelial permeability, and inhibit leukocyte interactions by modifying cell adhesion molecules and cell junctions [36, 74, 75]. Engineered MSCs overexpressing Ang-1 further reduce protein content, albumin, and immune cells in BAL [35, 76, 77]. MSC culture in proinflammatory conditions enhances their capacity to produce Ang-1. Also, when Ang-1 was blocked with siRNA, MSCs no longer prevented epithelial permeabilization [73].

Keratinocyte growth factor (KGF), also known as fibroblast growth factor 7 (FGF7), is a critical factor for epithelial repair and stimulating epithelial cell proliferation [41]. Animal models of ALI, such as by administration of α-naphthylthiourea [37, 52] *P. aeruginosa* [78] or ventilator-induced lung injury [39], have shown the ability of KGF to reduce alveolar edema. Engineered MSCs overexpressing KGF improve microvascular permeability, reduce proinflammatory cytokines (IL-1β

and TNFα), and increase the anti-inflammatory response (IL-10). The underlying mechanism is not completely understood but may be attributed to the promotion of AEC-II cells and the production of surfactant [41].

Hepatocyte growth factor (HGF) was found to preserve integrity of pulmonary endothelium though the inhibition of Rho GTPase, prevent the actin stress fiber formation, and preserve the gaps between endothelial cells [39, 42]. Sphingosine-1-phosphate (S1P) enhances the resistance of the endothelial barrier. This mechanism seems to be dependent of the capacity to inhibit leukocyte permeability as well as reduce levels of TNFα [47, 48]. Vascular endothelial growth factor (VEGF) has been described to reduce lung permeability, protect endothelium from apoptosis, control inflammation, and facilitate VE-cadherin recovery. Knockdown of VEGF in MSCs confirms these activities as these cells present reduced therapeutic activity on ALI models [51].

11.4.2 Alveolar Fluid Clearance, AFC

Alveolar fluid clearance (AFC) is the capacity of the lung epithelium to remove alveolar fluid during pulmonary edema. This process is mediated through sodium channels, aquaporin, and sodium-potassium adenosine triphosphatase Na-K-ATPase [43]. Many conditions such as high-volume mechanical ventilation, live bacteria, or proinflammatory cytokines can reduce AFC [79, 80]. The compromised capacity of AFC is used as prognostic value to determine morbidity and mortality [80]. Both AEC-I and AEC-II are involved in AFC during pulmonary edema. AEC-I has the highest permeability to water, potentially through aquaporin, which supports its role in ion transport [81–83]. Na-K-ATPase is expressed in both AEC-I and AEC-II and has a critical role in alveolar fluid reabsorption, where the sodium transport is followed by outflux of water in an isosmolar manner [84, 85].

Studies with intra-bronchia administration of LPS in ex-vivo perfused lungs revealed a marked decrease in AFC, and mechanisms dependent on the blood presence suggest that immune cell are required for the injurious effect of the LPS. MSCs, or their conditioned media (CM), normalized AFC in a KGF-dependent manner as siRNA for KGF reduced the therapeutic effect of the MSCs [86]. KGF increases fluid transported across the alveolar epithelium through increased trafficking of sodium transport proteins to the cell surface [87, 88]. KGF also has important functions reducing the expression of aquaporin 5 and avoiding the transdifferentiation of AEC-II toward AEC-I [38]. Another group described that KGF effects on AFC are mediated through an increased rate of epithelial repair, cell adherence, and migration [40].

Epithelial growth factor (EGF) stimulates the proliferation of epithelial cells, increases sodium channels and Na-K-APase function in in vitro alveolar epithelial cultures [54]. An in vivo rat model with aerosolized EGF showed an increase in active sodium transport, Na-K-ATPase activity, and lung fluid clearance [53]. Rat models with instilled TGF-β increase alveolar fluid clearance in a time-dependent and dose-dependent manner. This increase of alveolar clearance is driven by an increased activity of the sodium channels [55].

11.4.3 Immune Response Regulation

MSCs constitutively do not exert their immunomodulatory properties but instead have to be “primed” by inflammatory mediators [89]. In the context of ARDS, the acute inflammation drives this “priming” of the MSCs, activating their immunomodulatory properties [24]. MSCs were found to protect tissue damage from extraordinary inflammation by downregulate the expression of proinflammatory cytokines as IL-6, IL-8 IL-1β, IFNɣ, and TNFα and through the production of anti-inflammatory cytokines such a IL-4, IL-10, or IL-13 [71, 79, 90, 91]. Additionally, MSCs produce IL-1rN which is a cytokine that competes with IL-1β receptor binding, thus inhibiting its effects [62].

Several in vitro and in vivo studies have demonstrated that MSCs have several effects over the innate immune system. They can influence the

maturation of the dendritic cells (DC) [92], increase the phagocytic capacity of monocytes [93–95], neutrophils [96–98], and modify macrophage toward immunomodulatory M2 phenotype [99, 100]. MSCs secrete prostaglandin E2 that then stimulates macrophages and monocytes to produce the anti-inflammatory cytokine IL-10 [101]. IL-10 has been reported to inhibit the rolling, adhesion, and transepithelial migration of neutrophils, thus resulting critical downstream in the MSC therapeutic effects [13, 102]. MSCs produce KGF that induces the secretion of granulocyte–macrophage colony-stimulating factor that increases alveolar macrophage phagocytosis [103]. KGF also inhibits macrophage apoptosis through a downregulation of the β-catenin pathway that increases Bcl-2 and decreases BAX and caspase-3 [104].

MSCs also can modulate the adaptive immune response. MSCs suppress B cell proliferation and terminal differentiation [105], suppress T cell proliferation [102, 106, 107], induce a switch from Th1 proinflammatory response to Th2 response, and finally increase the number of T regulatory cells [108]. MSCs secrete idoleamine 2,3-dioxygenase (IDO) upon stimulation with IFNɣ. IDO activity results in tryptophan depletion and kynurenine production that inhibits T cell proliferation [109]. TNF-stimulated gene protein 6 (KGF6) is another contributor to the immunomodulatory effects of the MSCs. In lung injury models, MSCs upregulate KGF6 that binds IL-8, blocking its function [66]. Blockage of KGF6 by siRNA completely reverses MSCs' anti-inflammatory properties [65].

It is important to note that while MSCs secrete an extensive range of anti-inflammatory cytokines, they also have the capacity to produce several proinflammatory cytokines such as IL-6 and IL-8. The production of these cytokines has been associated with poor outcomes in ARDS patients [110, 111]; however, there is new evidence that suggests a role of these cytokines in the MSC therapeutic effect. IL-6 is usually implicated in proinflammatory responses, but apparently, it could have promiscuous functions [112–114]. It is not clear how IL-6 induces therapeutic effects, but its inhibition in lung injury models diminished the MSC therapeutic effect [115]. The role of IL-8 is not clear but there is evidence suggesting that IL-8 promotes the production of VEGF that promotes angiogenic effects [50].

11.4.4 Enhancement of Bacterial Clearance

MSCs attenuate bacterial sepsis directly by antimicrobial peptides secretion and by enhancement of macrophage phagocytosis [116]. MSCs stimulated by the presence of bacteria released β-defensin 2 (BD2) [67], LL-37 [68, 69] and lipocalin-2 [71]. BD2 is upregulated in the presence of bacteria through TLR2 and TLR4 pathways, inhibiting bacterial growth in vitro [67]. LL-37 is a cathelicidin peptide with antibacterial, antifungal, and antiviral properties [68, 117]. Lipocalin-2 is known to act on mucosal cells during pulmonary infection to regulate chemokines such as CXCL9 to reduce inflammation in front bacterial infections [70, 72, 117].

CM from stimulated MSC was found to contain high levels of antimicrobial peptides and to inhibit bacterial growth. However, when treated with inhibitors of these peptides, the effect was abolished. Animal models of lung injury show a reduced bacterial growth in the lung homogenates on the animals treated with MSCs. This effect was decreased if a neutralizing antibody against LL-37 was administered with the MSCs [92]. Similar observations were done blocking the TLR2 and TLR4 and thus the production of BD2 [67].

11.4.5 Transfer and Rescue of Mitochondrial Function in Target Cells

MSCs could contribute to rescue mitochondrial function in epithelial cells containing nonfunctional mitochondria [118, 119]. In vitro imaging reveals the formation of connexin-43-gap junctions between MSC and alveolar epithelial cells, allowing the transport of mitochondria to the LPS-injured epithelium. This resulted in increased ATP levels that rescued the surfactant

secretion by AEC-II leading to reduce alveolar permeability and reduced mortality. An endotoxin lung injury model treated with instilled MSCs confirmed this mitochondrial transfer to the injured epithelium, leading to the restoration of its function. This rescue mechanism was abrogated when MSCs with dysfunctional mitochondria were instilled, supporting its role in restoring the lung epithelium [25]. As described in more detail in Chap. 4, another group found that the mitochondria were transferred through nanotubes, which was upregulated by the Rho-GTPase Miro1 [120].

Mitochondrial transfer to macrophages has also been described in vitro through the formation of tunneling nanotubes. Mitochondrial transfer enhanced macrophage oxidative phosphorylation and phagocytosis. The blockage of the tunneling nanotubes formation completely abolished the MSC effect on macrophages [121, 122].

11.5 Preclinical Models of MSCs in ARDS

11.5.1 Animal Models of ARDS Treated with MSCs

The therapeutic efficacy of MSCs in the treatment of lung injury has been demonstrated in numerous animal models. Most of the studies have been performed in mouse or rat models and large animal models using swine [145, 146] or sheep [147, 148]. Acute lung injury is mainly induced through infection with endotoxin or live bacteria, and via cecal ligation and puncture driving sepsis. Rat models are preferred for the study of ventilator induced lung injury. There is lot of variability in dose, timing, route of administration, and source of MSCs, but all the studies found an improvement of the injury. Table 11.2 shows the main findings in animal models of ARDS using MSCs.

Animal models have shown the potential of using MSCs as a treatment therapy for ARDS. A recent study compared the therapeutic potential and the distribution of the MSCs with different administration routes in a large animal model of acute lung injury. Endobronchial and intravenous administration of MSCs have similar rates of lung retention at 5 h post administration and recovery of arterial oxygenation in equal extent [28].

Most of the studies performed showed histological improvements upon MSC treatment with reduction of the inflammation, edema, and lung injury with reduced collagen deposition [29, 65, 69, 71, 94, 108, 123–138]. At the endothelial–epithelial level, the alveolar barrier is better maintained in the animals treated with MSCs, with lower levels of protein in the BAL [65, 123, 129, 135–137, 139, 140]. In vivo, MSCs display immunomodulatory properties modifying the lung immune response. At molecular levels, MSC are able to shift the cytokine profile from the pro-inflammatory status in ARDS toward a more anti-inflammatory one [124, 128, 131, 133, 135, 136, 138, 140, 141]. At the cellular level, MSCs promote a T regulatory lymphocyte response [108], increasing phagocytic activity of macrophages [69, 101, 127, 129] and reducing the neutrophil numbers [8, 125, 127, 131, 136]. All these modifications of the immune response control the inflammation and preserve the lung tissue integrity. Also, MSC perform antimicrobial properties promoting phagocytosis and bacterial killing by the secretion of LL37 peptide [69] or lipocalin [71, 94]. In addition, MSC have showed antioxidant effects in vivo, preserving plasma levels of cysteine and glutathione redox state after endotoxin administration [126, 149].

11.5.1.1 Development of Genetically Modified MSCs

MSCs' capacity to migrate to the sites of inflammation makes them an attractive factor for gene-based therapy [150]. Overexpression of specific genes in MSCs can be used to enhance their therapeutic effects [25]. Most of the approaches are directed to increase their regenerative potential or their immunomodulatory capacity.

Administered MSCs overexpressing KGF improve pulmonary microvascular permeability, reducing the lung injury in the model of LPS-induced lung injury [41]. VEGF-overexpressing MSCs protect the endothelium from apoptosis, reducing the permeability and edema [51]. The

Table 11.2 Main therapeutic findings in animal models of ARDS

Lung findings	BAL findings	Plasma findings
Physiological changes		
Prevention of lung inflammation, injury, and edema [29, 65, 69, 71, 94, 108, 123–138] Increased adhesion molecule-1 and vascular cell adhesion molecule-1 [134, 139] Increased antioxidant and reduced glutathione [126] Reduced elastase [129, 135–137, 139] Reduced collagen [129] Increased KGF [139] Mitochondrial transfer [25]	Reduced protein content [65, 123, 140]	Improved oxygenation [135, 137]
Anti-inflammatory		
Cytokines: IL-1β, TNFα, and IL-6 [124, 128, 131, 133, 135, 136, 138, 140, 141] Increased anti-inflammatory cytokines: IL-10 and IL-1RN [130, 131, 133, 135, 138, 140] Reduced neutrophils [8, 125, 127, 131, 136] Reprogramming of macrophages to M2 [69, 101, 127, 129] Increased Tregs [108]	Cytokines: TNFα, MIP2, IL-1β, and IL-6 [65, 123, 124, 130, 137, 139, 140, 142] Increased IL-10 [123, 142] Reduced neutrophils [65, 124, 137] Reduced cell count [130, 137, 139, 140, 142]	Decreased expression of IL-1β, TNFα, and MIP2 [8, 29, 123, 124, 132, 141, 143] Increased expression of IL-10 [8, 143] Increased phagocytic activity of monocytes [141]
Antimicrobial		
Promotion of phagocytosis and bacterial killing [68, 69, 124, 141] Reduced viral load [13, 144] Increased LL37 in rat lungs [69]	Reduced bacterial growth [68] Increased LL37 [68] Enhanced bacterial clearance by production of lipocalin-2 [71, 94]	Reduced bacterial counts [141] Increased LL37 [69]

expression of HGF makes MSC to protect adherent junctions, VE-cadherin of the epithelium and reduce apoptosis, preserving lung architecture [46]. β-Catenin-overexpressing MSC improves alveolar permeability, promoting the differentiation of lung precursors into AEC-II [32]. Engineered MSCs targeting the immune system have been modified to express anti-inflammatory molecules or to alter the expression of chemokines. MSCs overexpressing prostaglandin receptor [63], IL-10 [151], or IL-1rl1 [152] induce a strong shift in the cytokine profile toward an anti-inflammatory response, reducing lung inflammation and edema. Finally, MSCs with altered expression of chemokine receptors show a reduced accumulation in the lung of inflammatory cells and mediators [153].

All the previous studies described the effects of the MSC in acute lung injury when administered at the initiation of the injury, but administration of MSCs once the injury is already performed did not show significant therapeutic effects [154]. MSCs overexpressing the microRNAs let-7d (antifibrotic) or miR-154 (profibrotic) were administered 7 days after bleomycin instillation. Mice treated with let-7d expressing MSCs were found to recover quicker from the initial weight loss, while those untreated or treated with miR-154 had the lowest survival rate, although no fibrotic differences were found in the lung tissue. The effect was more immunomodulatory, altering the pattern of cytokines and the leukocyte infiltration [155].

11.5.1.2 Pretreatment of the MSCs to Enhance Their Potential

MSCs' immunomodulatory effects vary depending on the immune microenvironment [156, 157]. The therapeutic effect achieved varies greatly between different studies and one possible explanation could be the resting status of the MSC. The immunosuppressive function of the MSC is enhanced by the presence of pro-inflammatory cytokines such as IFNγ, TNFα, IL-1α, or IL-1β

[158], while the presence of anti-inflammatory cytokines like IL-10 abrogates their suppressive effect and even induces the production of pro-inflammatory cytokines [159–161].

An early study assessing the role of the micro-environment in the treatment of acute lung injury with MSCs showed the importance of the expression of TLR4 on the MSCs. TLR4 expression of MSCs is essential for the release of prostaglandin E2 upon activation by LPS or TNFα. MSCs lacking the genes for TLR4 or their downstream mediators are unable to produce prostaglandin E2 and activate macrophages to produce IL-10 in a mouse model of acute lung injury [101].

Rojas et al. showed that MSC treatment with serum from ARDS patients containing pro-inflammatory cytokines increases their immunomodulatory function with higher production of IL-10 and IL-1β receptor agonist. Pretreated MSCs have an enhanced protective capacity, reducing lung injury, edema, and accumulation of pro-inflammatory cells and cytokines in a mouse model of acute lung injury [130].

The time point at which MSCs are administered seems to be crucial. Only MSCs injected at the time of inducing the lung injury have shown to have therapeutic effect, even though they could lose all the antifibrotic properties when administered long after the injury [154]. One of the possible mechanisms responsible for the shift in their function is the microenvironment of the injured lung. In a mouse model of irradiated lungs, TGF-β expression was incremented in the damaged lungs, and in vitro the cytokines released by the lung injured cells inhibited the differentiation of the MSC into epithelial cells [162]. A recent study of acute lung injury by acid instillation revealed that treatment with MSC worsens the acid effects, driving a fibrotic process. This process seemed to be mediated by the presence of IL-6 and fibronectin in the microenvironment, which could be driving a senescence-associated phenotype on the MSCs. The fibrotic effect was reversed when the MSC were engineered to overexpress IL-10 or HGF neutralizing both in vivo in mouse models of HCl and ventilator-induced lung injury [163].

Another factor that could influence the therapeutic effect achieved is the aging status of the MSCs. MSCs isolated from aged individuals have reduced expression of cytokine and chemokine receptors that impair their migration and activation, failing to reduce inflammation in a mouse model of acute lung injury [164].

11.5.1.3 Alternative Sources of MSCs Than Bone Marrow

Traditionally, MSCs obtained from bone marrow have been used in most of the preclinical and translational studies. However, there is a growing number of studies using adipose and umbilical cord blood (UC-MSC) as potentially more plentiful sources. MSCs from different sources exhibit different receptors and immunomodulatory properties, which may cause differential therapeutic effect on ARDS, but overall, animal models have shown beneficial outcomes [165–171].

UC-MSCs have shown higher proliferative rates and lower expression of senescence markers than BM-MSCs which could reflect a more multipotent capacity [165]. UC-MSCs have great immunomodulatory capacity in mouse models of acute lung injury, inducing a shift toward a regulatory immune response with increasing levels of IL-10 and phagocytic macrophages [60, 64]. At the regenerative level, UC-MSCs attenuate lung injury, preserving vascular permeability and protecting from apoptosis [56, 172, 173].

Adipose MSCs have the advantage of their availability and easy isolation. Adipose MSCs have shown immunomodulatory properties in animal models of acute lung injury reducing inflammation, leukocyte infiltrate and modifying the cytokine profile toward an anti-inflammatory response [59, 174–176]. Recently, other studies have appeared using stem cells from pulp and periodontal ligament or menstrual stem cells, showing improvement of alveolar epithelial permeability and reducing pro-inflammatory cells and cytokines in LPS-induced ARDS models [177, 178].

11.5.1.4 Use of Soluble Factors Generated by MSCs

Microvesicles (MV) are small circular membrane fragments that are shed from the cell surface or released from the endosomal membrane and play an important role in cell communication. This communication system has emerged early during

evolution and serves as template in the further development of intercellular interaction mechanisms. MV can transfer specific genes, miRNAs, or small organelles, including mitochondria, from the MSC to the injured target cell through the connexin-43 gap junction channels.

MSC have demonstrated to have therapeutic effects in in vivo animal models but little is known about their long-term side effects, including the possibility of becoming tumorigenic. Given that the MSCs' therapeutic effect depends on the release of soluble factors, the in vivo use of MV represents an alternative and safer approach. Analysis of the RNA of microvesicles derived from MSCs revealed mRNAs associated with transcription, proliferation, immune cell regulation, and microRNiAs as well.

Intra-tracheal instillation of MSC-derived MV reduces edema and alveolar protein levels in mouse models of acute lung injury [179]. MV also show anti-inflammatory properties reducing neutrophils and inflammatory macrophages. A partial therapeutic effect of MSC MV depends on KGF, as KGF siRNA pretreatment of MSCs partially eliminated their therapeutic benefits [180]. Further studies show that the MV-dependent activity is mediated by CD44 receptors, promoting internalization of MV into monocytes, resulting in a decreased expression of inflammatory cytokines [181].

11.5.2 Ex-Vivo Lung Perfusion (EVLP) Models

Ex-vivo lung perfusion (EVLP) offers a unique opportunity for in situ testing of the effects of MSCs. EVLP was originally developed as a treatment to increase the number of lungs available for transplantation. Nowadays it is not only a way to improve unacceptable lungs for transplantation, but it also represents an excellent research tool as a preclinical model for in situ testing [182]. Several groups have developed models of acute lung injury [86, 103, 183] in which the effect of MSCs was studied [86, 103, 184, 185].

A study conducted on pigs looked at the optimal route and dose for the MSCs. Intravascular delivery of MSC showed better outcomes that intratracheal administration and the optimal dose was of 5×10^6 MSC per kilogram of animal [184]. Early studies using MSCs in human lung grafts were focused on testing their capacity to restore the AFC in lungs that were unsuitable for transplantation. Intravenous administration of MSC restored AFC in injured lungs in a mechanism dependent on KGF [185].

A couple of groups have developed models of ARDS to study the disease in human lung grafts. Treatment with MSC reduces edema, improves AFC, and restores epithelial barrier permeability in ex vivo perfuse human lungs injured with *E. coli* endotoxin. The beneficial effect on endotoxin injured lung was almost abolished when the MSCs or their conditioned media (CM) was treated with KGF siRNA [86]. The treatment with MSC in a model of pneumonia using live bacteria restored AFC, reduced inflammation, and increased bacterial killing through increased macrophage phagocytosis. KGF was shown as one of the main factors protecting monocytes from apoptosis and increasing bacterial clearance [103]. A recent study used MSC in combination with extracorporeal membrane oxygenation (ECMO) after inoculation of *E. coli* endotoxin in a sheep model. The combination of the MSC and the ECMO treatment showed better histopathology changes with less inflammation [186].

The use of MSCs microvesicles has a positive result as well in the treatment of injured lung grafts. Human MSCs derived microvesicles have been used to recover lungs rejected for lung transplantation by increasing AFC and improving airway and hemodynamic parameters [187]. In another study, treatment of ex-vivo lung perfusion model of bacterial pneumonia with MSC microvesicles increased AFC, reducing protein permeability and bacterial load [188].

In summary, since the description of the protective effect of the administration of MSCs to mice with induced ARDS [29, 123], several research groups have confirmed this observation on small and large animal models and more recently in the EVLP in which ARDS is induced in human lungs. The proposed mechanisms by which MSCs can induce protection are multiple,

some described in the present chapter. However, there is consensus that engraftment in the lung and differentiation into lung cells does not occurs. More recently, data generated from approved clinical trials, conducted by several academic institutions, demonstrated that the use of MSCs is safe and, in some cases, with demonstrated protection in patients with ARDS. There is still more research needed to determine the appropriate source of MSCs, route and time of administration, and the generation of modified MSCs in which the protective potential is enhanced.

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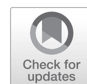

12 The Safety and Efficiency of Addressing ARDS Using Stem Cell Therapies in Clinical Trials

Emanuele Rezoagli, Emma J. Murphy, John Laffey, and Daniel O'Toole

12.1 ARDS

Acute Respiratory Distress Syndrome (ARDS) is a complex and debilitating disease of the lungs, which continues to have a high mortality rate and huge disease burden on patients. Incidence is rising, possibly due to greater awareness leading to more diagnoses rather than a change in the underlying rate. It arises from multiple etiologies, though pathogenic infection, termed pneumonia, is the most prevalent and widely studied. The distinct pathophysiology and rapid evolution of ARDS makes it uniquely challenging with regard to therapeutics development and, to date, no medicines are licensed for specific therapy. Antibiotics, ventilation, and other organ support remain intervention standards.

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12.2 Definition and Diagnosis

In 1967 Ashbaugh and colleagues [1] recognized a specific clinical pattern characterized by an acute onset of elevated respiratory rate, hypoxemia resistant to high FiO_2, bilateral lung infiltrates on chest X-ray in the absence of cardiogenic edema and the presence of a heterogeneous number of risk factors that can lead to the same syndrome [2]. The first formal definition of ARDS was developed at the American-European Consensus Committee in 1994 [3].

1. Acute onset.
2. Presence of bilateral infiltrates at the chest X-ray.
3. Pulmonary wedge pressure ≤ 18 mmHg or no clinical evidence of high left atrial pressure (to rule out a cardiogenic cause of lung edema).
4. Hypoxemia, regardless of the applied levels of positive end expiratory pressure.

The levels of hypoxemia were used to stratify the severity of lung injury as ALI ($PaO_2/FiO_2 \leq 300$) or ARDS ($PaO_2/FiO_2 \leq 200$). An updated and improved version was proposed in 2012 during a task force meeting of experts in Berlin, from which the last ARDS definition takes its name [4].

1. Rapid onset of symptoms that cannot be attributed to any underlying cause.
2. Bilateral infiltration of leukocytes from surrounding tissue to the airspace, as identified by chest X-ray.

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3. Exclusion of hydrostatic causes of edema leading to respiratory failure.
4. Impaired blood oxygenation as assessed by arterial:alveolar oxygenation ratio (PaO_2/FiO_2), with relative levels denoting mild (≤300), moderate (≤200), or severe (≤100) ARDS.

Beyond initial diagnosis criteria, scoring systems have also been devised to assess degree of injury, including APACHE [5] and Murray [6] scales for adults and PRISM [7] and PIM [8] scales for pediatric patients. It was subsequently shown that the Berlin definition criteria of ARDS are adaptable also for the pediatric population [9, 10].

ARDS can arise from pneumonia, sepsis, and overaggressive ventilation strategies, while other less common causes include smoke inhalation, near-drowning, and poisoning [11, 12]. Only very recently has an accurate picture of in-hospital ARDS incidence been attained [13, 14], although prevalence as regards to the general population is still somewhat unclear.

12.3 ARDS Management

ARDS is an acute condition, generally arising within a week of an inciting event (e.g., pneumonia) essentially occuring and is resolved over a matter of days to weeks, and has a distinct acute hyperinflammatory phase [15–17]. The high and imminent mortality means issues such as eventual chronic fibrosis development may be secondary considerations compared to immediate restoration of lung function, specifically adequate blood oxygenation. Broad-spectrum antibiotics, given as early as possible where infection is known or suspected to be present, as is support of gas exchange, usually via assisted ventilation or in more severe cases, if available, extra-corporeal membrane oxygenation (ECMO) support [18]. The core treatment of ARDS is based on supportive measures that primarily aim at gaining time to allow the antibiotic treatment or the patient immunologic system to defeat the primary cause of ARDS. Mechanical ventilation strategy includes: the use of low tidal volume ventilation, inspiratory pressure, higher positive end expiratory pressure and using prone positioning, and administering neuromuscular blockers in higher severity ARDS [19–21]. Current guidelines supporting protective mechanical ventilation is aimed at preventing the risk of ventilator-induced lung injury [22] in patients with ARDS. The pathophysiologic reason behind this is based on the concept of the “baby lung” [23], which has reduced compliance caused by the severe decrease of lung aerated volumes, and is defined based on CT scans over the course of initial ARDS diagnosis (0–7 days) and the subsequent fibroproliferative response (15–20 days) [24, 25].

It might also be argued that despite the era of protective mechanical ventilation [26], large tidal volumes are still fundamental for mechanical ventilation daily management [13], and adjunctive measures such as proning [27] and neuromuscular blockers [28], while proven to have a positive effect on outcome, have not been fully implemented yet [13].

Furthermore, ARDS mortality remains high, despite considerable advances in terms of antibiotic stewardship and antibiotic treatment options [29–32] and fluid management [31, 33] to face up the two main risk factors leading to ARDS, pneumonia, and sepsis [13].

The multimodal nature of ARDS also necessitates a multimodal approach to treatment, which cannot be met with traditional small molecule or recombinant protein medicines. A wide variety of anti-inflammatory pharmacologicals, proteins, and antibodies have demonstrated promise in the laboratory but have failed in clinical trial (reviewed in [34]). Of relevance here is the fact that injury and repair responses are intimately linked phenomena at the cell signaling and transcriptional level, and blanket inhibition of inflammation may delay or even prevent essential regenerative processes that restore lung tissue to normal function [35–37]. An idealistic treatment of ARDS should target multiple mechanisms and biologic pathways instead of aiming at a single exclusive target. This hypothesis is supported by: (1) the heterogeneity of the mechanisms involved in the lung injury, (2) decades of negative randomized clinical trials

with pharmacologic and other therapies and (3) the new upcoming evidences about the role of biologically distinct pathways and response to treatment in specific subsets of ARDS patients – as recently observed by the identification of specific endotypes and phenotypes [38–40].

In light of these considerations, cell-based therapies with mesenchymal stem/stromal cells (MSCs) have been proposed as novel therapeutics in the treatment of ARDS, due to their broad immunomodulatory effect during inflammation, their enhancement of host defense through antimicrobial mechanisms, and their lung healing potential through the activation of repairing mechanisms [41].

12.4 Epidemiology

ARDS occurrence remains as high as 10.4% of all ICU admissions, rising up to 23.4% of mechanically ventilated patients. ARDS still appears to be an under-recognized syndrome, with up to 40% as recently reported in the Large observational study to Understand the Global impact of Severe Acute respiratory FailurE (LUNG SAFE) study [13]. Furthermore, ARDS mortality is still estimated as high as 40%.

12.5 Pathology

The pathophysiologic hallmark of ARDS is the well-known diffuse alveolar damage (DAD), which leads to the characteristic protein-rich non-hydrostatic pulmonary edema during ARDS [33, 42–44]. Considering that a match among clinical signs and pathology findings is far from being perfect, it is relevant that DAD is highly associated to the category of nonresolving ARDS [45] and that DAD predicts higher mortality in ARDS compared to patients with non-DAD ARDS [46]. Excess alveolar fluid blocks gas exchange at the alveolar epithelial surface, while surfactant inactivation by infiltrating albumin and other substances [47–49] damages fluid tension leading to alveolar collapse. Even for patients undergoing ventilation, eventually ventilation/perfusion (V/Q) mismatch occurs where oxygen delivered to the pulmonary space fails to reach the bloodstream and there is impairment of CO_2 clearance, leading to systemic hypoxemia and hypercapnia.

At the cellular and molecular level, the initial phase of ARDS arising from pulmonary infection involves resident pulmonary macrophage defenses being overcome, and the epithelial cell layer lining the alveolus begins to produce proinflammatory cytokines [50]. These signals initiate the recruitment of circulating leukocytes, including neutrophils, macrophages, and B-cells, to migrate up a chemokine concentration gradient to the lung tissue, where these cells release matrix metalloproteinases that enzymatically digest connective tissue facilitating entry to the airspace [51]. Recruited infiltrating cells produce a wide range of noxious substances including superoxide radicals [52–58], leukotrienes [59–64], and antimicrobial peptides [65–67] and further cytokine cocktails in an attempt to destroy the infectious agents. In ARDS, a section of these substances is uncontrolled and induce damage to the lung tissue itself leading to fluid buildup and surfactant loss. Finally as the alveoli fill with liquid and the integrity of the epithelial and endothelial barrier is compromised, gas exchange deteriorates and hypoxemia and hypercapnia result. In later phases of ARDS during systemic inflammatory response syndrome (SIRS), patients may suffer from functional immune-suppression and have heightened susceptibility to additional infection [68–70]. While ARDS presents an acute onset after the exposure to the causing factor, its evolving process starts with acute exudation and infiltrates of acute inflammatory cells into the alveoli within 1 week. During the second week a subacute deposition of collagen fibers are produced by fibroblasts. The syndrome typically resolves through a chronic stage characterized by alveolar macrophage infiltration into the alveoli and a fibrotic repairing process of the lung parenchyma [11].

12.6 MSCs for ARDS: A Promising Potential Therapy

MSCs have generated interest for a wide range of regenerative medicine applications and appear to

have broad immunomodulatory properties rendering them attractive for autoimmune and other inflammatory disorders. Initial safety has been amply demonstrated [71, 72], although some questions remain over hypersensitivity to repeat dosing in non-ARDS models which will only be answered over time [73, 74]. As further conditions are likely to have MSCs licenses as a medicinal therapeutic, this will be of added relevance where MSC therapy may be employed in the same patient for sequential separate disease instances.

MSCs accumulate in the lungs initially after IV administration [75–77] and can remain viable there for up to 24 h [78] after which this time MSCs disappear, suggesting any therapeutic effect has already been conferred to the host; however, it is not clear what happens to MSCSs after they have left the lungs. Interestingly, recent stem cell therapy research in other diseases has indicated the lung is crucial to licensing of MSC to ultimately allow their beneficial effects [79–81], suggesting an immunomodulatory effect that outlasts the MSC's presence in the body.

The MSC's responsiveness to the injury milieu [82–85] and diverse range of effects on multiple pathological and repair processes has made them, theoretically, an ideal candidate for ARDS interventional studies [86] (Fig. 12.1). Indeed, many of the leukocyte subpopulations involved in ARDS pathology have been shown to have direct interaction with MSCs [87–89], while direct antibacterial activity [65] is to be considered an added bonus. Also, the fact that most patients with ARDS require tracheal intubation to permit support of lung function opens up the possibility of direct delivery to the lung airspace of MSC or MSC derivatives [90], although since patients are also almost certain to have IV access obtained and there have been, to date, no demonstrated efficacy advantages observed in delivery of MSC intratracheally over IV [91] in ARDS models, this remains a point of urgent future investigation.

Inviting as MSC therapy may appear, the timecourse and whole body nature of ARDS demand a specific set of considerations around preparation, storage, and administration to be resolved before deployment of MSCs to the clinic. Translation of experimental findings from animal models to the patient are also problematic, with uncertainty regarding dose scaling and testing of the human MSCs destined for patients in nonhuman models, where DNA/RNA sequence and protein/ligand binding incompatibilities warrant extra attention.

12.7 Ex Vivo Human Lung Models

In a first report in 2009, Lee et al. explored the potential role of allogenic human MSCs in the treatment of ARDS induced by *E. coli* endotoxin in an ex vivo perfused human lung [92]. The authors administered allogeneic human MSCs or MSCs-derived conditioned medium at 1 h after the injury induction. Fluid balance was normalized by the decrease of the extravascular lung water, restoring the alveolar fluid clearance (AFC) and by improvement of lung endothelial barrier permeability. The alveolar epithelial fluid transport was in part coordinated by the keratinocyte growth factor (KGF), secreted by the MSCs, which restored the correct function of the amiloride-dependent sodium transport.

Some years later, it was observed that clinical-grade MSCs, administered via the lung perfusate or directly into the right middle lobe, could decrease neutrophil influx and inflammation, effectively cleared bacteria, confirming the contributing role of the KGF, and restored the clearance of the alveolar fluid, with a relevant improvement of the lung histology [93]. In 2014, it was observed in a follow-on study that intravenous administration of clinical-grade allogenic human MSCs could increase the AFC at 4 h. The role of KGF in the AFC was confirmed by the study of a neutralizing antibody of KGF that could decrease the AFC activity [94]. Recently, the same group explored the effects of microvesicles (MVs) released by human mesenchymal stem cells in their established ex vivo human lung perfusion model of bacterial pneumonia. The investigators reported positive results highlighting the beneficial effects of MSC MVs in increasing lung antibody forming cells, in decreasing the lung permeability, and improving the bacterial clearance, particularly when MSCs were pre-

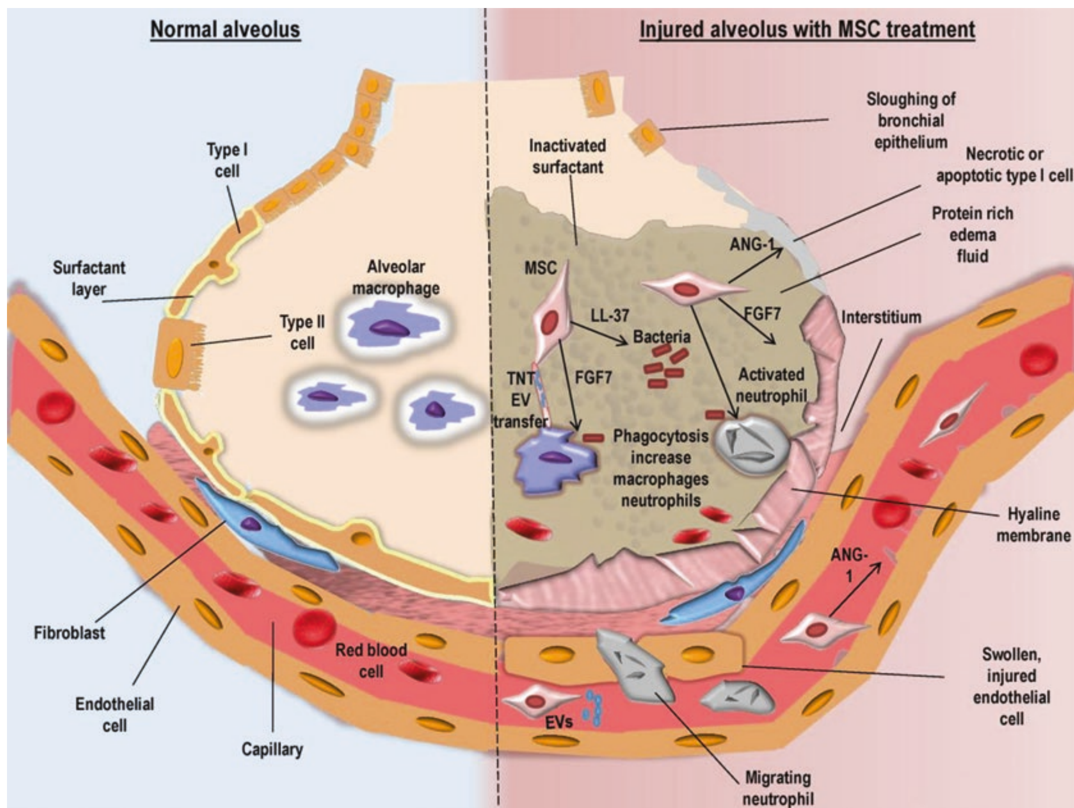

Fig. 12.1 Possible mechanisms of action of the MSC in the ARDS alveolus and surrounding vasculature. Left side: healthy alveolus. Right side: during injury, protein-rich edema fluid and inflammatory cells permeate the alveolus. MSCs have been demonstrated to alleviate the pathophysiological symptoms of ARDS through the secretion of paracrine factors, cell-to-cell contact and mitochondrial transfer (TNT and EV release). Indirect and direct contact of MSCs has been shown to reduce the permeability of alveolar epithelium and increase fluid clearance. Through the secretion of ANG-1, endothelial and epithelial repair is increased. Reduction of neutrophil migration also improves endothelial and epithelial barriers. Release of KGF promotes an increase in alveolar fluid clearance. Bacteria clearance is achieved through the direct release of the antimicrobial peptide LL-37 or through increased phagocytosis by neutrophils and macrophages mediated by the release of cytokines including FGF-7 or by transfer of micro vesicles through TNTs. Mitochondrial transfer to epithelial cells also increases surfactant release. A few mechanisms of action of MSCS in ARDS have been displayed in this diagram. *EVs* extracellular vesicles, *TNT* Tunnelling Nanotubules, *ANG-1* angiopoietin-1, *PGE2* prostaglandin E2, *KGF* keratinocyte growth factor, *LL-37* peptide β-cathelicidin

treated before isolation of MVs with a Toll-like receptor 3 agonist, polyinosinic:polycytidylic acid (Poly(I:C)) [95].

12.8 Mechanistic Considerations for Clinical Therapy

Allogenic MSCs have the ability to avoid detection of the immune system and it is assumed that this evasion is due to the low expression of the major histocompatibility complexes (MHC) I and II, while MSCs also do not express CD80 and CD86 which are identified T-cell stimulators [96]. Therefore autologous MSC administration is considered a viable therapeutic option as the likelihood of an immune response is extremely low.

The routes of administration will influence the MSCs ability to differentiate, their immunogenic

effect, and ultimately their survival [97]. Some studies have contradicted the MSC's proposed ability to evade immunological detection. MHC II protein expression analysis on MSCs has been shown to be higher than originally documented [98–100]. In vivo studies have also shown that allogenic MSCs are not immune privileged and have the potential to cause an immune response, while other research has contradicted findings and stated MSCs are immune privileged [101–104].

MSC efficacy has been demonstrated in multiple preclinical models of ARDS [90, 93, 94, 105–111], while MSC products including conditioned medium and extracellular vesicles have also shown promise [91, 94, 107, 112–117]. These are interesting in that they avoid safety and cryostorage issues associated with whole cell delivery and may be more compatible with direct delivery to the airspace by nebulizer. MSCs have been shown to reduce inflammation and improve bacterial clearance [107] through direct antimicrobial peptide release such as LL-37 and indirectly through the modulation of phagocytic activity in BAL monocytes [108] as well as alveolar resident monocytes specifically [93].

More recently, MSCs have been documented to protect from injury via direct interaction through tunneling nanotubules (TNT) or formation of connexin 43 gap junctions [118–125]. It has been demonstrated that mitochondrial transfer from MSCs to alveolar epithelial cells improved survival after endotoxin injury [118], while in a rat model of COPD, iPS-derived MSC mitochondrial transfer to bronchial epithelial cells was also observed [126]. MSCs have been shown to transfer mitochondria to macrophages in vitro and in vivo, improving macrophage function and enhancing phagocytosis [127]. In a mouse model of *E. coli*-induced pneumonia it was reported that therapeutic effect was dependent on transfer of MSC mitochondria to alveolar macrophages through TNT, enhancing antimicrobial activity and phagocytosis [128]. While it is unclear whether the mechanism of action in vivo is due to an enhancement of a normal mitochondrial function or restoration of dysfunctional mitochondria, there is some evidence for damaged mitochondrion, e.g., downregulated NDUFB8 (complex I) and ATP synthase (complex V), in ARDS meaning the latter is a distinct possibility [129, 130].

12.9 Production Considerations for Clinical Cell Therapies

For ARDS, autologous stem cell therapy is not an option as there is insufficient time to isolate and expand patient MSCs while the rapid onset nature of ARDS demands a cryopreserved MSC product. Cryoprotective agents are used to protect the cellular components from crystal formation and osmotic shock and membrane damage during the slow freezing process, preserving the fine structures of cells [131]. For clinical applications, MSCs are typically frozen to at least −150 °C at a controlled rate of 1–5 °C per minute in 5% or 10% dimethyl sulfoxide in an electrolyte solution and added protein, typically human serum albumin [132]. Despite extensive optimization, the process can cause damage and affect cell viability [133–135] and inadequate insight into how MSCs function after systemic infusion remains an issue [134, 136–139].

Freeze-thawed MSCs, in comparison to cells harvested from continuous cultures, have diminished immunomodulatory properties as well as a reduced responsiveness to proinflammatory cytokines [140]. The immunomodulatory effects of MSCs is affected by cryopreservation, launching a heat shock protein response [141]. In vivo experiments have shown that cryopreserved are less well tolerated. In a clinical application where predominant indications included graft versus host disease (GvHD) and tissue injury in hemorrhagic cystitis, therapeutic properties of freeze-thawed and freshly harvested MSCs were compared. A 100% response rate was observed in patients treated with fresh cells at a low passage compared to patients treated with cryopreserved freeze-thawed cells at a higher passage, with cryopreserved MSCs eliminated faster by compliment after exposure to recipient blood [140]. The thawing process can damage cell surface proteins and this abnormality attracts the binding of complement initiating clearance by phagocy-

tosis [142–144]. After complement exposure, there is an 80% decrease in cell viability in cryopreserved cells compared to a 50% decrease in fresh MSCs [141, 145]. However activation of complement may not be negative as recognition of opsonized MSCs are hypothesized to induce an M2 phenotype, producing anti-inflammatory mediators [140]. Macrophages can display various phenotypes, with the two being described as M1 and M2. M1 phenotypes are generated by the classical pathway [146, 147] and produce abundant inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-12, and reactive oxygen species. M2 phenotypes are generally activated by the alternative pathway and express a variety of lectins, protein, and scavenger receptors [146–148].

While various research have demonstrated that umbilical cord (UC) [149–152] and adipose tissue (AT)-derived [153] MSCs have a faster population doubling time than bone marrow (BM) sourced, attaining human-sized doses of the order of 10^9 cells is still a daunting task. Additionally, there is currently no data published on whether there is an upper limit on MSC population doubling that still retains therapeutic efficacy. Kern et al. compared the senescence ratio of AT-MSC to BM-MSC and found that BM-MSC had a growth threshold of passage 7, whereas AT-MSC had a threshold of 8 [154], but efficacy itself may be lost long before senescence arises. In addition, MSCs isolated from patients with advanced age [155, 156], diabetes [157], rheumatoid arthritis [158], or indeed ARDS itself [159] have decreased activity, including lower regenerative and differential potential and therefore autologous MSC therapy in patients with significant chronic comorbidities may not be a promising approach in any case. Downregulation of inflammatory marker receptors may render MSCs isolated from such patients less responsive to the injury microenvironment and hence of lower overall therapeutic value [146, 147, 159].

Beyond the conventional MSC therapeutics, human embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) as new cell types have also been investigated in immunoregulation and have shown encouraging results [160–163]. iPSCs are immunomodulatory in a mouse model of allergic inflammation [164], while their systemic administration inhibited serum levels of IgE and TH2 cytokines (IL-4, IL-5 or IL-13) with better survival and engraftment rate after transplantation compared to adult tissue derived MSCs [163, 165]. There are also, variations in age related to DNA methylation levels, which is correlated to differential abilities, with ESC-derived iPSC having a higher proliferation and regenerative capacity [166, 167]. However, caution is still required as genetic abnormalities remain an area of concern in iPSCs [168]. Clinical studies using iPSCs found some cells in the study contained genetic abnormalities, and were consequently not used. Cells taken from elderly patients to be reprogrammed for administration can come with increased risks of genetic abnormalities [168], demonstrating the need for screening of cells before infusion if autologous cells are ever to be used in elderly patients.

12.10 Clinical Trials—Demonstrating Safety in ARDS

Clinical trials utilizing MSCs for ARDS patients are in phase 1-2 studies and focused on safety, tolerability and feasibility concerns. We are far from being able to claim MSC therapy is a viable option for ARDS. However, during the last decade, promising preclinical evidence supports the hypothesis of a potential benefit in treating ARDS patients with MSCs [169]. Despite the many studies into the potential clinical benefit of MSCs in ARDS that have been proposed over the last years and posted on clinicaltrials.gov, most of them are still currently ongoing or they lack a status update. Furthermore, few studies have disclosed the initial safety results (Table 12.1). The first clinical trial where MSCs were used to treat ARDS was reported in 2014 (NCT01902082), where Zheng and coworkers administered 1×10^6 AT-MSCs/kg of body weight or saline in a 1:1 fashion in 12 patients with moderate and severe ARDS. The investigators reported that the administration of allogeneic MSCs was feasible and

Table 12.1 List of clinical trials reported on clinicaltrials.gov about mesenchymal stem/stromal cells in ARDS—current status and key findings

Study	NCT	Reference/url	Design	MSC type	Dose	Population	Status	Findings
Mesenchymal stem cells (MSCs) for treatment of acute respiratory distress syndrome (ARD) in patients with malignancies	02804945	https://clinicaltrials.gov/ct2/show/results/NCT02804945	Phase 2—open label	Allogeneic human Mesenchymal stem cells (hMSCs)	3×10^6 cells/kg one time on day 1 over about 1–2 h	ARDS $PaO_2/FiO_2 \leq 200$	Recruiting	In progress
Adipose-derived Mesenchymal stem cells in acute respiratory distress syndrome	01902082	https://clinicaltrials.gov/ct2/show/NCT01902082	Phase 1—"Triple blinded" (participant, care provider, investigator)	Allogeneic adipose-derived hMSCs	1×10^6 cells/kg body weight within 48 h of enrollment	ARDS $PaO_2/FiO_2 < 200$	Unknown	Published: no infusion toxicities or serious adverse events related to MSCs administration
Human umbilical cord Mesenchymal stem cells (MSCs) therapy in ARDS (ARDS)	03608592	https://clinicaltrials.gov/ct2/show/NCT03608592	Early phase 1	Umbilical cord derived mesenchymal stem cells (UCMSCs)	60×10^6 cells suspended in 100 ml normal saline after randomization in 30–60 min	ARDS $PaO_2/FiO_2 < 200$	Not yet recruiting	In progress
Human Mesenchymal stem cells for acute respiratory distress syndrome	01775774	https://clinicaltrials.gov/ct2/show/NCT01775774	Phase 1—open label	Allogeneic Bone marrow-derived hMSCs	3 cohorts with 3 subjects/cohort who receive doses of 1, 5 and 10×10^6 cells/kg predicted body weight (PBW)	ARDS $PaO_2/FiO_2 < 200$	Completed	Published: No prespecified infusion-associated events or treatment-related adverse events
Human mesenchymal stem cells for acute respiratory distress syndrome (START)	02097641	https://clinicaltrials.gov/ct2/show/NCT02097641	Phase 2—Triple masking (participant, care provider, investigator)	Allogeneic Bone marrow-derived hMSCs	A single dose of 10×10^6 cells/kg PBW over approximately 60–80 min	ARDS $PaO_2/FiO_2 < 200$	Completed	Published: No predefined MSC-related

Mesenchymal stem cells for multiple organ Failure after cardiac surgery	03552848	https://clinicaltrials.gov/ct2/show/NCT03552848	Pilot—open label	Mesenchymal stem (stromal) cells (MSC)	1×10^6 cells/kg of body weight intravenously once every 4 days for four times	Failure >2 organs and: (1). In case of ventilation $PaO_2/FiO_2 < 100$,100% oxygen demand (2). Sequential organ failure assessment (SOFA) ≥ 10, multiple organ dysfunction (MOD) ≥ 10	Not yet recruiting	In progress
Mesenchymal stem cell in patients with acute severe respiratory failure (STELLAR)	02112500	https://clinicaltrials.gov/ct2/show/NCT02112500	Phase 2—open label	Bone marrow derived mesenchymal stem cells	Not reported	ARDS $PaO_2/FiO_2 < 200$ Not clear if AHRF or ARDS?	Unknown	
Repair of acute respiratory distress syndrome by stromal cell administration (REALIST)	03042143	https://clinicaltrials.gov/ct2/show/NCT03042143	Phase 1 dose escalation pilot study /phase 2—Quadruple masking (participant, care provider, investigator, outcomes assessor)	Orbcel-C (human umbilical cord derived CD362 enriched MSCs)	Dose not specified - maximum tolerated dose from the phase 1—Single infusion	ARDS $PaO_2/FiO_2 \leq 27$ kPa	Recruiting	In progress
Human umbilical-cord-derived mesenchymal stem cell therapy in acute lung injury (UCMSC-ALI)	02444455	https://clinicaltrials.gov/ct2/show/NCT02444455	Phase 1/2—open label	Umbilical-cord-derived mesenchymal stem cell (UC-MSC)	5×10^5/kg once a day, a total of three times—maximum tolerated dosage without side effects	ARDS oxygenation index:$200 < PaO_2/FiO_2 \leq 300$ mmHg	Unknown	
Using human menstrual blood cells to treat acute lung injury caused by H7N9 Bird Flu virus Infection	02095444	https://clinicaltrials.gov/ct2/show/NCT02095444	Phase 1/2—open label	Menstrual blood stem cells	$1–10 \times 10^7$ cells/kg, two times a week, 2 weeks for infusion	H7N9 infection and critical lung tissue injury	Unknown	

(continued)

Table 12.1 (continued)

Study	NCT	Reference/url	Design	MSC type	Dose	Population	Status	Findings
A phase 1/2 study to assess multi stem therapy in acute respiratory distress syndrome (MUST-ARDS)	02611609	https://clinicaltrials.gov/ct2/show/NCT02611609	Phase 1/2—Quadruple masking (participant, care provider, investigator, outcomes assessor)	MultiStem	Low versus high multistem dose	Moderate to severe ARDS	Recruiting	Active, not recruiting
Human umbilical-cord-derived mesenchymal stem cell therapy in paraquat poisoning induced lung injury (UCMSC-PQLI)	02444858	https://clinicaltrials.gov/ct2/show/NCT02444858	Phase 1/2—single masking (participant)	Umbilical-cord-derived mesenchymal stem cell (UC-MSC)	5×10^5 cells/kg, once a day, a total of three times	Paraquat-induced lung injury	Unknown	
Treatment of severe acute respiratory distress syndrome with Allogeneic Bone marrow-derived mesenchymal stromal cells	02215811	https://clinicaltrials.gov/ct2/show/NCT02215811	Phase 1—open label	Allogenic bone marrow-derived mesenchymal stromal cells (BM-MSC)	Not reported	ECMO patients with viral-induced ARDS	Unknown	
A pilot study using placenta derived Decidual stromal cells for toxicity and inflammation with special focus to the allogeneic hematopoietic cell transplantation setting	02175303	https://clinicaltrials.gov/ct2/show/NCT02175303	Phase 1/2—open label	Decidual stromal cell therapy	1×10^6 cells/kg, at one or more occasions at weekly intervals dependent on clinical response	Unclear criteria including: — acute lung injury — Decidual stromal cells–stem cell transplantation —inflammation	Unknown	

safe with no infusion toxicities or serious adverse events related to MSCs in the treatment group and with no differences in terms of adverse events and biomarkers of lung injury between the MSCs and the placebo group [170].

In 2015, Wilson J.G. and coworkers tested the safety of BM-MSCs in a multicenter phase 1b dose-escalation study in patients with moderate-severe ARDS, with PaO_2/FiO_2 less than 200 mm Hg, a positive end-expiratory pressure (PEEP) ≥ 8 cmH_2O, and bilateral infiltrates at the frontal chest X-ray (NCT01775774). Nine patients were enrolled and three groups of three received three doses of MSCs intravenously (1, 5, or 10×10^6 MSC/kg ideal body weight). The investigators reported no significant difference in biomarkers of inflammation (IL-6, IL-8), lung epithelial (receptor for advanced glycation end products—RAGE) and endothelial injury (Ang-PT2) among the groups. MSC administration was safe and the authors reported neither infusion-associated events nor serious adverse events. The viability of the MSCs infused ranged from 50–63% [171].

The same investigators recently reported the findings of a double-blind multicenter randomized phase 2a clinical trial testing the safety of BM-MSCs versus placebo in ventilated patients with moderate-severe ARDS, with $PaO_2/FiO_2 < 27$ kPa and PEEP ≥8 cmH_2O. Patients randomly received in a 2:1 fashion 1×10^6 BM-MSCs/kg ideal body weight or placebo. The primary objective of this investigation was the safety of MSCs in an intention to treat analysis (NCT02097641). No patient in the MSCs treatment group experienced any adverse respiratory or hemodynamic events. The treatment group had higher APACHE III score, minute ventilation, and PEEP compared to placebo. No statistically significant 28-day mortality difference was observed among treatment and placebo group, even after adjustment with the APACHE III score, while a trend in a lower number of ICU-free days to day 28 was reported in the treatment group compared to placebo. Of importance, there was higher absolute 28 day and 60 day mortality in the MSC group, although it is unclear at this point if there is any clinical significance to this result or if it was related to variability in MSC viability on administration or some other quality issue.

Furthermore, a higher severity of illness at baseline—quantified by the SOFA and APACHE III scores—was present in the MSC group compared to the placebo group and mortality in the MSC group and in the placebo group was lower and higher than anticipated, respectively.

However, this RCT was not powered for efficacy, as per the Food and Drug Administration mandate to clearly demonstrate safety before targeting lung oxygenation or compliance as in a phase 2b trial. The viability of the MSCs infused ranged from 36–85% [71].

The range in MSC viability was unanticipated and only discovered after study completion. The authors reported a significantly higher MSCs viability after centrifugation when MSCs were thawed compared to when the cells were washed to remove dimethyl sulfoxide during preparation. Based on these findings the investigators conducted a post-hoc analysis and observed that plasma angiopoietin-2 levels in the intermediate and highest tertiles of MSCs viability were significantly lower in the MSCs treatment group at 6 h after administration compared to placebo, and albeit nonsignificantly, the oxygenation improved at day 2.

These results suggest that the administration of MSCs with a high viability is required to target an improvement in efficacy. Recent experimental data on the comparison of different cell products reports that fresh BM-MSCs are 14% more viable compared to cryopreserved ones [172]. Furthermore, delivery of MSC immediately upon thawing instead of thawing and washing the MSCs could enhance MSC viability, as observed by Matthay MA et al [71]. This is an unusual finding, as washing of MSC in physiological buffer has not been considered traditionally to have any impact on viability, and warrants further investigation.

Simonson and colleagues reported data on the clinical outcomes of two patients with severe ARDS who received allogenic BM-MSCs. MSC administration was safe and no adverse events were reported during infusion. The investigators reported a decrease of plasma and BAL proinflammatory cytokines, chemokines, miRNAs,

and biomarkers of epithelial apoptosis and alveolar-capillary fluid leakage. One patient developed pneumonia 5 days after cell administration, which resolved after antibiotic therapy and the patient was subsequently extubated 4 weeks after MSCs administration. The second patient was extubated 12 days later [92].

Very recently, Athersys disclosed in a press release the positive results for the MUST-ARDS study about MultiStem® Cell Therapy in patients with moderate-severe ARDS (NCT02611609). After an initial dose confirmation phase ($n = 6$), Athersys confirmed the tolerability and the safety profile of the MultiStem® treatment ($n = 20$) with no adverse events during administration, and lower levels of inflammatory biomarkers compared to the control group ($n = 10$). Furthermore, despite the study was not powered for efficacy outcomes, MultiStem® cell therapy was associated with better short term prognosis, as shown by a lower mortality rate (25% versus 40%), higher ventilator-free (12.9 versus 9.2) and ICU-free days (10.3 versus 8.1) compared to control. Further findings will be unveiled at end of the collection of the 1-year follow-up data, as aimed according to the study design. (http://www.athersys.com/news-releases/news-release-details/athersys-announces-positive-results-its-exploratory-clinical).

All the studies currently ongoing in the field of MSCs and ARDS are safety studies (Phase 1, 2, 1/2) (Table 12.1). At the moment there are still additional issues that need to be overcome: (1) improvement of MSCs bioavailability by the optimization of the cell preparation and storage [71]; (2) the modulation of the microenvironment [173]; (3) the characterization of the specific phenotypes/endotypes of ARDS potentially more suitable to respond to cell therapy [39, 40]. This might enhance the likelihood of success in subsequent efficacy (Phase 3) studies.

12.11 Future Directions

12.11.1 Patient Stratification

ARDS is classified by the Berlin definition into different severity categories, according to the degree of hypoxemia, and each associated with increasing mortality rates. However, other evidence suggests that: (1) either the etiology (i.e. pulmonary versus extrapulmonary ARDS) [174–177], or (2) the macroscopic ARDS presentation at radiological imaging [178] or (3) the levels of different inflammatory biomarkers contributing to different biological patterns of ARDS might play a key role in stratifying the outcome of this syndrome [179]. Pulmonary ARDS was associated with longer total ventilation time and longer ICU stay compared to extrapulmonary ARDS [180]. ARDS patients with a higher epithelial injury, as observed by higher levels of soluble form of the receptor for advanced glycation end product (sRAGE), showed a specific nonfocal CT lung pattern, which was associated with higher mortality compared to the focal pattern [178].

The ARDS Network proposed a novel classification of ARDS with two distinct subphenotypes, which included different clinical and laboratory characteristics [39]. Interestingly, in a secondary analysis of the ARMA [26, 181, 182] and the ALVEOLI trials [183], the investigators could identify a specific pattern of ARDS that the investigators named hyperinflammatory subphenotype, phenotype 2. Phenotype 2 showed higher plasma concentrations of inflammatory biomarkers greater prevalence of vasopressor use and lower serum bicarbonate concentrations than phenotype 1. The hyperinflammatory subphenotype could differentiate a subgroup of patients with a higher mortality rate.

In light of the heterogeneity of ARDS, attempts have been made to optimize treatment regimens [173], and stratification parameters are emerging among recipients of MSC therapy which may be of relevance to ARDS patients [184].

12.11.2 Large-Scale Cell Manufacture

As detailed earlier, generation of human-sized doses of GMP quality MSCs, for the numbers of patients needed for large-scale clinical trials (and subsequent clinical therapy), is not a trivial undertaking. Preclinical studies typically use between 1×10^6 and 10×10^6 MSCs per kg of

bodyweight or ideal bodyweight of the patient, and clinical trials have been designed with these doses in mind. In light of the observed relatively low cell viability in ongoing trials, production of the order of 10^9 MSC may be required to reach the upper doses when allowance for dead cells is calculated. Coupled with the lack of information with regard to passage or population doubling at which efficacy is lost, this will likely necessitate pooled donor batches to enter the MSC isolation and production process. Considerable work is being concentrated on this area both academically and industrially to optimize and automate, including the utilization of xeno-free media that allays fears of contamination with viruses or other as yet unknown contaminating factors.

The preponderance of preclinical work with MSCs has involved freshly harvested MSCs and this will remain an impractical and unlikely therapeutic for the clinic. Despite the development of rapid shipping solutions from manufacturing facility to clinical site, and research into supportive media to extend the MSCs' effective lifespan in suspension prior to administration, it is probable that a cryopreserved MSC will become the choice in the long term. Cryopreservation at the clinical site or expedited transport from manufacturing facilities will be required, but it remains to be determined what further equipment such as centrifuges and viability assessment assays will be needed to prepare the MSC dose and allow quality control prior to administration to the patient.

12.11.3 Lack of Clearly Defined Mechanism of Action

Despite a myriad of possible effector mechanisms by which the MSC may alleviate ARDS severity, including secreted antimicrobials, cytokines, extracellular vesicles, and other factors, and the observed influence MSCs have on leukocytes, it has remained difficult to ascertain which of these mechanisms are of importance to the MSCs' efficacy. Indeed several studies that have sought to replicate the various mechanisms proposed through administration of MSC-produced factors have failed or not reproduced the efficacy of the MSC itself, indicating critical gaps in our knowledge of MSC action in ARDS and suggesting interpretation of unsuccessful or even successful follow on clinical trial will be difficult. If the mechanism remains unknown, then interactions with other drugs or comorbidities will always be unpredictable.

12.11.4 Lack of MSC Potency Assay

Related to both production and mechanism, a critical limiting factor in successful deployment of MSC therapy to the clinic is the lack of defined assays to accurately predict MSC potency in the ARDS patient. Many cell manufacturers and research groups have proposed small, easily quantified molecules such as aldehyde dehydrogenase (ALDH) [185] or indoleamine 2,3-dioxygenase (IDO) [186], which correlated well with in vitro tests such as T-cell expansion inhibition or in vivo tests in ARDS animal models. However, as the MSC's mechanism of action in ARDS remain unclear, these factors can only be considered correlative and not conclusive proof of likely efficacy in the human patient.

12.11.5 Beyond the MSC?

Determining the mechanism(s) of action of the MSC specifically, however, will lead to us a question: do we need the cell at all? A suite of effectors produced by MSC cultures, or indeed by similar cell types engineered to replicate or improve upon the MSC secretome while being more open to manipulation and expansion, could replace cell therapy entirely. Also, as alluded to already, these factors will be likely easier to analyze, store, and deliver than the MSC they are derived from. Presuming cell-contact dependent mechanisms such as TNTs are the sole means underlying the MSC's efficacy in ARDS, we may ultimately see an MSC product cocktail available in stable, off-the-shelf format that can be delivered IV or intratracheally by nebulizer that will reproduce the efficacy initially demonstrated with the IV-delivered cryopreserved whole cell.

12.12 Conclusions

ARDS has been a stubbornly challenging syndrome to address clinically for decades. Despite gradual improvement in supportive care for the patient, specific therapies have proven elusive. The MSC is an exciting prospect, as it is a real paradigm shift from traditional approaches, due to its ability to respond to the level and nature of injury, having both direct and immunomodulatory properties, and a multimodal mechanism of action that targets multiple pathologies seen in the ARDS patient. Issues around dosing, MSC production, and potency reproducibility remain but are being addressed. We look forward to the conclusion of the many current and planned clinical trials to determine the true therapeutic potential of MSCs for those suffering from this devastating disease.

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Part III

Stem Cell Delivery Systems and Devices

Stem Cell Delivery Systems and Devices - Spraying

13

Sally Yunsun Kim and Wojciech Chrzanowski

13.1 Introduction

For clinical translation of stem cell therapy, several challenges need to be addressed including the optimal source of cells, cell dose, dosing intervals and route of administration [1]. The most commonly investigated delivery route is intravenous for the ease of administration in pre-clinical studies; however, the optimal route of cell delivery should be specific for disease and patient circumstances. It has long been established that the most direct route of delivering therapeutics to the lung is through the trachea [2–4]. There is growing evidence that intratracheal administration of stem cells positively influences lung repair processes in a number of lung injury and repair models [3, 5, 6]. Lung injury models used for intratracheal administration of stem cells include acute lung injuries such as naphthalene-induced injury [7, 8], acute respiratory infection [9], as well as for other models such as asthma [10–13], emphysema [14–16], ventilator induced lung injury [17, 18], pulmonary hypertension [19, 20], bronchopulmonary dysplasia [21–23] and bleomycin-induced pulmonary fibrosis [24, 25]. Therapeutic benefits of stem cells delivered via the airway have also been investigated for neonatal lung injury models [22].

Direct intratracheal delivery of cells to the lung offers obvious benefits over the indirect intravenous route. Intratracheal delivery of cells directly to the airway yields significantly greater numbers of cells reaching the lungs compared to the intravenous delivery route [7]. A comparison of transtracheal delivery and intravenous delivery of bone marrow-derived epithelial-like cells in a mouse model of naphthalene injury found that approximately 10% airway progenitor cells were detected in the lung after transtracheal delivery compared to about 1% after intravenous delivery [26]. Intravenously delivered cells are also found in other organs such as liver, spleen and bone marrow, while no cells were observed in these sites following transtracheal delivery [26–28]. In the case of therapeutic drugs, direct delivery to the lung via intratracheal administration is known to be superior to indirect intravenous delivery because the drug reaches the target organ efficiently without entering the systemic circulation [29, 30]. Even in the case of drugs

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with low bioavailability and limited metabolism, such as curcumin, direct drug administration to the lungs of rats by inhalation had high therapeutic efficiency in the treatment of lung cancer [31].

Although administered stem cells have low levels of cell engraftment or retention in the lung after delivery of cells, therapeutic benefits of stem cells are more likely due to paracrine mechanisms [14, 32–38]. While tissue regeneration may occur through the incorporation of cells into the damaged tissue and subsequent cell differentiation and extracellular matrix protein production for replacement of structure and function [39], a combination of complex paracrine mechanisms are involved. Mesenchymal stromal cells (MSCs) administered intratracheally to elastase-injured mice reduced the development of elastase-induced emphysema, which was attributed to the paracrine effects of the MSCs [14]. In support of the paracrine effect, intratracheal delivery of MSC-conditioned medium enhanced lung repair and attenuated inflammation following ventilator-induced lung injury [17].

While the traditional instillation of cells to the airway is a common method of intratracheal cell delivery in animal models, cells could instead be administered by spraying to yield a more uniform distribution of cells across the whole lung (Fig. 13.1). Despite potential benefits of aerosolised cell delivery for direct therapeutic action in the lung, there are limited number of studies that compare the benefits of traditional instillation versus aerosolisation. Studies that investigated the possibility to spray or aerosolise cells confirmed that this route of administration is feasible for delivering stem cells with high viability [40–42]. There are numerous parameters that need to be considered for successful direct pulmonary delivery of sprayed cells, such as the type of cells, cell number, and devices for cell spraying. The details of these will be discussed in this chapter with the aim to contribute to the growing knowledge of alterative yet potentially clinically relevant options for stem cell delivery for lung diseases.

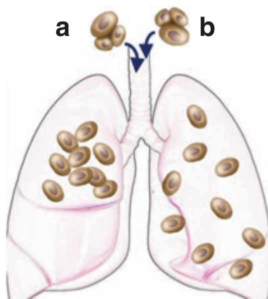

Fig. 13.1 Schematic diagram of airway delivery of cells to the lung using: (**a**) traditional instillation or (**b**) aerosolisation, leading to uniform distribution of cells in the lung

13.2 Types of Cells for Direct Stem Cell Delivery

Intratracheally delivered stem cells have been shown to be well-accepted without ensuing inflammatory reactions [43]. The most commonly used cell type for investigating direct cell delivery to the lung is MSCs from various tissues including bone marrow [44], adipose tissue [11], umbilical cord [45, 46], amniotic fluid [39], amniotic membrane [41], placenta [24] and lung [47]. A positive therapeutic potential of intratracheally delivered MSCs has been reported in various lung disease models. MSCs delivered intratracheally to tumour-bearing mice inhibited lung metastases and prolonged the survival of mice without causing inflammation or other apparent adverse effects [43].

Irrespective of the route of administration, MSCs interact with injured resident alveolar epithelial or lung endothelial cells to promote repair in the lung [44]. Other repair mechanisms of MSCs are attributed to secretion of growth factors such as vascular endothelial growth factors which have cytoprotective properties [48]. Human MSCs administered intravenously and intratracheally both reduced the severity of acute lung injury in a mouse model of pneumonia, improving pro-inflammatory responses, fluid balance and survival [49]. Administration of bone marrow-derived MSCs before inducing injury were effective in reducing ventilator-induced lung injury in a rat model regardless of intravenous or intratracheal route [18, 44]. Similar protective

effects of MSCs were observed regardless of the route of injection in bleomycin or lipopolysaccharide models of acute lung injury [49–51].

Other cell types used for direct pulmonary cell delivery in animal models include alveolar type II (ATII) cells [25, 52, 53], airway epithelial cells [3, 54] and a population of cells expressing Clara cell secretory protein (CCSP) in mouse and human bone marrow that demonstrated high pulmonary retention and regenerative potential for lung epithelial diseases [26]. Intratracheally instilled ATII cells in rats were shown to enhance recovery from acute lung injury induced by the combination of hydrochloric acid and lipopolysaccharide (LPS, *E. coli* O55:B5) [52]. In this model, ATII cells reduced the levels of IL-4 and IL-13 significantly and reduced inflammation by secreting soluble paracrine factors that contributed to modulation of alveolar macrophages to an anti-inflammatory phenotype [52]. Intratracheal transplantation of ATII has also been shown to reverse bleomycin-induced lung fibrosis [25].

In a mouse model of asthma, intratracheal administration of MSCs positively modulates airway remodelling, reduce inflammation and improve function [10, 12]. Because of the limited tissue retention of exogenous cells in the lung, the functional impact of MSCs may be attributed to their immunomodulatory response combined with the interference of the neuropeptide system activation and tissue remodelling [45].

Another important consideration should be around the handling of the stem cells prior to administration. Due to their responsiveness to the surrounding microenvironment, cells should be cultured or handled appropriately during the preparation stage prior to administration [55]. Different physicochemical cues can stimulate stem cell responses and can be used to precondition the cells. These include: mechanical stimulation [56] including substrate stiffness [57], topography of the substrate on which cells are cultured (e.g. micro- and nano-topographies) [58], dynamic culture conditions (e.g. media flow) [59], functional groups immobilised on the substrate that facilitate cells adhesion, migration, differentiation [60] and culture model (e.g. 2D versus 3D) [61]. Furthermore, variation of the composition of the cell culture conditions can be used to pre-condition cells (±FBS). Preconditioning and pre-stressing could be used to stimulate the cells differentiation prior to delivery, which is typically done to accelerate their activity at the injury site. However, stress can be induced non-intentionally during the delivery, which can impact cells negatively and potentially irreversibly. Subsequently, the stress experienced by stem cells during the delivery can lead to their undesired differentiation. Therefore, conditions of stem cells delivery must be precisely controlled and the potential impact on cells must be assessed to enable optimal tissue repair and avoid potential adverse and undesired effects.

While the therapeutic benefits of intratracheally delivered cells in modulating lung repair are established in many studies, a limited number of studies have compared traditional instillation and aerosolisation of cell suspensions. One of the most important benefits of intratracheal administration compared to the intravenous route is that the cells are mainly delivered to the lung instead of being distributed to the other organs through the systemic circulation. However, when cells are delivered by the traditional instillation method, cells are confined to certain areas of the lung due to the larger droplet sizes not being able to reach the smaller airways. Achieving smaller droplet sizes through aerosolisation enables cells to be more uniformly distributed across the whole lung. The next section will discuss a range of devices that are used for spraying cells and factors to consider in intratracheal aerosolisation delivery.

13.3 Devices for Spraying Stem Cells

13.3.1 Overview of Devices Used for Spraying Cells

The choice of a suitable device for spraying cells is not a trivial task, as cells may not survive through the aerosolisation process. Nebulisers used for delivering many medications in hospitals are

suitable and promising for vector administration in gene therapy [62]; however, nebulisation of cell suspensions will shred the cells. Similarly, other devices such as PennCentury IA-IB used for delivering many therapeutics via aerosolisation are not suitable for delivering cells as the small nozzle size does not allow whole cells to pass through [41, 63]. On the other hand, devices that produce larger droplets are inappropriate as they do not allow efficient delivery to the lower airways and cells are likely to be positioned at the edge of the droplet, where shear stresses are much higher than in the centre of the droplet [64, 65]. Cells exposed to high levels of shear stresses may result in reduced viability or altered differentiation capacity [66]. Furthermore, this can introduce heterogeneity in the stress level that cells are expose to, which is undesired. Thus it is ideal to use devices that generate particle sizes just exceeding the cell size [64].

Many cell types (including epithelial cells, keratinocytes, fibroblasts, stem cells and progenitor cells) have been sprayed using a variety of devices, including both commercially available and in-house designed systems [67–70]. Commercially available devices used to spray cell suspensions include bio-airbrush such as Duploject [64] and DuploSpray systems (Baxter AG) [71, 72], SkinGun™ (RenovaCare) [73] and ReCell® kit (Avita Medical) [74].

Bio-airbrushes such as the Duploject and DuploSpray are clinically approved for the clinical application of fibrin glue and have been repurposed for cell spraying [64, 71, 72]. In the last decade there has been increased usage and commercialisation of cell spraying technologies for accelerated regeneration of skin in patients with burns [73, 74].

The use of 3D printing is another feasible method to generate a custom-made nozzle to spray cells. The feasibility to spray human bone marrow-derived MSCs using a spray nozzle fabricated using a 3D computer-aided design software and produced using 3D printing has already been demonstrated [71].

13.3.2 Devices for Pulmonary Delivery of Stem Cells

The use of commercially available drug delivery aerosolisation devices for cell spraying is increasingly investigated for future treatment of lung diseases. In parallel, the repurposing of available devices for use in cell delivery to the lung is also increasingly observed. For example, MicroSprayer® Aerosoliser (PennCentury Inc) and LMA MAD780 (Teleflex Medical Australia) are commercially available for the delivery of therapeutics to animals and humans and they have both been used to develop technologies that enable an aerosol-based delivery of cells for intratracheal administration directly to the lung [40, 41, 63]. The sprayed cells have similar apoptosis profiles compared to seeded cells and retain normal morphology and actin cytoskeleton structure compared to seeded cells as confirmed in in vitro studies (Fig. 13.2a, b). In another study, uniform distribution of cells and minimal loss of cells were demonstrated in a rabbit model to which airway epithelial cells and skin-derived fibroblasts were delivered using the MicroSprayer® Aerosoliser [40]. The repurposing of currently available devices or modifying parts of available devices are promising options for advancing the technology closer to clinical translation.

13.3.3 In-House Designed Devices for Pulmonary Delivery

Compared to the increasing volume of research in stem cells and their paracrine factors for lung regeneration, there are far fewer studies on the fabrication of devices for efficient cell delivery to the lung. An overview of key developments of in-house-designed devices is presented in this section.

A microjet airway spray device for both ex vivo and in vivo direct cell delivery applications was developed by Skolasinski et al. [70]. Multiple prototype sprayers were fabricated with variations at

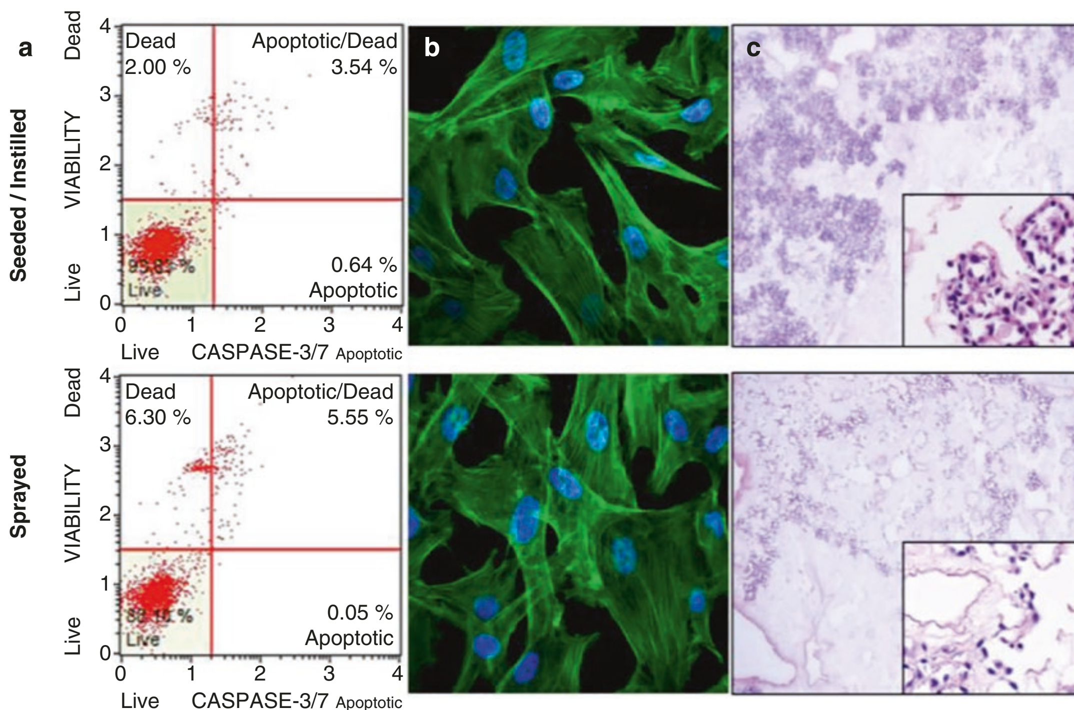

Fig. 13.2 Comparison of seeded (upper panel) and sprayed (lower panel) MSCs: (**a**) Apoptosis profiles (Caspase-3/7); (**b**) Cytoskeleton structures– actin (green), nuclei (blue); (**c**) The histology of decellularised rat lungs reseeded with instilled or sprayed MSCs (H&E, 4× & 40× magnification). (Panels A and B are reproduced with permission [41])

the spray generation tip for altering droplet size, fluid flow rate and spray velocity [70]. Through the assessment of cell viability and morphology, optimal cell survival was demonstrated when larger droplets were produced with lower velocity and low flow rate. The sprayed cells delivered by this in-house-designed microjet sprayer targeted areas of the lung that could not be reached otherwise [70]. One of the most important points to consider when designing devices of cell spraying therefore seems to be optimisation of spray nozzle designs [64]. Nozzle design optimisation should aim to achieve monodispersal droplet sizes and reduced impact velocities for successful cell deposition in the lung [64].

A novel high-frequency acoustic nebulisation platform was developed by Alhasan et al. to enable inhaled MSCs therapy [6]. The nebulisation uses surface acoustic waves that have been previously validated for pulmonary delivery of proteins [75] and nucleic acids [76]. These surface acoustic waves operate at high frequencies (10–100 MHz) to ensure that cells are exposed to only short vibrational excitations, while operating at low powers enabling miniaturised operation using a battery-operated portable handheld device [6]. This study presented a novel nebulisation platform as an attractive tool that enables stem cell delivery with high viability yet with low cost and portability.

Spraying respiratory epithelial cells with high viability through the use of a clinically used bronchoscope and a commercially available catheter was achieved by Thiebes et al. [77]. This spraying system was designed for tissue engineering purposes, enabling uniformly distributed, thin layer application of cells onto stents or implants [77]. In this study, the fluid dynamics of a cell-spraying device were studied for the first time and found majority of droplets had diameters larger than cells (typical size <20 μm). The authors suggested other nozzle designs to improve the cell

delivery to substrates and proposed potential use of the device for cell therapy applications [77].

These devices have great potential to play an important role in advancing bioengineering technologies as well as for in vivo administration of stem cells for pulmonary regeneration.

13.4 Other Considerations in Stem Cell Delivery by Spraying

13.4.1 Number of Cells for Intratracheal Delivery

One of the greatest challenges in cell therapy is determining the number of cells required [55]. The dose or number of stem cells used for beneficial effect varies largely between studies. This information is summarised in Table 13.1.

For optimisation of cell delivery parameters using in vitro studies, the number of cells used is determined according to the downstream assays. When cells are sprayed in a three-dimensional platform, 1 million cells are incorporated per mL for a final concentration of 250,000 cells per 500 μl volume [71]. In addition to the number of cells, careful considerations of passage number and consistency for each batch are essential as stem cells including MSCs are likely to undergo a dynamic fluctuation between differentiated and stem like cells [78]. Alternatively, cell lines that retain the same differentiation capacity with similar stem cell-like properties may be utilised, which may be especially useful for optimisation studies [79].

It is also important to optimise the volume of cell suspension for aerosol delivery for each species. Delivering large volumes of aerosol could also induce a localised inflammatory reaction in the area where the majority of volume is deposited [62], and thus this should ideally be minimised by using minimal volumes of liquid for aerosolisation. On the other hand, in a study that delivered fluorescently labelled MSCs for in situ visualisation, administration by instillation instead of aerosolisation, enabled cells to be selectively delivered to different regions of the lung by altering the volume of liquid used in the delivery [80]. In this study, instilling larger volumes of cell suspension led to more cells in the distal lung regions while smaller volumes led to cells being delivered to the larger airways. Whilst the aerosolisation method delivers cells more uniformly across the whole lung, the volume of cell suspension should be altered according to preliminary optimisations as illustrated by the work from Kim et al [80].

13.4.2 Mechanical Stress

When majority of exogenously delivered stem cells were not observed in the lung within hours

Table 13.1 Details of intratracheal stem cell administration in selected studies

Stem cell type	Number of cells	Volume of cell suspension (μl)	Model	Reference
Mouse lung-derived MSCs	5×10^4	50	Mouse model of elastase-induced emphysema	[47]
Rat bone marrow-derived MSCs	5×10^6	300	Rat model of ventilator-induced lung injury	[44]
Human amniotic fluid stem cells	2.5×10^5	Unknown	Mouse model of naphthalene injury	[39]
Human term placenta-derived fetal membrane cells	1×10^6	100	Mouse model of bleomycin-induced lung injury	[24]
Mouse adipose-derived MSCs	1×10^6	30	Mouse model of ovalbumin-induced asthma	[11]
Human umbilical cord blood-derived MSCs	1×10^5	50	Mouse model of *E. coli*-induced acute lung injury	[46]
Human umbilical cord blood-derived MSCs	5×10^3, 5×10^4 and 5×10^5	50	Neonatal rat model of hyperoxia-induced lung injury	[45]

of intravenous injection, it was once thought that cells could be damaged by shear forces upon intravenous injection [27]. However, as longer survival of exogenous cells are observed, it is accepted that the disappearance of cells is more likely to be related to removal of cells by immune cells rather than cell death caused by mechanical stress of infusion or other delivery mechanisms [27]. Nevertheless, as cells undergo at least some degrees of mechanical stress during delivery, especially when passing through small nozzles during the spray process, it is critical to consider this aspect when delivering stem cells by spraying. It is known that even small changes in the nozzle diameter and air velocity influence the shear and elongation stresses on cells [77, 81]. Hence the geometry of the spraying device must be well designed, given the implications for cell viability and functionality are dependent on air velocity, outlet area of the air flow, nozzle diameter and distance travelled by the cells, among others [77, 81]. The use of extracellular matrix or hydrogel to support the cells during the delivery process is suggested to maintain cell viability, retention and function during the cell spraying process [41, 82].

13.5 Assessment of Successful Delivery

There are various methods for assessing whether cells were effectively delivered, including cell viability, distribution and functionality. It is recommended to use a combination of these parameters, as no single method will ensure a complete picture of cell health. For instance, in the examination of cell distribution, a fluorescence label or luciferase enzyme activity may also be retained in dead cells or even in macrophages that phagocytosed the labelled cells [27]. Therefore, to correctly assess the efficiency of cell delivery, a combination of viability and functional studies should always accompany the comparison of cell distribution between delivery routes or devices. Many of these methods for assessing cell delivery are not relevant for clinical studies, not only due to safety reasons but also since human recipients are likely to have a more functional immune system compared to the immuno-compromised animals employed in various injury models [27]. Evidence of immune cells lysing MSCs has been observed in a number of in vitro studies [83, 84], while a study found that the majority of MSCs become apoptotic and do not survive for long after administration [85]. In summary, it is important to characterise the exogenous cells after delivery to interrogate the mechanisms of action of stem cells, especially in order to advance the aerosol technology for spraying cells forward towards clinical studies. The details of each method typically used for determining the successful delivery of stem cells are included in this section.

13.5.1 Cell Survival

For immediate assessment of cell viability after spraying in vitro prior to delivering cells ex vivo or in vivo, a simple trypan blue staining may be used [70]. When cells are delivered in a three-dimensional platform or when cell viability is determined in suspension form immediately following spraying, a live/dead cell viability assay is conducted to determine cell survival [64, 71]. For monitoring cell viability over a longer time period after being sprayed, metabolic assays (e.g. alamarBlue®, CCK-8 assay) combined with the measurement of deoxyribonucleic acid (DNA) content (e.g. Quant-IT PicoGreen® dsDNA kit) are recommended [6, 71].

13.5.2 Gene Expression Analysis

The influence of the spraying process on the ability of the cells to have a comparable gene expression to non-sprayed control group is an important functional analysis. After pre-determined time points post-spraying, total RNA could be isolated from the sprayed cells to conduct real-time polymerase chain reaction (RT-PCR) to observe changes in gene expression compared to non-sprayed cells (control group). Multi-lineage differentiation markers present in MSCs such as CD29, CD44 and CD106 could be used to assess

whether the pluripotent phenotype and integrity of MSCs are maintained after spraying [6]. In the case of chondrocytes, the chondrogenic genes usually assessed are aggrecan (ACAN), type II collagen (COL2A1) and type I collagen (COL1A1) [71].

Since the therapeutic actions of stem cells are attributed to immunomodulatory properties, comparing the expression of immunomodulatory genes before and after spraying is a method to analyse whether there are significant changes in stem cell functionality as a result of the spraying process. The immunomodulatory genes such as TGF-β, CSCL-10 and IDO are some examples [86].

13.5.3 Fluorescence Imaging

Through the use of transfection or fluorescent labelling, the location of exogenously delivered cells can be identified in the lung tissue by confocal laser scanning microscopy. Although this type of imaging is only possible after sacrificing the animal, it provides useful information about how the exogenous cells integrated into the microenvironment of the lung tissue. Green fluorescent protein-positive (GFP+) cells can be used for measurement of cell engraftment in the lung after delivery, as previously used in elastase-induced emphysema and pulmonary hypertension models [14, 19]. With the use of GFP+ cells, cell engraftment can be measured in the lung after intratracheal delivery and compared to those of other delivery routes. In fact, GFP+ cells can also be tracked in vivo through intravital imaging, where multiphoton with pulsed infrared lasers enable tracking of cells in live animals [87, 88].

In the case of determining whether a population of cells that express Clara cell secretory protein (CCSP), a marker of airway progenitor and stem cells, are differentially retained in the injured airways, Wong et al. labelled cells with the commercial red fluorescent marker 5-(and-6)-([(4-chloromethyl)benzoyl]amino) tetramethylrhodamine (CMTMR) [26]. At pre-determined time points, harvested lungs were examined by fluorescence microscopy for CMTMR positive cells that enabled comparison between CCSP positive and negative cells as well as for intratracheal and intravenous delivery routes.

To determine whether the cells engrafted in the lung differentiate into lung epithelial cells, immunofluorescence may be used. However, often minimal number of cells are observed with alveolar epithelial cell type I or II markers such as aquaporin 5 and pro-surfactant protein C [14, 89]. This suggests that the action of MSCs reducing the development of elastase-induced emphysema and having anti-inflammatory effects is independent on the extent of cell engraftment or differentiation [14].

13.5.4 Assessing Cell Distribution by Histology and Bioluminescence Imaging

Prior to in vivo studies, the comparison between traditional instillation and the aerosolisation method can be investigated using decellularised ex vivo lung models [90, 91]. To obtain decellularised lungs, lungs are harvested and treated over 3 days using an optimised sequential instillation of detergents and rinsing through both trachea and the right ventricle [92, 93]. Since these lungs are decellularised and therefore do not contain any endogenous cells, exogenously delivered cells detected by histology in this model enables the assessment of cell distribution across the lung. For the purpose of comparing cell distribution when delivered by instillation or aerosolisation through the trachea, the hematoxylin and eosin stain can reveal the degree of uniformity in cell distribution (Fig. 13.2c). Other staining such as Alcian blue and Masson's Trichrome staining can devise important information about the cells and extracellular matrix [94–97].

In order to assess the overall picture of cell distribution, luciferase-transfected cells are delivered to decellularised lungs and visualised using bioluminescence imaging. The bioluminescence imaging system (IVIS® Spectrum, PerkinElmer) enables three-dimensional visualisation of the cell distribution in the lungs. Achieving high luciferase transfection efficiency of the cells is

important, however for stem/progenitor cells, it may be difficult to achieve high efficiency of transfection as well as high proliferation rates after treatment. For the purpose of assessing cell distribution upon delivery by aerosolisation, other easier-to-transfect cell lines may be used for the purpose of bioluminescence imaging, provided that the morphology and the size of the cells are comparable to the cells of interest. This may lead to a proof of concept study, comparing cell distribution upon delivery by traditional instillation versus aerosolisation in ex vivo lungs (Fig. 13.3). Some options for transfection include 4D-Nucleofector™ (Lonza), Lipofectamine (Thermo Fisher Scientific) and FuGENE® (Promega), which are all successful in obtaining transient transfection but stable selection is often not achieved although may be necessary depending on experiments. Tests are required whether the transfection is affecting the functionality of the cells and alternative methods of transfection may be required to obtain the desired limits. An advantage of bioluminescence imaging for the assessment of cell distribution over other methods is that it can be conducted in situ, without the need of harvesting the lungs [39].

13.6 Future Directions

Despite substantial progress in stem cell-based therapy in the last few decades, more extensive research is required in improving the efficiency of cell delivery. While the benefits of aerosol delivery of stem cells seem promising for clinical translation, many limitations exist as the findings from animal models cannot be extrapolated for application in humans. For example, intratracheal instillation is not a physiological route in humans and therefore the results obtained from those studies may not be transferrable for clinical studies [98]. Thus, an assessment of potential clinical translation will be possible only through rigorous in vivo pre-clinical testings combined with ex vivo human lungs, followed by clinical trials. Recruitment of potential patients may be challenging, and a further complication would be that patients will have varying levels of immune responses. The selection of a cell type that has the lowest immunogenicity will be critical. Prior to clinical trials, further studies of aerosol delivery of stem cells in large animal models and/or perfused ex vivo studies in human lungs would be necessary to optimise dosages and assess benefits. Currently the only clinical trials conducted

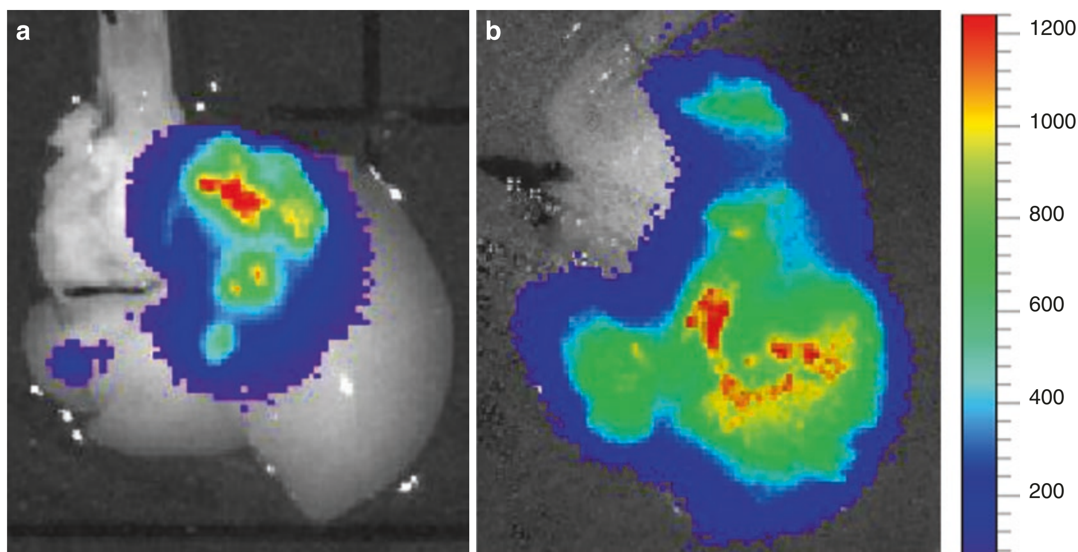

Fig. 13.3 Luciferase-transfected cells reseeded into decellularised rat lung by instillation (**a**) or aerosolisation (**b**); bioluminescence imaging

on intratracheal delivery of stem cells are for the use of MSCs in the treatment of bronchopulmonary dysplasia [99, 100].

Through future developments of aerosol delivery of stem cells and/or their paracrine factors, this approach could potentially become part of personalised medicine regimens. Instead of being used only in hospitals, cells and their paracrine factors may even be administered in patients' homes, in a similar manner to common aerosol medications such as salbutamol. In order to achieve this in the future, we need a standardisation of cell delivery techniques and assessment criteria for successful cell therapy.

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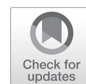
Part IV

Conclusion



Challenges and Opportunities for the Future of Stem Cell Therapy for Lung Diseases

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Irene H. Heijink and Janette K. Burgess

In this era of striving for personalized medicine approaches for complex diseases, preclinical studies have excited the field through their demonstration of the promise of stem cell therapy for the treatment of various lung diseases; with the most beneficial effects of stem cell-based strategies in preclinical settings originating from their antimicrobial, anti-inflammatory, and immunomodulatory properties. Although many lung disorders involve an inflammatory component, effective stem cell therapy in patients has not been realized yet for the majority of lung diseases and there are still multiple hurdles to be overcome. While beneficial effects have been realized in clinical studies of CF, results from clinical studies in other lung diseases have been disappointing to date. For instance, as described in Chap. 6 of this book, no improvements in lung function, quality of life, or exacerbation incidence were shown in a clinical trial on emphysema patients, with only a small reduction in levels of the inflammatory marker CRP. Importantly, the administration of stem cells was safe and no side effects were reported among recipients during a follow-up period of 2 years. Similarly, clinical studies in IPF have proven safe over a period of at least 6 months, but were again without any beneficial effects on lung function (described in Chap. 7). Also in ARDS, MSC treatment has proven safe and very recently, lower levels of inflammatory biomarkers and better short-term prognosis has been observed upon treatment with MultiStem® multipotent adult progenitor cells (Chap. 12). However, the mechanisms through which these beneficial effects were elicited remain largely unknown and therefore interactions with other drugs or comorbidities remain unpredictable. Thus, further preclinical studies, both in vitro and in vivo, for understanding the mechanisms of action enabling the effectiveness of cell therapies are warranted.

For enabling this field to move forward, studies involving healthy and diseased patient-derived stem/stromal cells from various origins will be

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essential to address questions relating to abnormalities in stem cells obtained from diseased patients for use in autologous treatment. Moreover, there is an urgent need to further characterize stem cell niches in the human lung and understand the interaction between stem/progenitor cells, stromal cells, the extracellular matrix, and the inflammatory microenvironment. The unique composition of the lung microenvironment becomes altered during pathological conditions, affecting MSC behavior. An increased understanding of the crosstalk between MSCs and their microenvironment will provide us with better understanding of the biological function of MSCs in health and disease, as well as leading to improved clinical strategies for treating lung disorders. Advanced three-dimensional models such as organoids, decellularized lung scaffolds, and lung-derived hydrogels will be vital for providing invaluable insight into cell–cell and cell–matrix interactions within the human lung. Nevertheless, we should realize that these models are still not able to fully recapitulate the complexity of lung architecture; comprising an intertwined network of multiple types of progenitor cells, differentiated multilineage epithelial cells, endothelial cells, and other cells forming blood vessels, inflammatory cells, nerves, multiple types of mesenchymal (stromal) cells, and extracellular matrix to form functional lung tissue. Most of the described progenitor populations and repair mechanisms characterized to date for the lung have been identified in animal models. While some of these may also exist in the human lung, there are also important species differences in the composition of the different cell layers in the human and animal lung. These essential differences need to be taken into account when translating findings from animal studies to humans.

In addition to the lack of understanding of stem cell mechanisms, insight with regard to the optimal source of stem cells, the best route to administer them, the timing of administration, the dosing (high numbers of cells may aggregate, leading to apoptosis) and frequency is shortcoming. Furthermore, we need to investigate how the engraftment, survival, and functioning of donated stem cells can be improved, e.g. by the use of bioactive scaffolds. As described throughout this book, various stem cells, including MSCs and iPSCs, have been shown to be capable of homing to the site of injury or disease, regardless of the route of administration, potentially due to the chemokines released upon lung injury. However, the actual engraftment of the stem cells into the niche of the injured lung has been less effective, possibly contributing to many of the poor clinical outcomes to date. A few murine studies have shown that only a very small number of the total population of administered cells engraft within 24 h (Chap. 12). Although cells particularly home to damaged tissue, stem cells require interaction with the extracellular matrix for their retention and survival, which may be impaired in various lung diseases especially those with extensive tissue damage and remodeling. In murine models, stem cell administration was followed by death of the majority of the stem cell population in less than 4 days, which may be driven by stem cell rejection. Although particularly MSCs have a low immunogenic potential, upon differentiation these cells may become more immunogenic. Therefore, the use of autologous cells may be more ideal. Notwithstanding, as described above, autologous stem cells may display disease-related abnormalities. Further, adult stem cells have limited growth and differentiation potential, and it is difficult to obtain the large population of cells currently thought to be needed for treatment. The age of the donor can also be a limiting factor in the expansion of stem cells, as higher age is associated with increased cellular senescence. Cellular senescence upon in vitro culture can result in stem cell exhaustion and even cause spontaneous transformation of the cells. Specific lung diseases, e.g. COPD and pulmonary fibrosis, have been associated with accelerated aging and cellular senescence, complicating the use of autologous stem cells. At the moment, defined assays to accurately predict MSC potency are lacking.

Beyond the conventional MSC therapeutics, embryonic stem cells and iPSCs have shown encouraging results. These cells have a tremendous potential to reprogram fully differentiated somatic cells and the use of iPSCs can also

overcome immune-mediated rejection by providing an autologous source of stem cells for transplantation. Preclinical models have shown that iPSCs have immunomodulatory potential, with better survival and engraftment rates after transplantation compared to adult tissue-derived MSCs (Chap. 12). Because of their in vitro expansion potential, iPSCs are also well suited for cell-based approaches using (epi)genetic editing, e.g. CRISPR-(d)Cas as described in Chap. 9, to overcome detrimental effects of mutations associated with disease. However, caution is still required as genetic abnormalities remain an area of concern in iPSCs and the pluripotent nature of both embryonic stem cells and iPSCs raises serious concerns with respect to teratogenic risks. Moreover, ethical issues arise with the use of embryonic stem cells as well as with genetic manipulation, and strict guidelines and regulations are required, posing an enormous challenge for biomedical researchers, bioethicists, and regulatory authorities.

Other complications with cell-based therapies include the potential contamination with other cell types that can have detrimental effects. The establishment of standardized isolation protocols for enabling preparation of pure progenitor cell populations are still a challenge for the field. Further, more extensive research is required in improving the efficiency of cell delivery systems. While the benefits of aerosol delivery of stem cells seem promising for clinical translation, as described in Chap. 13, limitations still exist. For example, intratracheal instillation is not a physiological route in humans. Standardization of cell isolation and delivery techniques and assessment criteria for successful cell delivery are required.

Cell-free products such as cell-conditioned medium and particularly extracellular vesicles may circumvent many of the challenges that arise with using cell-based strategies and have demonstrated promising results. Nevertheless, similar to cell-based strategies, clinical-grade manufacturing protocols will need to be established according to current Good Manufacturing Practices (GMP) guidelines. For the clinical-grade (GMP) and industrial-scale production of extracellular vesicles to be used in clinical trials, a number of technological and mechanistic issues must be resolved. These include the standardization of their preparation, harvesting, and storage as well as safety considerations, although such concerns will be considerably milder than those relevant to cell-based strategies. Compared to their parent cells, use of extracellular vesicles may have a superior safety profile, and they can be potentially be stored without loss of function. The challenges to translate this therapeutic strategy to clinical trials include the optimal timing, dose, and frequency of therapy, as well as solving the parental cell source.

We discuss the questions that should be addressed before considering administration of stem cells or their derivatives in a phase I studies. We should prevent stem cell tourism, where cell-based therapies are being marketed to extremely vulnerable patient populations and their caregivers. Unproven and often unsafe stem cell treatment practices can mislead patients into participating in often very expensive, unregulated, unethical, and unsafe treatments, which are not covered by insurance. Here, the education of patients, caregivers and of pulmonologists who are not familiar with the stem cell field will be of great value.