Abstract of thesis entitled

Mitochondrial transfer from induced pluripotent stem cell-derived mesenchymal stem cells to airway epithelial and smooth muscle cells attenuates oxidative stress-induced injury

Submitted by

LI, Xiang

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Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease characterized by persistent airflow limitation that is not fully reversible and is usually caused by cigarette smoke (CS). The disease is predicted to be the fourth leading cause of death by 2030, but none of the currently available treatments can alleviate the progressive decline in lung function.

Mesenchymal stem cells (MSCs) are fibroblast-like multipotent stem cells that can be isolated from various tissues such as bone marrow (BM-MSCs). Despite numerous reports of their efficacy in COPD-related pre-clinical models, BM-MSCs have not demonstrated efficacy in a clinical trial of COPD, highlighting the need for
improved MSC-based therapy. The in vitro derivation of MSCs from induced pluripotent stem cells (iPSCs) has provided a new source of MSCs. Compared to BM-MSCs, iPSC-derived MSCs (iPSC-MSCs) are a more abundant source, have a higher expanding capacity and are possibly not subject to the ageing-associated dysfunction seen in BM-MSCs.

In this study I determined the effects of human iPSC-MSCs in a rat COPD model using BM-MSCs as comparison. Rats were exposed to CS for 1 hr/day for 56 days. iPSC-MSCs or BM-MSCs were administrated at days 29 and 43. iPSC-MSCs demonstrated superior effects over BM-MSCs in attenuating CS-induced lung airspace enlargement, fibrosis, inflammation and apoptosis. In a mouse model of ozone-induced lung damage, intravenous administration of iPSC-MSCs 24 hours before ozone exposure for 3 hours alleviated airway hyper-responsiveness, inflammation and apoptosis in the lung.

There is increasing evidence demonstrating that mitochondrial dysfunction may play an important role in COPD pathogenesis, indicating mitochondria as a potential therapeutic target. Meanwhile, mitochondrial transfer from MSCs to injured airway cells has been reported as a novel mechanism of action for MSCs.

In this study mitochondrial transfer from iPSC-MSCs to the airway epithelium of CS-exposed rats was detected. iPSC-MSCs also transferred mitochondria to bronchial epithelial BEAS-2B cells and primary airway smooth muscle cell (ASMCs) in vitro in a direct co-culture system, an effect that was enhanced by CS medium (CSM). Direct
co-culture with iPSC-MSCs alleviated CSM-induced ATP deprivation in BEAS-2B cells, as well as CSM-induced mitochondrial reactive oxygen species (ROS), apoptosis and reduction of mitochondrial membrane potential ($\Delta \Psi_m$) in ASMCs. Administration of iPSC-MSCs also prevented ozone-induced mitochondrial ROS and $\Delta \Psi_m$ reduction in mouse lungs.

The paracrine effects of iPSC-MSCs were also investigated. iPSC-MSC-derived conditioned medium (iPSC-MSCs-CdM) protected BEAS2-B cells from CSM-induced apoptosis. The effect was reduced by depleting stem cell factor (SCF) from iPSC-MSCs-CdM. However, both iPSC-MSCs-CdM and trans-well inserts containing iPSC-MSCs were only able to alleviate CSM-induced mitochondrial ROS, but not $\Delta \Psi_m$ reduction and apoptosis, in ASMCs.

I demonstrated the capacity of iPSC-MSCs to alleviate oxidative stress-induced COPD phenotype in vivo. Mitochondrial transfer from iPSC-MSCs was able to alleviate oxidative stress-induced mitochondrial dysfunction and apoptosis in target cells. The full capacity of iPSC-MSCs to achieve these effects may rely on a combination of cell-cell contact and release of paracrine factors. These findings define iPSC-MSCs as a promising candidate for the development of MSCs-based therapy of COPD.

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By

LI, Xiang

M.Phil. H.K., B.Sc. H.K.

A thesis submitted in fulfilment of the requirements for

the Degree of Doctor of Philosophy

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Declaration

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to The University of Hong Kong or Imperial College London, or to any other institution for a degree, diploma or other qualifications.

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LI, Xiang
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Part of the results in this thesis has been published in the following publications:

Journal article


Abstracts


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<th>Description</th>
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<tbody>
<tr>
<td>AEC1s</td>
<td>type 1 alveolar epithelial cells</td>
</tr>
<tr>
<td>AEC2s</td>
<td>type 2 alveolar epithelial cells</td>
</tr>
<tr>
<td>AHR</td>
<td>airway hyper-responsiveness</td>
</tr>
<tr>
<td>ASM</td>
<td>airway smooth muscle</td>
</tr>
<tr>
<td>ASMC</td>
<td>airway smooth muscle cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BOLD</td>
<td>Burden of Obstructive Lung Disease</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BM-MSC</td>
<td>bone marrow-derived mesenchymal stem cell</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>COPD assessment test</td>
</tr>
<tr>
<td>CC10</td>
<td>Clara cell secretory protein 10</td>
</tr>
<tr>
<td>CCL</td>
<td>chemokine ligand</td>
</tr>
<tr>
<td>CdM</td>
<td>conditioned medium</td>
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<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CS</td>
<td>cigarette smoke</td>
</tr>
<tr>
<td>CSM</td>
<td>cigarette smoke medium</td>
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<tr>
<td>DAB</td>
<td>3,3′-diaminobenzidine</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger-associated molecular pattern</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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</table>
ELISA  enzyme-linked immunosorbent assay
ESC    embryonic stem cell
FACS   fluorescence-activated cell sorting
FEV₁   forced expiratory volume in 1 s
FISH   fluorescent *in situ* hybridization
FVC    forced vital capacity
GBD    Global Burden of Disease
GM-CSF granulocyte macrophage-colony stimulating factor
GOLD   global initiative for chronic obstructive lung disease
GPx    glutathione peroxidase
GRO    growth-related oncogene
GSH    glutathione
GSSG   oxidized glutathione
GVHD   graft-versus-host disease
HBSS   Hank’s Balanced Salt Solution
HNA    human nuclear antigen
HRP    horseradish peroxidase
HSC    haematopoietic stem cell
IFN    interferon
IL     interleukin
IP-10  IFN-γ-inducible protein 10
iPSC   induced-pluripotent stem cell
iPSC-MSC induced-pluripotent stem cell-derived mesenchymal stem cell
iPSC-MSCs-CdM iPSC-MSCs-conditioned medium
JC-1    5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanineiodide
KC     keratinocyte-derived chemokine
KIF5   kinesin motor protein-5
LABA   long-acting β2-agonist
Lₘ     mean linear intercept
<table>
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<th>Description</th>
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<tr>
<td>TIMP</td>
<td>tissue inhibitors of MMP</td>
</tr>
<tr>
<td>TL</td>
<td>telomere length</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNT</td>
<td>tunnelling nanotube</td>
</tr>
<tr>
<td>TSG-6</td>
<td>tumor necrosis factor-stimulated gene-6</td>
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<td>TUNEL</td>
<td>terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>ΔΨm</td>
<td>mitochondrial membrane potential</td>
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Chapter 1. Introduction
1.1. Introduction to Chronic Obstructive Pulmonary Disease (COPD)

1.1.1. Definition and diagnosis of COPD

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines chronic obstructive pulmonary disease (COPD) as ‘a common preventable and treatable disease’ which is ‘characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases’ (263). The chronic inflammation drives small airways disorder named obstructive bronchiolitis leading to structural alterations and narrowing of small airways, and emphysema characterized by destruction of parenchyma which causes separation of alveolar from the small airways and loss of lung elastic recoil (263). Obstructive bronchiolitis and emphysema together causes the persistent airway flow limitation in patients with COPD (Figure 1.1), but the contribution from each of them varies within individual patients (263).

COPD is diagnosed by post-bronchodilator spirometry to measure persistent airway limitation. Spirometry measures volume of air that a patient can exhale at a maximal force (as long and as quickly as possible) after a maximal deep inspiration (208). The total volume of air exhalation with greatest effort of the patient is defined as forced vital capacity (FVC) (208). The total volume of air exhalation with greatest effort of the patient in the first second is defined as forced expiratory volume in 1 s (FEV₁) (208). Persistent airway limitation is confirmed when the ratio of FEV₁ to
FVC is less than 0.7 (263) (Figure 1.2).

Figure 1. 1 Cause of airflow limitation in airways of patients with COPD. Compared to (A) healthy lungs, lungs with (B) COPD demonstrate parenchymal destruction leading to emphysema, mucosal and peribronchial inflammation and fibrosis leading to obliterative bronchiolitis, and mucus hypersecretion leading to luminal obstruction. As a result, patients with COPD develop irreversible airflow limitation. Adapted from Barnes PJ, N Engl J Med (22).

Figure 1.2 Measurement of airflow limitation by spirometry. Patients are asked to exhale as long and as quickly as possible. Exhalation volume from (A) normal lungs or (B) obstructive lungs is plotted against time. FVC: forced vital capacity; FEV$_1$: forced expiratory volume in the first second; Airway obstruction is defined if FEV$_1$/FVC is less than 0.7. The figure is reproduced based on plots by Ranu H et al, Ulster Med J (208).
Further assessment of COPD includes determination of levels of symptoms, severity of airflow limitation, exacerbation risk and comorbidities. The major symptoms of the disease include chronic and progressive dyspnea, cough and sputum production. The levels of symptoms can be assessed by questionnaires such as Modified British Medical Research Council (mMRC) questionnaire on breathlessness and COPD Assessment Test (CAT). The severity of airflow limitations is graded into four different stages by GOLD based on the post-bronchodilator FEV₁ (Table 1.1). The spirometric criteria for Mild, Moderate, Severe and Very Severe severities are 80%, 50% and 30% of predicted FEV₁. Exacerbations refer to short periods (at least 48 hours) of symptom worsening such as increased cough and dyspnea which is beyond normal variations (65, 263). Exacerbations lead to reduced quality of life, accelerated disease progression and increased risk of death (65, 71, 236). Exacerbations usually call for changes in medication by which their severity can be defined, including change of inhaled treatment (mild exacerbation), medical intervention such as antibiotic and oral steroids (moderate exacerbation) and hospitalization (severe exacerbation). A combined assessment of COPD which integrates levels of symptoms, severity of airway limitation and exacerbation history is suggested by GOLD. The assessment groups patients with COPD into four sub-groups demonstrating different burden of symptom and risk of exacerbation (Group A, low risk and less symptoms; Group B, low risk and more symptoms; Group C, high risk and less symptoms; Group D, high risk and more symptoms). The burden of symptoms is defined high or low by mMRC or CAT grade. The risk of
exacerbation is defined high if the severity of airway limitation is GOLD 3-4 or there are two or more exacerbations in the preceding year. In addition to the combined assessment approach, assessment of comorbidities is also important. COPD is highly associated with comorbidities including ischaemic heart disease, diabetes, skeletal muscle wasting, cachexia, osteoporosis, depression and lung cancer (65, 263). The comorbidities can increase the risk of admission to hospital and death (234).

**Table 1.1 GOLD Severity of airflow limitation in COPD (FEV₁/FVC<0.70)**

<table>
<thead>
<tr>
<th>GOLD stage</th>
<th>Severity</th>
<th>Post-bronchodilator FEV₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>Mild</td>
<td>FEV₁ ≥ 80% of predicted</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Moderate</td>
<td>50% ≤ FEV₁ &lt; 80% of predicted</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Severe</td>
<td>30% ≤ FEV₁ &lt; 50% of predicted</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Very severe</td>
<td>FEV₁ &lt; 30% of predicted</td>
</tr>
</tbody>
</table>

**1.1.2. Epidemiology of COPD**

The prevalence of COPD varies among different reports, maybe due to different assessing criteria and methods of survey and analysis (96). A recent meta-analysis report estimated that the worldwide COPD cases in people aged 30 or more had increased from 227.3 million in 1990 to 384.0 million in 2010, while the prevalence was 10.7% in 1990 and 11.7% in 2010 (3). The actual prevalence may be higher than the data from epidemiological studies as COPD is considered to be under-diagnosed in general (259). It is estimated that 60-85% patients with COPD may be undiagnosed,
especially for the mild and moderate severities (GOLD stage 1 or 2) (114).

COPD prevalence varies among different countries and different groups of people (e.g. gender and smoking history) (Figure 1.3). For example, the estimation of male population with COPD of GOLD 2 severity or above was estimated as 22.2% in Cape Town, South Africa, in comparison to 8.5% in Reykjavik, Iceland (168). Based on the data from two large multinational studies, the Burden of Obstructive Lung Disease (BOLD) study and the Latin American Project for Investigation of Obstructive Lung Disease (PLATINO) study, the prevalence of COPD in different countries ranges from 3% to 23% (39, 168). The prevalence of COPD has been stable in some developed countries, while in the United States, it is the only common cause of death that has increased over the last 40 year (23). In developing countries such as some African countries, the prevalence increased more dramatically (65). Variations among nations were also observed in mortality studies. For example, the death rate of patients with COPD was estimated to be 130.5/100,000 in China in comparison to 4.4/100,000 in Japan, although the prevalence is similar between the two countries (168). COPD was ranked as the 6th leading cause of death worldwide in 1990, and was predicted to be the 3rd leading cause by 2020. A more recent study estimated COPD as 4th leading cause of death in 2030 (172). Based on the data of 2010 Global Burden of Disease (GBD) study, COPD led to 2.9 million deaths, accounting for about 5% of total deaths globally (161, 181).

The economic burden associated with COPD is huge. For example, in 2003 the United States spent US$ 32.1 billion in total on COPD, US$ 18.0 billion of which on
direct healthcare costs and the rest on indirect morbidity and mortality costs (168). In 2010 the number increased to US$ 49.9 billion in total and US$ 29.5 billion on direct healthcare costs (49). In United Kingdom, in 2007 the healthcare cost of COPD is three fold higher than asthma and the consultation rate of COPD is two to four-fold higher than ischemic heart disease (23).

![Figure 1. 3 Prevalence of COPD (GOLD stage 2 or higher) in 2007. Combined results from BOLD study and PLATINO study are demonstrated in the figure. The data were collected from a city or region of the country thus do not represent national population. Adapted from Mannino DM et al, the Lancet (169).](image)

### 1.1.3. Risk factors of COPD

COPD results from the interaction between gene and environmental stimuli (65). The most common genetic risk for COPD is deficiency of $\alpha_1$ antitrypsin, a circulating inhibitor of serine proteases. Mutation of genes encoding matrix metalloproteinase-12 (MMP-12) (113) and transforming growth factor $\beta_1$ (47) have also been reported in association with COPD.
Cigarette smoking is the most common worldwide risk factor for COPD. Cigarette smoking is associated with an increase in prevalence of respiratory symptoms, lung function abnormalities, annual rate of FEV\textsubscript{1} decline, and COPD mortality rate (136). The association between development of COPD and cigarette smoking is dose-dependent (292), and smoking cessation reduces rates of morbidity and mortality in comparison to continued smoking (209). Besides normal cigarette smoking, passive exposure to cigarette smoke in the environment is also a risk factor for COPD (73). For example, in China where 30% of the world’s cigarettes are consumed (277), 12.1% of male and 51.3% of female nonsmokers are exposed to environmental smoke at home, while 26.7% of male and 26.2% of female non-smokers are exposed to environmental smoke in the work place in 2001 (92). In never-smokers with COPD, the passive exposure to cigarette smoke contributed to 1.9 million additional deaths in China (280). Maternal smoking during pregnancy may also increase the risk of COPD to the fetus (245). Although cigarette smoking is recognized as the major risk factor for COPD, COPD does not develop in some people with the same smoking history (263). In addition, COPD may also develop in some nonsmokers (48, 141), indicating the importance of other risk factors.

In addition to environmental smoke, other particles and gases in environment can also contribute to development of COPD, such as occupational exposure to hazardous dusts and fume (17) and indoor pollutant from biomass burning for family cooking and heating (36, 75, 191, 207).

Age is commonly considered as a risk factor for COPD as the prevalence,
morbidity and mortality increases along with increased age (38, 126, 137). Gender was regarded as one risk factor in the past with men presenting higher prevalence and mortality of COPD than women (233). However, the prevalence of COPD in men and women from high-income countries has become more similar now (39, 233).

Development of COPD can be also associated with many other risk factors including impaired lung development, asthma, bronchial hyper-responsiveness, asthma, infections and social status (168).

1.1.4. Pathogenesis of COPD

The most significant pathophysiological feature of COPD is airflow limitation resulting from narrowing of the small airway compartment and destruction of parenchyma, both leading to gas trapping and subsequently hyperinflation (186). Other pathophysiological changes that may present in patients with COPD include gas exchange abnormalities, mucus hypersecretion, pulmonary hypertension and other comorbidities (263). The pathological changes in patients with COPD mainly include infiltration of various inflammatory cells, structural changes of small airways such as thickening of walls and mucus overproduction, and destruction of parenchyma (60, 263). The pathogenesis of COPD, including the mechanisms driving the chronic inflammation, remodeling of small airways and parenchymal destruction is still not fully understood (65). Current evidence indicates a complex interplay between oxidative stress, inflammatory responses, protease and anti-protease imbalance, ageing, and changes in apoptosis, proliferation and tissue repair process (57). A
simple illustration of the factors that leads to emphysema is shown in figure 1.4. The details are described in following sections.

**Figure 1.4 Interplay of multiple events leading to emphysema.** Cigarette smoke exposure activates airway epithelial cells, which release early inflammatory cytokines that recruit macrophages and neutrophils. They can also activate dendritic cells and the subsequent formation of CD8+ or CD4+ T cells. Along the chronic inflammation, various cell types are activated by inflammatory mediators and in response their secretory function may also become activated. Their anti-oxidant system may become defective, while cells such as neutrophils will release ROS, leading to oxidant/anti-oxidant imbalance. The resulting oxidative stress can induce senescence and mitochondrial dysfunction. Mitochondrial dysfunction and accelerated ageing can further amplify the oxidative stress. Apoptosis can be induced by external cytokines such as perforin and granzymes, as well as by cytochrome c release due to mitochondrial damage. Both accelerated ageing and apoptosis may lead to airspace enlargement. The immune cells and structural cells, especially neutrophils and macrophages can release protease. When the anti-protease of the cells is not enough to counterbalance such effect, excess protease activity can lead to destruction of alveolar structures.
1.1.4.1. Oxidative stress

COPD is associated with persistent oxidative stress. Markers of oxidative stress, such as lipid peroxidation byproducts 4-hydroxy-2-nonenal, 8-isoprostan and malondialdehyde (MDA), are found increased in lungs of patients with COPD (135, 206). The exhaled breath or breath condensate collected from patients with COPD also contains increased levels of various markers of oxidative stress including hydrogen peroxide, carbon monoxide, myeloperoxidase (MPO), 8-isoprostan and MDA (27, 176, 194, 195). Exacerbations are associated with further enhanced oxidative stress (263).

Reactive oxygen species (ROS) are free oxygen radicals with unpaired elections that can oxidize various molecules such as proteins, DNA and lipids (133). Typical forms of ROS include superoxide radical (O$_2^-$), hydroxyl radical (OH$^-$) and hydrogen peroxide (H$_2$O$_2$). Under normal conditions, the major sources of ROS are respiration of mitochondria, xanthine oxidase activity and NADP oxidase activity (167). During cigarette smoking, the structural and immune cells in the lung are exposed to high levels of exogenous oxidants from inhaled cigarette smoke (CS). One puff of CS contains about $10^{15}$ oxidants in the gas phase and $10^{18}$ oxidants in the tar phase, as well as 3000 ppm of nitric oxide (NO) (57, 58). Exposure to CS leads to release of inflammatory mediators and ROS from epithelial cells and immune cells, leading to release of various inflammatory mediators and ROS (164). Although CS is an obvious source of oxidants and indeed a major risk factor for COPD, oxidative stress is persistent after smoking cessation in patients with COPD (160), indicating that the
cellular accumulation and release of ROS is an important source of oxidative stress during the progression of COPD. Increased release of ROS was reported in alveolar macrophages and peripheral neutrophils from patients with COPD (115, 220). The increased production of ROS may be due to the mitochondrial ROS overproduction and altered xanthine oxidase activity. Increased mitochondrial ROS was reported in airway smooth muscle cells from patients with COPD (273), and products of xanthine oxidase activity were detected in bronchoalveolar fluid (BALF) from patients with COPD (202). Although the increased ROS may be mainly in the form of less potent radicals such as superoxide anion and hydrogen peroxide, they can be converted to more potent and destructive ROS such as peroxynitrite (ONOO') by rapid reaction between superoxide radical and NO (261), and hypohalous acids (HOCl and HOBr) by MPO-catalyzed reactions between hydrogen peroxide and Cl' or Br' (224). MPO levels in neutrophils are reported to be elevated in patients with COPD (1).

ROS generation is balanced by the activity of anti-oxidant defense in the normal body. The first anti-oxidant barrier against the noxious particles or gases is the epithelial lining fluid containing anti-oxidants such as vitamin C, vitamin E and uric acid (134). Reduced levels of vitamin C and vitamin E in the lung were associated with declining lung function in patient with COPD (134). Regarding cellular anti-oxidant activity, one of the most important reactions is the synthesis of glutathione (GSH), an anti-oxidant that depletes oxidants via conversion to oxidized glutathione (GSSG). Under acute oxidative stress, epithelial cells from healthy subjects presented elevated level of glutamate-cysteine ligase which is an enzyme
involved in GSH synthesis (228). Such anti-oxidant defense was impaired in bronchial epithelial cells and alveolar macrophages from patients with COPD, as indicated by lower expression of glutamate-cysteine ligase (228). Another anti-oxidant defense system is mediated through superoxide dismutase (SOD) which converts superoxide radicals into hydrogen peroxide (167). Hydrogen peroxide can be further converted to oxygen and water directly by catalase (53) or by reaction with GSH mediated by glutathione peroxidase (GPx) (232). As mentioned earlier, under excess ROS production, superoxide anions can react rapidly with NO to form peroxynitrite. Peroxynitrite can inhibit SOD, thus reducing the anti-oxidant defensive activity (261). The persistent high ROS burden and defective anti-oxidant defense lead to oxidative stress of the cells.

1.1.4.2. Chronic airway inflammation

Chronic and persistent airway inflammation is observed in patients with COPD, and higher grades of COPD severity is associated with higher severities of inflammation (108). Infiltrated inflammatory cells include neutrophils, macrophages, B-cells, lymphoid aggregates and T cells (107). In addition to the increased numbers in the lung, the function of inflammatory cells may also change in line with the pathogenesis of COPD (109).

The number of macrophages increases in bronchial submucosa along with the severity of airflow limitation in patients with COPD (108). Macrophages can contribute to oxidative stress, destruction of structural cells and further progression of
inflammatory responses by releasing ROS, extracellular matrix proteins, leukotrienes, multiple cytokines, chemokines and matrix metalloproteinases (MMPs) (57). One primary function of macrophages in normal lungs is bacterial clearance through phagocytosis, which may be defective in patients with COPD (72). Ex vivo studies have demonstrated a defective phagocytotic ability against bacteria commonly detected in airways in both alveolar macrophages (29) and monocyte-derived macrophages (250) from patients with COPD. Moreover, the secretion of inflammatory mediators is elevated in alveolar macrophages from smokers and ex-smokers (101).

Increased number of neutrophils in sputum has been reported to associate with higher severity of airflow obstruction and faster decline of lung function in patients with severe COPD (187). Neutrophils are distributed in various sites particularly in bronchial epithelium, bronchial glands (217) and in the airway smooth muscle (20). When recruited to the airways, neutrophils become activated and release ROS, inflammatory cytokines, neutrophil elastase, MMPs and MPO (240). In vitro data suggest that the phagocytic ability of neutrophils can be impaired by cigarette smoke extract (242).

In general, infiltrating neutrophils and macrophages are considered to be the major players in the innate immune response in COPD. However, increased numbers of eosinophils have also been reported in sputum, BALF and the airway wall (140, 142, 156). Levels of pro-eosinophil cytokines IL-4 and IL-5 are also reported to be increased in plasma cells of patients with COPD (298). The exact function of
recruited eosinophils and its contribution to the development of COPD remains unclear (57).

There is also an adaptive immune response to the inflammatory responses in COPD. The number of dendritic cells has been reported to increase in epithelium and adventitia of small airways of patients with COPD compared with never-smokers and healthy smokers and correlated with the severity of airflow limitation (66). Dendritic cells can present both self-antigens from tissue destruction and foreign antigens to naive T cells, and numbers of both CD4+ and CD8+ T cells have been reported to increase in lungs of patients with COPD (175, 213, 216). CD8+ T cells from lungs of patients with COPD have been shown to release higher levels of inflammatory mediators including tumor necrosis factor (TNF)-α, interferon-γ and granulocyte macrophage-colony stimulating factor (GM-CSF).

1.1.4.3. Protease and anti-protease imbalance

Parenchymal destruction is one of the most significant pathological changes in the lungs of patients with COPD. The resulting loss of elastic recoil leads to the airflow limitation. Destruction of connective tissue, particularly lung elastin, by excess protease activity is believed to play an important role in this process (25). Active neutrophils can release serine proteases including neutrophil elastase, cathepsin G and proteinase 3 (25). Various cysteine protease cathepsin can also be released by different cells, including cathepsin S from airway smooth muscle, cathepsin B, K and L from alveolar macrophages and cathepsin W from CD8+ T cells
Alveolar macrophages can provide another important protease family, MMPs, particularly MMP-9. The alveolar macrophages from healthy smokers express more MMP-9 than normal subject, while alveolar macrophages from patients of COPD express an even higher level (154, 214).

The protease activities are balanced by anti-proteases in normal lungs. Serine protease activity can be counteracted by two major serine protease inhibitors in the lung, α₁ antitrypsin and secretory leukoprotease inhibitor (SLPI). Genetic variability of α₁ antitrypsin that leads to a deficiency in circulating levels is a risk factor for COPD. Cigarette smoking may lead to oxidized and impaired α₁ antitrypsin activity, resulting in lack of counterbalance against the activity of neutrophil elastase (46). SLPI activity may also be reduced by oxidative stress (25). As for the other protease family MMPs, their activity can be counteracted by tissue inhibitors of MMPs (TIMP). Alveolar macrophages from normal subjects release increased amounts of TIMPs after pro-inflammatory stimulation, indicating a counterbalance to the release of MMPs. The increased release of TIMPs is reduced in the alveolar macrophages from COPD patients, which may lead to excess activity of MMPs (214).

1.1.4.4. Apoptosis

In addition to the excess protease activity, the parenchymal destruction may also be contributed to by increased cellular apoptosis in lung (67). Apoptosis and proliferation are well equilibrated in healthy tissue. Increased apoptosis in endothelial cells, alveolar epithelial cells, interstitial cells, neutrophils and lymphocytes has been
reported in tissue sections of the lung from patients with COPD compared to healthy
subjects (223). Another study demonstrated that lung tissue from patients with COPD
exhibited increased apoptosis of alveolar epithelial cells, endothelial cells and
mesenchymal cells in association with an increased amount of the active subunit of
caspase-3, a central mediator of apoptosis (116). Moreover, persistent apoptosis of
alveolar epithelial cells and T-lymphocytes in bronchial bushings and bronchoalveolar
lavage has been observed after smoking cessation, as indicated by comparison
between ex-smokers with COPD, current smokers with COPD and healthy
never-smokers (103). Increased apoptosis in alveolar cells in the absence of adequate
compensation by proliferation leads to the gradual destruction of alveolar walls in the
lung (67). Apoptosis of alveolar wall and endothelial cells are reported to induce
emphysema even without infiltration of inflammatory cells in an animal model (67).

The increased apoptosis in the lung may be driven by multiple mechanisms which
are currently not well understood. Oxidative stress-induced attenuation of vascular
endothelial growth factor (VEGF) activity may be a mechanism of the elevated
apoptosis in COPD. It has been reported that increased apoptosis in epithelial and
endothelial alveolar septal cells in patients with emphysema is associated with
reduced expression of VEGF and VEGF receptor 2, which may lead to the endothelial
septal death (127). The level of VEGF in induced sputum from patients with COPD
has also been reported to decrease in severe COPD, in association with increased
levels of oxidative stress (125). In animal models, apoptosis induced by inhibition in
VEGF receptor (128, 199, 255) or VEGF (249) leads to emphysema even without
obvious inflammation. The relationship between oxidative stress and apoptosis may also be through mitochondrial dysfunction. Mitochondria are the major source of cellular ROS production of cells under oxidative stress (134). Airway smooth muscle cells (ASMCs) from patients with COPD have demonstrated increased mitochondrial ROS compared to healthy subjects (273). In general, mitochondrial dysfunction can be a potent driving force in inducing apoptosis through the mitochondrial pathways (90), but more evidence is required to reveal the exact relationship between mitochondrial damage and apoptosis in COPD. The inflammatory characteristic of COPD may also contribute to apoptosis. The phagocytic ability of alveolar macrophages against apoptotic airway epithelial cells is impaired in patients with COPD (90), leading to insufficient clearance of apoptotic airway epithelial cells. The recruitment of CD8+ cells into the lung may also induce an increase in apoptosis of structural cells, by secretion of cytotoxic mediators such as perforins, granzymes and TNF-α (26, 158). Indeed, increased infiltration of CD8+ T cell to alveolar walls is associated with increased apoptosis in the lung (166).

Cell death, including apoptosis, is constantly counterbalanced by proliferation under normal physiological conditions (67). However, whether the proliferative activity is able to counterbalance increased apoptosis remains unclear (67). Increased proliferative activity has been reported in the alveolar wall of patients with emphysema (116, 287), which may indicate a compensatory mechanism to the increased apoptosis. However, another report demonstrated similar proliferation of the alveolar septal cells between patients with emphysema and healthy subjects with
increased apoptosis of alveolar epithelial cells in patients with end-stage emphysema (44).

1.1.4.5. Ageing

Age is a risk factor for COPD. On the one hand, age is associated with longer exposure history to noxious gas or particles, on the other hand, the process of ageing may also contribute to the pathogenesis of COPD (119). Lung function starts to decline approximately after the age of 30 as a result of normal ageing (198), but in patients with COPD the decline is accelerated (79, 163). The decline in lung function of ageing lungs may be explained by progressive distal airspace enlargement, which leads to loss of lung elastic recoil (120). Although the airspace enlargement is not as destructive as is the case in lungs of patients with COPD (262), the similarity of the functional and structural changes between ageing lungs and lungs of patients with COPD suggests a possible relationship between them. COPD is characterized by persistent oxidative stress, while oxidative stress-induced DNA damage is believed to play an important role in cellular senescence according to the free radical theory (99). Senescence of cells is associated with shortening of telomere length (TL) (218). Indeed, it has been reported that TL of circulating lymphocytes is shortened in smokers in a dose-dependent manner, but the effect was not amplified in smokers with COPD (178). Another study demonstrated TL shortening in circulating leucocytes from patients with COPD compared to healthy subjects of any age range (110, 219). TL of alveolar epithelial cells, endothelial cells (254) and fibroblasts (180) are also shortened in patients with emphysema compared with non-emphysematous subjects.
Whereas oxidative stress may drive an accelerated ageing process, the ageing process itself may also contribute to oxidative stress and inflammation, which is evident from multiple animal studies. Acute CS exposure of mice has been reported to induce a five-fold increase of GSH levels in BALF in two-month old mice, indicating an anti-oxidant response. In mice of increasing age, these effects gradually diminished, in association with increased inflammatory marker TNF-α and oxidative stress marker 8-hydroxy-2-deoxyguanosine in BALF (88). Another study suggested that older mice had more neutrophils, inflammatory mediator keratinocyte-derived chemokine (KC, a mouse homolog of IL-8) and MIP-2 in lungs after 9 days of cigarette smoke (177).

1.1.4.6. Bronchial epithelial cells and airway smooth muscle cells in COPD

Bronchial epithelial cells are the first cellular defensive barrier against noxious gases and particles in lung. The epithelial injury caused by CS leads to the onset of oxidative stress and inflammation in the lung. The direct exposure to CS induces the intracellular release of endogenous molecules named danger-associated molecular patterns (DAMP) which can be identified by pattern-recognition receptors (PRR) e.g. Toll-like receptor 2 and 4 (190). This process activates epithelial cells to synthesize and secrete early inflammatory mediators such as TNF-α, interleukin (IL)-1β and IL-8 (65). The release of these chemokine leads to recruitment of inflammatory cells such as macrophages and neutrophils. Bronchial epithelial cells can also facilitate the onset of the adaptive immune response in COPD. Dendritic cell migration, maturation and activation are reported to be regulated by bronchial epithelial cells by releasing
various chemokines such as macrophage inflammatory protein (MIP)-3α and chemokine ligand 20 (CCL20) (66, 201). In addition to the initiation of the inflammatory responses, bronchial epithelial cells also contribute to the persistent inflammation in COPD by releasing multiple cytokines and chemokines including IL-1β, TNF-α, IL-6, IL-8 and GM-CSF. For example, epithelial cells from smokers with COPD express higher levels of mRNA and protein of monocyte chemotactic protein 1 (MCP-1) and IL-8 compared to the cells from smokers without COPD (64). Bronchial epithelial cells also play an important role in the anti-oxidant system in the lung. As mentioned in previous sections, epithelial cells from patients with COPD demonstrated reduced ability to elevate GSH production in response to oxidative stress (228). In addition, bronchial epithelial cells also serve as a source of the anti-protease SLPI (25).

Patients with COPD present with an increased mass of airway smooth muscle (ASM) in small airways (37, 59), correlating with declining lung function (57). The thickened airway smooth muscle layer contributes to a thicker airway wall, which plays an important role in the airflow obstruction of COPD (56). Due to its contractile properties, ASM is the major modulator of bronchomotor tone in the airway. Exaggerated constriction of the airways is a feature of patients with COPD, and relaxation of contracted ASM by bronchodilators is a major therapeutic intervention in the treatment of COPD (19). The role of ASM in the pathogenesis of COPD is not limited to its contractile properties. Similar to epithelial cells, ASM cells (ASMCs) also release inflammatory cytokines and chemokines including IL-6, IL-8, MCP-1,
MCP-2, MCP-3, growth-related oncogene (GRO)-α, IFN-γ-inducible protein (IP)-10 and GM-CSF (56). In addition, increased levels of transforming growth factor-β (TGF-β) in patients with COPD can lead to reduced expression of catalase and manganese-SOD in ASMCs, resulting in reduced anti-oxidant activity (174).

Unlike bronchial epithelial cells, ASMCs are usually not directly exposed to noxious particles or gases, although this may happen when the epithelial barrier is severely damaged by CS (102, 226). Therefore, the release of inflammatory mediators from ASMCs is most likely not the result of a direct response to CS, but rather induced by the effect of cytokines released by neighboring cells, e.g. epithelial cells. As mentioned earlier, CS causes direct injury to bronchial epithelial cells leading to release of inflammatory mediators. Amongst them, IL-1β and TNF-α can induce the release of IL-8 from ASMCs (121), and IFN-γ and TNF-α can induce the release of IP-10 from ASMCs (98). Moreover, bronchial epithelial cells may also contribute to the increased TGF-β levels in COPD. An in vitro study demonstrated that epithelial cells from smokers and patients with COPD secreted higher levels of TGF-β than the cells from normal subjects (248). As mentioned earlier, high levels of TGF-β can reduce the anti-oxidant activity in ASMCs. Therefore the secretory activities of cytokines and a defective anti-oxidant system in ASMCs may partly result from an interaction with injured epithelial cells.

1.1.4.7. Mitochondria and COPD

Mitochondria are double-membrane organelles with a unique structure and
important function. They are composed of an outer membrane, inner membrane inter-membrane space and matrix (11). The inner membrane is highly folded into cristae, and is impermeable to ions. Respiratory chain complex proteins reside on the inner membrane where they mediate electron transport chain and the subsequent accumulation of protons in the inter-membrane space (170). The proton gradient between the two sides of the inner membrane leads to the flux of proton back to matrix through adenosine triphosphate (ATP) synthase (Complex V) and drives the synthesis of ATP (170). The electrochemical gradient across the inner membrane is indicated by the mitochondrial membrane potential ($\Delta \Psi_m$) (170). The electron leakage from oxidative phosphorylation is believed to be a major source of ROS production in cells. It has been estimated that 0.2-2.0% of oxygen consumption by mitochondria results in formation of superoxide radicals (149, 165). Leakage of electrons from the electron transport chain takes place at Complex I towards the matrix and at Complex III towards both sides of the inner membrane (97, 149, 165). Leaked electron leads to partial reduction of oxygen. The resulting superoxide radicals can act as precursors for many other more active ROS, or they can be converted to hydrogen peroxide by SOD1 (superoxide dismutase 1) in the matrix or SOD2 (superoxide dismutase 2) in the inter-membrane space (97, 149, 165). Superoxide is believed to be short-lived while hydrogen peroxide is more stable and permeable to other part of mitochondria and cells (149, 170). The level of hydrogen peroxide has been estimated to be 100-fold higher than the level of superoxide radicals in mitochondria (43). In general, mitochondria are not rich in catalase, and
the reduction of hydrogen peroxide relies on the reaction with GSH catalyzed by glutathione peroxidase (149) (Figure 1.5). As the major source of ROS, mitochondria are also a target of ROS-mediated damage. Excess mitochondrial ROS may induce damage and mutations of mitochondrial DNA (170, 281). ROS may also induce carbonylation and dysfunction of various mitochondrial proteins, including the complex proteins leading to defective mitochondrial respiration, and the mitochondrial carrier proteins regulating molecular transport across the membrane, and the protein assembly of permeability transition (PT) pore which may trigger signals of apoptosis (170).

ΔΨm indicates the electrochemical gradient across the inner membrane, which results from the activity of the electron transport chain (170). As ROS is a byproduct of the electron transport chain, under the normal conditions where there is an ROS/anti-oxidant equilibrium, ΔΨm and ROS should be positively correlated. While this is supported by several studies (149, 165, 188), under conditions of cellular damage ΔΨm reduction is associated with increased ROS (9, 273). Reduction of ΔΨm may be a result of impaired proton transfer due to an interrupted electron transport chain, or be caused by opening of PT pores resulting in increased permeability.

Mitochondria are highly dynamic organelles under constant cycles of fission and fusion (183). Fission refers to the splitting of mitochondria leading to short and less branched morphology while fusion is the merging of mitochondria leading to an elongated and a more branched network (149). These morphological dynamics are very important for normal function of the mitochondrial network in a cell.
Interruption of either the fusion or fission process causes dysfunction of mitochondria (183). This might be because the exchange of mitochondrial DNA in matrix during the fission and fusion can alleviate the effect of mutation (183).

In addition to their role in energy supply and redox regulation in the cells, mitochondria play a central role in the regulation of apoptosis, senescence and autophagy (5, 11, 149, 170). Excess ROS may induce mitochondrial PT pores into a high-conductance state, leading to rising permeability for small molecules (83). This event is reflected by loss of ΔΨm. As the inner membrane is highly folded, the fluid influx lead to swelling of the inner membrane, resulting in further opening of mitochondrial PT pores and sometimes rupture of outer membrane (123). During this process cytochrome c is released from mitochondria, which triggers activation of caspase-9 and leads to apoptosis (90). In addition to the effects on PT pores, oxidative stress can also lead to peroxidation of cardiolipin, a lipid that immobilizes cytochrome c in the inner membrane, which further promotes the release of cytochrome c from mitochondria (124, 193).

There is an increasing focus on the involvement of mitochondria in the pathogenesis of COPD (11). Most direct evidence has come from primary cells from the patients with COPD. It has been demonstrated that primary bronchial epithelial cells from ex-smokers with severe COPD exhibited swelling and fragmented mitochondrial morphology which is similar to that seen in CS medium (CSM)-treated immortalized bronchial epithelial cells (BEAS2-B) (105). Another study on ASMCs has been reported by Wiegman and colleagues with a more comprehensive evaluation
of mitochondrial function (273). In this study ASMCs from patients with COPD demonstrated elevated mitochondrial ROS levels, reduced ΔΨm, reduced amount of Complex I, III and V compared to healthy controls. Moreover, mitochondrial respiration was impaired in ASMCs with COPD as indicated by reduced basal and maximum respiration levels, and respiratory reserve capacity. Healthy smokers also demonstrated mitochondrial dysfunction compared to healthy controls, but the effects are less pronounced compared to patients with COPD. Several other studies also suggested mitochondria of respiratory striated muscle and skeletal muscles are damaged in patients with COPD (5).

Mitochondrial damage is also observed in other COPD-related animal models or CSM-treated *in vitro* models. It has been reported that CS exposure can impair energy metabolism in mouse lung by switching glucose metabolism to the pentose phosphate pathway and reducing the substrate supply to mitochondria (4). In a chronic ozone-induced mouse model of COPD, elevation of mitochondrial ROS and reduction of ΔΨm is observed, which can be alleviated by the mitochondria-targeted anti-oxidant MitoQ (273). The alleviation of ozone-induced mitochondrial ROS leads to attenuation of ozone-induced airway hyper-responsiveness (AHR) and inflammation, indicating a potential strategy to target mitochondrial ROS for the treatment of COPD. Another study has demonstrated that wood smoke exposure to guinea pigs can induce impaired oxygen consumption activity and complex protein enzyme activity (89). CSM was also reported to alter the fission-fusion activity of mitochondria *in vitro* in both airway epithelial cells (105) and ASMCs (10), leading to
mitochondrial fragmentation and increased mitochondrial ROS.

Figure 1. 5 Generation of mitochondrial ROS from electron transport chain. Leakage of electron from electron transport chain leads to generation of superoxide radicals, which can be converted to hydrogen peroxide by SOD. I-V: complex proteins; Q: coenzyme Q; Cyto c: cytochrome c; SOD: Superoxide dismutase, GPx: Glutathione peroxidase. The figure is plotted based on Li X et al, J Hematol Oncol (149).

1.1.5. Current therapies

For patients with COPD who are current smokers, smoking cessation is the most effective approach that can influence the natural history of COPD. Smoking cessation can be facilitated by nicotine replacement therapies such as nicotine gum, inhaler, nasal spray, transdermal patch, sublingual tablet and lozenge, as well as supportive pharmacotherapy including varenicline, bupropion and nortriptyline (263).

Pharmacologic therapies for COPD include bronchodilators, corticosteroid and
phosphodiesterase-4 inhibitors (263). The choice of each medications or the combination of them is patient specific. Bronchodilators refer to the medications that induce the dilation of bronchi and bronchioles leading to increase in FEV$_1$ (263). There are three classes of bronchodilators, $\beta_2$-adrenoreceptor agonist, anticholinergics and methylxanthine (82). The $\beta_2$-adrenoreceptor agonists are further classified as short-acting $\beta_2$ agonist (SABA) and long-acting $\beta_2$-agonist (LABA), both working through induction of cellular cyclic adenosine monophosphate (cAMP). Bronchodilators are usually administrated through inhalation, and they are the central medication to manage the symptoms of COPD. Combination of different classes of bronchodilators may improve efficacy and reduce the side effects compared to a single class. Corticosteroid treatment is mainly adopted for the severe conditions. Oral corticosteroids aim to suppress the chronic inflammation, but they are generally associated with high risks of adverse effects (263). Inhaled corticosteroids in combination with bronchodilator may improve efficacy compared to single treatment, but the long-term safety and adverse effects of inhaled corticosteroids is largely unknown (263). Phosphodiesterase-4 inhibitors are a relatively new medication for COPD. They are able to reduce inflammation by inhibiting cAMP breakdown, and should be used with long-acting bronchodilators (263).

COPD is a complex, chronic disease resulting from long term interaction between genes and environmental stimuli (65). The pathogenesis of the disease is far from understood and the development of effective pharmacologic therapy has not been easy, especially for the control of chronic inflammation which is corticosteroid-resistant in
COPD (24). To date, the pharmacologic therapy is able to reduce COPD symptoms, exacerbations, and improve health status and exercise tolerance (263). However, none of current treatments is able to alleviate the progressive decline of lung function in COPD (263).

1.2. Introduction to Stem Cells

1.2.1. Definition and classification

Stem cells are cells that are able to self-renew and differentiate into other cell types (251). In contrast, somatic cells are usually able to self-renew but cannot differentiate, while progenitor cells are able to differentiate into somatic cells, but with poor ability of long-term self-renewal (251). Stem cells and progenitor cells act as sources of new somatic cells as a normal turnover or as a replenishment to increased somatic cell death after injury (251).

Stem cells can be classified as totipotent, pluripotent and multipotent according to their potency that is their capacity to differentiate (2, 251). Totipotent stem cells have the highest differentiation potential with capacity to develop into new individuals from a single cell. Although not able to form new individuals, pluripotent stem cells are capable of differentiating into cell types of all three germ layers (ectoderm, mesoderm and endoderm), which basically means they can generate almost any cell types except for extra-embryonic tissues such as trophectoderm (2, 251). Multipotent stem cells can form limited cell types, usually of one single lineage (2, 251). In general, the potency of stem cells gradually decreases along the development process.
(2, 251). Totipotent stem cells only exist as zygotes and cells from very early embryos, while stem cells derived from blastocyst named embryonic stem cells (ESCs) represent the major form of pluripotent stem cells (2, 251). Stem cells in the adult body, or adult stem cells, are multipotent stem cells (2, 251). They are usually distributed in specific microenvironments called niches of the parent tissues and generate somatic cells of the same origin (2, 251). The progenitor cells have similar differentiation features as the adult stem cells. They also reside at niches of tissues and differentiate into specific cell types (85).

Utilising stem cells therapeutically is not a recent idea. Haematopoietic stem cells (HSC) have been used in bone marrow transplantation for treatment of leukaemia for more than 40 years (41). Other cells that have been tested in clinical trials include mesenchymal stem cells (MSC) and muscle satellite stem cells (251, 269). When it comes to stem cell-based therapy, the stem cells can be alternatively classified as endogenous stem cells which are the adult stem cells in their niches in the body, and exogenous stem cells which are the isolated and expanded adult stem cells ex vivo (251).

1.2.2. Endogenous stem cells in lung

The delineation of endogenous stem cells in individual organs is not an easy task. The most convincing approach is to examine the capacity of the cells to reconstitute the entire organ. For example, HSCs can rebuild the entire haematopoietic system after transplantation into irradiated animals (251). However the approach requires
destruction of organs which is rather difficult in most cases. An alternative approach is to genetically trace the lineage of cells in the organs, which can provide evidence of the differentiation of specific cell types into other cells. Intestinal stem cells (260) and skin stem cells (34) were identified through this approach.

The lung is a complex organ with a very low turnover rate during normal homeostasis compared with intestine or skin tissues (106). The regenerative capacity of human lung is mostly demonstrated upon injury, such as pneumonia and severe respiratory infections (28, 42, 131, 138). Whether there is a defective regenerative function of endogenous lung stem cells in airway diseases is still unclear (268). In fact, the identity and roles of stem cells and progenitor cells in the lung remains unresolved due to the lack of markers for each cell type and lineage tracing approaches (268). Various putative airway and alveolar epithelial stem and progenitor cells are identified by mounting data from animal and human studies, including airway submucosal glands, keratin-14 expressing tracheal basal cells, Clara cells in association with neuroendocrine bodies or bronchoalveolar duct junction, and type 2 alveolar epithelial cells (AEC2s) (85). However, there is also controversial data from other studies and there is not yet uniform agreement on the identity of lung stem cells (268).

As COPD is associated with progressive destruction of the alveolar structure, the identification of stem cells in alveoli, and further investigation of their role in COPD, may provide valuable information on the disease. Alveoli are mainly composed of type 1 alveolar epithelial cells (AEC1s) and AEC2s. AEC2s which express high levels of surfactant protein C (SFTPC) can function as local stem cells (21). Lineage tracing
in mice are demonstrated that AEC2s can differentiate into AEC1s during homeostasis, and bleomycin-induced injury promoted such activity (21). The self-renewal ability of SFTPC+ AEC2s was also observed for at least one year. Moreover, when co-cultured with a mesenchymal population, the SFTPC+ AEC2s can differentiate into alveolar structures consisting both AEC2s and cells with AEC1 markers (21). In combination with the self-renewal property in vitro, these findings demonstrated the capacity of AEC2s for self-renewal and differentiation into other cells both in vivo and in vitro.

1.2.3. Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent stem cells from stromal origin (269). Originally described as a fibroblastic-like population of bone marrow (BM) cells, MSCs now refer to similar cells found in a variety of tissues, including BM, adipose tissue, cord blood and placenta (269). MSCs can differentiate into chondrocyte, adipocyte, osteoblast or other lineages such as epithelial and endothelial cells (269). The general criteria to define MSCs include the cell marker profile, self-renewal property and the capacity of differentiating into osteoblasts, adipocytes and chondrocytes (251). Although MSCs isolated from different tissue share common features regarding these parameters, different gene expression, lineage tendencies and other properties have been demonstrated amongst them (15, 132). Moreover, individual MSC types may still present a heterogeneous group of cells of different subtypes (200). The functional and therapeutic differences between MSCs from different sources have been increasingly investigated (269).
Cells with features of MSCs have been isolated from adult mouse lungs, human nasal mucosa, and lungs of human neonates and lung transplant recipients (269). The functions of these cells in the lung remain unclear. They have been reported to play a potential role in fibrotic diseases and in the bronchiolitis obliterans following lung transplantation (14, 61, 210, 264).

MSCs express very low levels of major histocompatibility complex (MHC) class I and class II molecules, which makes them highly immunologically-tolerant (80, 132, 144, 269). Exogenous administration of allogeneic MSCs usually lead to low levels of immune rejections and does not require immunosuppression (251). MSCs are commonly used to develop exogenous stem cell-based therapy for lung diseases. There are numerous studies on this topic, which will be introduced in the later sections.

1.2.4. Induced-pluripotent stem cells (iPSCs)

As described earlier, in nature, cells demonstrate a unidirectional reduction in differentiation potency with development. Therefore, the access to pluripotent stem cells had relied on the isolation of ESCs until the discovery of induced-pluripotent stem cells (iPSCs) (247). iPSCs are pluripotent stem cells derived from somatic cells through transcriptional factor-induced reprogramming in vitro (e.g. through induction by Oct4, Sox2, Nanog and Lin28). Deriving iPSCs from somatic cells was first achieved by Takahashi and Yamanaka using mouse fibroblasts in 2006 (247). Human iPSCs were later generated also from fibroblasts in 2007 (246, 289). In theory, this
A robust approach can generate iPSCs from any somatic cell type.

Compared to the isolation of adult stem cells from the relevant tissue, the reprogramming of somatic cells is less restricted to the type of somatic tissue. Moreover, the high proliferation capacity of iPSCs reduces the amount of somatic tissue required. Therefore, iPSC provides an abundant supply of stem cells from each particular individual. However, the therapeutic application of iPSCs is limited by pluripotency. They are so potent that direct administration of iPSCs may lead to formation of teratomas (122).

Although iPSCs may not be a very good candidate as a direct therapy for airway disease, they can serve as a great source to generate other cell types that may be effective in airway diseases. Indeed, the derivation of lung progenitor cells or epithelial cells from iPSCs has been reported by several studies (91, 111, 179, 278, 279). AEC2 is the most common and prominent cell type derived from iPSCs, while AEC1 can also be generated. The iPSC-derived epithelial cells can be used in ex vivo tissue engineering of lung, providing cellular source to re-populate decellularized whole lung scaffolds (86, 159). However, information of their effects as direct source of cell-based therapy for airway disease is still lacking.

1.2.5. iPSC-derived MSCs

Given the numerous studies on the therapeutic potential of MSCs in airway disease models, deriving MSCs from iPSCs provides a promising strategy. This task was achieved by Lian and colleagues in 2010 (153) (Figure 1.6). The derivation was
through incubation of iPSCs in pro-MSC induction medium (detailed methodology can be found in Chapter 2). MSC-like cells were identified as CD24- and CD105+ cells. The resulting cell culture exhibited fibroblast-like morphology which is typical of MSCs. To acquire homogenous populations, single-cell derived colonies were generated from these cells. In addition to the fibroblast-like morphology, the derived cells demonstrated other features of MSC. Firstly, they presented an MSC-like surface marker profile (CD44+, CD49a+ CD49e+, CD73+, CD105+, CD166+, CD34−, CD45−, and CD133−). Secondly, the differentiation capacity was demonstrated by efficient adipogenesis, osteogenesis and chondrogenesis. Lastly, they were able to self-renew without loss of differentiation potential. In fact, they were far more expandable than BM-MSCs, with an ability to keep their differentiation capacity up to passage 40. As they fulfil all criteria of MSCs, these cells were named iPSC-derived MSCs (iPSC-MSCs). Compared to iPSCs, iPSC-MSCs demonstrated reduced expression of pluripotency-associated genes such as Oct4, Nanog and Sox2. When subcutaneously injected into combined immunodeficient (SCID) mice, iPSC induced teratoma while no teratoma was induced by iPSC-MSCs for up to four months.

The in vitro generation of iPSC-MSCs from iPSCs brings potential opportunities to overcome several limitations of the adult MSCs as seen in BM-MSCs. The amount of BM-MSCs available from BM is limited which makes ex vivo expansion important (269). However, BM-MSCs become senescent after 20 doublings during ex vivo expansion with loss of differentiation potential (69). Their genetically stability in vitro also draws concerns as they may develop abnormal karyotypes during expansion
The limited in vitro expansion capacity restricted the resource for cell therapy. In addition, one of the future directions of MSCs-based therapy is to genetically manipulate the cells. As MSCs can be recruited to injured tissue, by this approach they can express and release desired molecules at the site of the injured tissue (63, 122). Such genetic manipulation also calls for high stability and expanding capacity of the MSCs. In contrast to BM-MSCs, iPSC-MSCs demonstrated normal karyotypes during expansion (153). They can be expanded for 120 doublings with stable surface marker profile for MSCs (153). Another limitation of BM-MSCs is that when isolated from aged subject or patients with ageing-related disorders, they may present reduced survival and differentiation ability (87, 100, 267, 294). In theory, as the safety of allogeneic MSCs has been well demonstrated, this issue can be avoided by selection of the donor. Nevertheless, availability of personalized MSCs is appealing for future development of MSCs-based therapy. iPSC-MSCs may hold hope as a way of overcoming the ageing-associated impairment of BM-MSCs. When generating iPSC-MSCs, somatic cells were firstly reprogrammed to an embryonic stem cell-like state, which may help to avoid the problem of senescence. According to the same study by Lian and Zhang et al., the telomerase activity of iPSC-MSCs was 10 times higher compared to BM-MSCs (153).

As a relatively new type of MSCs, beneficial effects of iPSC-MSCs have already been demonstrated in rodent models of limb ischemia (153), allergic airway inflammation (244) and cardiomyopathy (293).
1.3. MSCs-based Therapy

1.3.1. Efficacy of MSCs in animal models

MSCs are the most studied candidates for the development of cell-based therapy against lung diseases. Efficacy of MSCs was demonstrated in a broad spectrum of animal models including acute lung injury and bacterial lung infection, COPD, asthma, bronchiolitis obliterans, bronchopulmonary dysplasia, fibrosing pulmonary injury, pulmonary hypertension, pulmonary ischemia re-perfusion injury, obstructive sleep apnea, radiation-induced lung injury, sepsis and burns (269). The studies of MSCs in COPD-related models are listed in Table 1.2. In general, these models can...
exhibit histological changes (primarily airspace enlargement) in the lung mimicking the lungs of patients with COPD, which are alleviated by MSCs from various sources including BM, adipose tissue, lung and amniotic fluid. The major approaches for inducing emphysema in animals are by administration of elastase and by chronic exposure to CS. Elastase can cause excess protease activity rapidly leading to direct destruction of alveolar structures, while CS exposure can induce inflammation very quickly but with the airspace enlargement developing much later (usually months after exposure). However, as cigarette smoking is the major risk factor of COPD, the CS-exposed model may be more relevant to the pathogenesis of COPD. CS-exposed animals have been demonstrated to develop inflammation, oxidative stress and excess protease activity both in lung and systemically (51, 52). The capacity of alleviating CS-induced inflammation is demonstrated in MSCs from BM (94, 112), amniotic fluid (151) and adipose tissue (222). Moreover, the MSCs from BM and adipose tissue also inhibited CS-induced apoptosis in the lung.
<table>
<thead>
<tr>
<th>Studies</th>
<th>Experimental Model Route, and Timing of Treatment</th>
<th>Source of MSCs</th>
<th>Outcome Compared to Injury Effects</th>
<th>Potential Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shigemura 2006 (229)</td>
<td>Rat: elastase. i.v. ASCs 1 week after elastase.</td>
<td>Rat adipose Plastic adherent</td>
<td>• Decreased apoptosis, improved histological repair</td>
<td>HGF secretion</td>
</tr>
</tbody>
</table>
| Yuhgetsu 2006 (290)  | Rabbit: elastase. i.t. MSCs 24 hours after elastase.                                                                    | Rat BM - mononuclear cells from Ficoll gradient of total BM | • Improved histology and lung function  
• Decreased BALF inflammation  
• Decreased tissue MMP2 and MMP9 expression                                                                 | None specified (soluble mediators)     |
| Zhen 2008 (295)       | Rat: papain. i.v. MSCs after papain (timing not specified).                                                            | Rat BM Plastic adherent mononuclear cells from Percoll gradient | • Decreased histological injury  
• Decreased alveolar cell apoptosis                                                                 | None specified             |
<table>
<thead>
<tr>
<th>Authors</th>
<th>Species</th>
<th>Treatment</th>
<th>Source Cells</th>
<th>Findings</th>
<th>Control Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhen 2010 (296)</td>
<td>Rat: papain. i.v. MSCs 2 hours after papain</td>
<td>Rat BM Plastic adherent mononuclear cells from Percoll gradient</td>
<td>• Decreased histological injury • Partial restoration of VEGF expression in lung homogenates</td>
<td>None clarified (soluble mediators)</td>
<td></td>
</tr>
<tr>
<td>Katsha 2011 (130)</td>
<td>Mouse: i.t. elastase. i.t. MSCs 14 day after elastase.</td>
<td>Mouse BM Plastic Adherent</td>
<td>• Decreased histological injury • Decreased IL-1β • Transient increase in lung EGF, HGF, and SLPI</td>
<td>None clarified (soluble mediators)</td>
<td></td>
</tr>
<tr>
<td>Huh 2011 (112)</td>
<td>Rat: CS exposure for 6 months. Retrobulbar MSCs at the beginning of smoke cessation.</td>
<td>Rat BM</td>
<td>• Decreased histological injury • Increased numbers of small vessels and reduced RVSP • Increased proliferation of AEC2 cells and endothelial cells • Decreased apoptosis</td>
<td>Soluble mediators Akt signaling suspected</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Cell Source and Treatment</td>
<td>BM Source and Processing</td>
<td>Findings</td>
<td>Toxicant Specified</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------</td>
<td>--------------------------</td>
<td>--------------------------------------------------------------------------</td>
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<td></td>
</tr>
</tbody>
</table>
| Schweitzer 2011 (222) | Mouse: CS exposure for 4 months. i.v. ASCs every other week for 3rd and 4th months of CS exposure. | Human or mouse adipose plastic adherent | • Decreased histological injury and apoptosis (caspase activation)  
• Decreased BALF inflammation  
• Decreased CS-induced MAPK signal transduction  
• Decreased weight loss  
• Decreased BM suppression | None specified |
|                  | Immunodeficient NOD-SCID mouse: VEGFR blockage induced emphysema. Human ASCs 3 days after VEGFR blockade | Human adipose plastic adherent | • Human ASCs decreased histological injury and caspase 3 activation following VEGFR blockade  
• Emboli if used > $5 \times 10^5$ MSCs or if > passage 3 | None specified |
| Hoffman 2011 (104) | Mouse: i.t. elastase i.v. MSCs 6-7 weeks after elastase | Mouse BM Lung-MSCs from primary explants cultures | • Higher retention of lung MSCs  
• Decreased histological injury | None specified |
<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Treatment</th>
<th>Organ</th>
<th>Findings</th>
<th>Comments</th>
</tr>
</thead>
</table>
| Ingenito  | Sheep   | i.t. elastase (5 doses/20 weeks) | Sheep lung | • Increased lung tissue mass and perfusion  
• Increased histological cellularity, cell retention and extracellular matrix  
• Improved lung mechanics | Paracrine effects. Increased epithelial proliferation in *in vitro* co-culture. |
| Guan      | Rat     | CS exposure for 11 weeks, sacrificed after 4 weeks cessation. i.t. MSCs on the first day of week 7 | Rat BM | • Decreased histological injury  
• Increased lung function  
• Decreased lung apoptosis  
• Decreased TNF-α, IL-1β, MCP-1, IL-6, MMP9 and MMP12 in lung  
• Increased VEGF, VEGF receptor-2 and TGFβ1 in lung | Non specified                           |
| Song      | Rats    | CS exposure for 7 weeks, sacrificed after 4 weeks cessation. i.t. MSCs at week 8 | Rat BM | • Increased histological injury  
• Increased lung function  
• Decreased inflammatory cells in BAL  
• Decreased levels of IL-1β, IL-6, TNF-α, MDA and increased TGFβ1 in lung | Non specified                           |
| Tibboel   | Mouse   | i.t. elastase i.t. or i.v. MSCs before or after elastase | Mouse BM | • Increased lung function only by i.v. injection prior elastase  
• No significant histological difference | Non specified                           |
<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Study Design</th>
<th>Treatments</th>
<th>Outcomes</th>
<th>Control/Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li X 2014 (150)</td>
<td>Rat: CS exposure for 56 days i.v. MSCs at day 29 and 43</td>
<td>Human iPSC-MSCs</td>
<td>• Decreased histological injury</td>
<td>Mitochondrial transfer</td>
</tr>
<tr>
<td>Li Y 2014 (151)</td>
<td>Rat: CS exposure for 12 weeks plus i.t. LPS at week 4 and 8. i.t. MSCs after CS exposure period</td>
<td>Rat amniotic fluid</td>
<td>• Decreased histological injury • Increased surfactant protein A and C • Decreased AEC2 apoptosis</td>
<td>Differentiation into AEC2-like cells Anti-apoptotic mechanism unknown</td>
</tr>
<tr>
<td>Gu 2015 (93)</td>
<td>Rat: CS exposure for 12 weeks sacrificed after 4 weeks cessation. i.t. MSCs started at week 7, twice/week for 5 weeks</td>
<td>Rat BM</td>
<td>• Decreased histological injury • Decreased inflammation • Decreased PGE-2 and IL-6 and increased IL-10 in BALF and serum • Decreased COX-2 and PGE2 in lung</td>
<td>Inhibition of COX-2/PGE2 in alveolar macrophages by p38 MAPK pathways</td>
</tr>
</tbody>
</table>

**Abbreviations:**
- **AEC2**: Type 2 Alveolar epithelial cell
- **ASC**: adipose stem cell
- **BALF**: bronchoalveolar lavage fluid
- **BM**: bone marrow
- **COX-2**: cyclooxygenase-2
- **CS**: cigarette smoke
- **EGF**: epidermal growth factor
- **HGF**: hepatocyte growth factor
- **IL**: interleukin
- **iPSC-MCS**: induced pluripotent stem cell-derived mesenchymal stem cells
- **i.t.**: intratracheal
- **i.v.**: intravenous
- **MAPK**: mitogen-activated protein kinase
- **MCP-1**: monocyte chemoattractant protein-1
- **MDA**: malondialdehyde
- **MMP**: matrix metalloproteinases
- **PGE2**: prostaglandin E2
- **RVSP**: right ventricle systolic pressure
- **SLPI**: secretory leukoprotease inhibitor
- **TGF-β**: transforming growth factor-β
- **TNF-α**: tumor necrosis factor
- **VEGF(R)**: vascular endothelial growth factor (receptor)

The list before (including) 2012 is adapted from two reviews by Weiss *et al* (269, 270); studies after 2012 are described from corresponding references.
1.3.2. Clinical trials

The safety and efficacy of MSCs have been tested in acute myocardial infarction and several inflammatory and autoimmune diseases such as graft-versus-host disease (GVHD), autoimmune diabetes and Crohn’s disease (269). The first licensed MSCs-based therapy has been approved in Canada, as a medication for GVHD (8, 269). The intensive clinical study in these fields showed an important fact that MSCs may be well tolerated even by patients with very severe illness (8, 269).

The mounting evidence supporting the efficacy of MSC in COPD-related animal models led to the initiation of the first multicenter, double-blind, placebo-controlled phase II clinical trial of a systemic-administrated BM-MSC preparation (PROCHYMAL, Osiris Therapeutic Inc, Columbia, MD, USA) for moderate or severe COPD (271). 62 patients participated in the clinical trial, during which they received 4 monthly infusions of BM-MSCs (100x10⁶ cells/infusion) or vehicle controls and were monitored in a subsequent 2-year period. End points included comprehensive safety evaluation, pulmonary function testing (PFT), and quality-of-life indicators including questionnaires, 6-minute walk test, and assessments of systemic inflammation. The trial proved the safety of BM-MSCs in patients with moderate or severe COPD. No pulmonary emboli formation or other infusional toxicity was observed for every single infusion among all the 4 infusions of all patients. No mortality or serious adverse effects were observed in the two-year follow-up. However, the administration of BM-MSCs did not improve the lung function of the patients compared to the placebo, as well as the quality of life.
indicators such as 6-minute-walk evaluation and quality of life questionnaire. The only effect observed was on the systemic C-reactive protein (CRP) levels, which may indicate a modulation of systemic inflammation. Among the patients with high systemic CRP levels (above 4 mg/L) at the start of the trial, infusion of BM-MSCs significantly reduced the systemic CRP levels within the first month and the trend persisted for 2 years although the statistical significance was lost over time.

A multicentre, open-label, dose-escalation, phase I clinical trial on treatment with BM-MSCs in moderate to severe acute respiratory distress syndrome (ARDS) in USA has been reported recently (276). Nine patients were included, and three dosages were tested (10^6, 5x10^6, 10x10^6 cells/kg predicted body weight). The findings demonstrated safety of the single infusion into patients with ARDS, with no adverse effects or mortality attributable to the infusion. The trial has entered a phase II stage for examination of efficacy. Another placebo-controlled study demonstrated safety of adipose-derived MSCs infused in patients with ARDS (297). However, no significant efficacy was observed in this study.

To date two non-randomized phase I clinical trials in patients with idiopathic pulmonary fibrosis have been performed. In the first placenta-derived MSCs were administrated intravenously (50) and in the second endobronchial infused adipose derived stromal cells-stromal vascular fraction were used (256). Both these phase I trials demonstrated good safety profile of MSCs in patients with idiopathic pulmonary fibrosis.

Although the efficacy of MSCs in lung diseases has not been demonstrated in any
studies to date, the above studies proved strong evidence of the safety of MSCs infusions. There are many variables regarding the potential functions of MSCs, including dosage, preparation, and tissue source, and more investigation is required to define its clinical role. The lack of efficacy in the clinical trial in contrast with the numerous reports on the efficacy in animal models also calls for better pre-clinical models and more information about the mechanisms of action of MSCs.

1.3.3. Mechanisms

1.3.3.1. Engraftment and regeneration

While intratracheal administration definitely leads to the delivery of MSCs to the lung, systemic-administrated MSCs have also been reported to localize in the lung vascular bed initially, and the number of localized cells can be enhanced by lung injury (269). The retention ability of systemic-administrated MSCs in lung varies in different studies. The effects of the MSC properties such as size, expression of specific integrins and proteoglycan patterns on the retention ability remain unclear. MSCs from different sources present different retention capacities. For example, MSCs from umbilical cord blood have been reported to be more easily cleared by the lung compared to BM-MSCs (185). In CS-exposed models, adipose stem cells have been reported to retain in lung tissue after 7 days or 21 days (222), while another study only detected 2-13 male BM cells per entire lung from 1 day to 1 month by fluorescent in situ hybridization (FISH) (112). Higher retention rate of iPSC-MSCs in the lung compared to BM-MSCs has also been reported 14 days after administration
The capacity of BM-MSCs and adipose-MSCs to differentiate into cells expressing markers of alveolar or epithelial cells has been demonstrated in vitro (162, 171, 243, 282), which represents an early motivation for research on MSCs-based therapy: to let the MSCs differentiate into progenitor or somatic cell types, replenish the damaged cells and thus regenerate the injured tissue. Indeed, early reports provided evidence of structural-engrafted HSCs, MSCs or endothelial progenitor cells differentiating into airway and alveolar cells or pulmonary vascular or interstitial cells (129, 270). However, later research suggested some major technical limitations of these early studies and current understanding regarding the issue is that the differentiation of MSCs into structural cells in the lung is rare and with unlikely physiological significance (270).

Although the retention of MSCs does not lead to differentiation, it may induce other activities. The retention is possible to modulate paracrine function of MSCs, such as secretion of the anti-inflammatory cytokine tumor necrosis factor-stimulated gene-6 (TSG-6) (145). The retention may also facilitate the direct contact between MSCs and local cell types, which may induce the onset of other mechanism such as mitochondrial transfer.

1.3.3.2. Immunoregulation

Immunoregulation is a very significant function of MSCs. As mentioned above, treatment of the autoimmune disease GVHD using BM-MSCs has already been
approved for clinical use.

The role of endogenous MSCs in immunoregulation is still largely unknown. Endogenous MSCs are found to reside perivascularly, and they may represent a portion of the pericytes (182). At this site they can interact with HSC and may affect immune responses. The immunoregulating capacity of exogenous MSCs has been widely reported in vitro. They have been demonstrated to inhibit various functions of immune cell such as dendritic cell differentiation, B cell proliferation, cytolytic potential of natural killer cell, and proliferation and activation of T cell (33, 235, 269, 270). MSCs also facilitate the development of the M2 phenotype of macrophages, which is believed to be anti-inflammatory (81, 266, 286). In addition, MSCs are able to reduce the release of inflammatory mediators from these immune cells such as IFN-γ and promote secretion of anti-inflammatory cytokines such as IL-10. The full mechanism of the immunomodulation is unclear but the paracrine release of immunomodulating agents including of TGF-β1, HGF, prostaglandin E₂ (PGE₂), IL-10 and nitric oxide from MSCs may play an important role in the process (2, 235, 270).

1.3.3.3. Mitochondrial transfer

Mitochondrial transfer was first observed in vitro in 2006 by Spees and colleagues (238). In their study they damaged mitochondrial DNA in a lung epithelial cell-line A549 by pre-treatment with ethidium bromide, leading to impaired aerobic respiration and proliferation. The mutated A549 cells only survive in a permissive medium containing uridine and pyruvate used to supplement anaerobic glycolysis.
However, after co-culture with BM-MSCs, these cells re-acquired the capacity to proliferate in normal medium, indicating restoration of aerobic respiration. Mitochondrial function was recovered as indicated by ATP levels, and mitochondrial transfer was identified from BM-MSCs to mutated A549 cells both by DNA measurement and microscopy. Following this study, mitochondrial transfer from BM-MSCs to other cell types including endothelial cells, cardiomyocytes and osteosarcoma cells *in vitro* has also been reported (55, 192, 204).

In 2012, Islam and colleagues reported the first *in vivo* study demonstrating the occurrence and functional significance of mitochondrial transfer from exogenous BM-MSCs to host airway epithelial cells (118). In the study acute lung injury was induced by intratracheal infusion of lipopolysaccharide (LPS), followed by intratracheal infusion of BM-MSCs. They found BM-MSCs attached to the alveolar epithelia at connexin 43-containing gap junctions. Mitochondrial transfer was observed within 24 hours from BM-MSCs to alveolar epithelial cells, which is mediated by connexin 43-dependent generation of nanotubes and microvesicles. The mitochondrial transfer elevated alveolar ATP concentrations. The therapeutic efficacy of BM-MSCs on the acute lung injury, including reduction of alveolar leukocytosis and protein leak, elevation of surfactant secretion and reduction of mortality, were eliminated in BM-MSCs with mutated connexin 43 or dysfunctional mitochondria.

Current understanding on the mechanisms of mitochondrial transfer is still very limited. Tunnelling nanotubes (TNTs) are identified as routes through which mitochondria move intracellularly in most *in vitro* studies. TNTs were first discovered
in vitro by Rustom and colleagues in 2004 as thin structures connecting single cells over long distance. TNTs are characterized with continuity of membrane connection between the two cells as well as continued cytoskeleton in the tubes (13). F-actin is found in most TNTs spanning along the entire length, and inhibition of F-actin polymerization lead to blockage of TNT formation (40). Machinery of intracellular movements of mitochondria in neuronal cells has been studied for many years. In neuronal cells, mitochondrial Rho GTPase 1 (Miro-1, also named Rhot-1), a calcium-sensitive adaptor protein, can bind mitochondria to kinesin motor protein-5 (KIF5) with the aid of accessory proteins Milton/TRAK (155). Kinesin can then carry the mitochondria to move along microtubes (155). It has been proposed that this mechanism may also mediate the intercellular movement of mitochondria along the TNTs (Figure 1.7). A recent study demonstrated that over-expressing of Miro-1 in BM-MSCs can promote mitochondrial transfer to epithelial cells and enhance efficacy in vitro, while Miro-1 knock-down leads to lack of efficacy (6). Over-expression of Miro-1 also enhanced the efficacy to attenuate rotenone-induced lung injury and allergic airway inflammation in vivo. This finding provided a possible approach to selectively block mitochondrial transfer, which may be very helpful for future research about the role of mitochondrial transfer in MSCs action. Nevertheless, the mechanisms regulating TNT formation or the Miro-1-involved movement of mitochondria remain unclear. Further understanding on the topic may help address the important feature of mitochondria transfer such as the direction selectivity and enhanced activity upon injury.
Figure 1. Proposed mechanism of mitochondrial transfer. BM-MSCs transfer mitochondria to damaged epithelial cells through tunneling nanotubes (TNTs). Miro-1 loads mitochondria on KIF5 with aid of accessory protein Milton/Trak. KIF-5 moves along microtubes in the TNTs and thus transfers mitochondria between cells. The image showing the enlarged region is adapted from Sheng ZH et al, Nat Rev Neurosci (227).
1.4. Aims of Study

The overall hypothesis for this thesis is that iPSC-MSCs are able to alleviate oxidative stress (CS or ozone)-induced lung damage (histological changes, hyper-responsiveness, inflammation, apoptosis and mitochondrial dysfunction) in vivo as well as mitochondrial dysfunction and apoptosis in bronchial epithelial cells and ASMCs in vitro, through multiple mechanisms including paracrine effect and mitochondrial transfer.

Based on previous sections, the background of the study can be summarized as follows:

1. COPD is a chronic and complex disease. It is a rising cause of death associated with huge social and economic burden. There are no current medications that can slow the progression of the disease. Cigarette smoking is the major cause of COPD. Age is also an important risk factor.
2. Mitochondrial dysfunction may play an important role in the pathogenesis of COPD. Targeting mitochondria may be a novel strategy to develop new treatment for the disease.
3. Efficacy of MSCs isolated from BM, adipose tissue and cord blood on CS-induced emphysema models has been demonstrated. BM-MSCs have been proven to be safe in clinical trials but without efficacy in COPD patients.
4. iPSC-MSCs have several advantages over BM-MSCs including more abundant resource, high capacity of expanding without loss of differentiation potency and
potential to overcome ageing-associated impairment.

5. The mechanisms of action of MSCs are not fully understood. Paracrine effects and mitochondrial transfer represents two possible mechanisms.

Based on the above knowledge, my Ph.D study focuses on the effects of iPSC-MSCs on airway cells in a COPD or oxidative stress-related context. The aims of the study include:

1. To compare effects of iPSC-MSCs and BM-MSCs on CS-induced damage in the rat lung in vivo and in human bronchial epithelial cells in vitro with an emphasis on apoptosis. Both mitochondrial transfer and paracrine effects were examined.

2. To study the capacity of iPSC-MSCs to alleviate mitochondrial dysfunction in CS-treated human ASMCs in vitro and to examine the relevant contribution of mitochondrial transfer and paracrine effects to the efficacy.

3. To investigate the capacity of iPSC-MSCs to alleviate oxidative stress-induced AHR, lung inflammation, mitochondrial dysfunction and apoptosis in an acute ozone-exposed mouse model.

These studies should provide evidence to demonstrate the potential of iPSC-MSCs as a candidate for cell-based therapy for COPD.
Chapter 2 Material and Methods
2.1 Materials

2.1.1 Reagents and chemicals

Chemicals, reagents and recombinant proteins were supplied by Thermo Fisher Scientific (Carlsbad, CA, USA), Sigma-Aldrich (St. Louis, MO, USA), Amresco (Solon, OH, USA), Bio-Rad Laboratories (Richmond, CA, USA), Cayman Chemical (Ann Arbor, MI, USA), Life Technologies (Eugene, OR, USA) and Roche (Mannheim, Germany). Cell culture reagents were purchased from Invitrogen (Thermo Fisher Scientific) and Sigma-Aldrich. Cigarettes including Camel (11 mg TAR and 0.8 mg nicotine, R.J. Reynolds, Winston-Salem, NC, USA) or Marlboro Red (10 mg TAR and 0.8 mg nicotine, Philip Morris International, New York City, NY, United States) were obtained from commercial retailers.

2.1.2 Experimental animals

For the in vivo study performed in Hong Kong, the procedures of the project and the personnel involved were under licenses from Department of Health, Hong Kong SAR and approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong. Male Sprague-Dawley (SD) rats (weighted 170-200 g) were purchased from and kept in the Laboratory Animal Unit, the University of Hong Kong.

For the in vivo study performed at Imperial College London, the procedures of the project and the personnel involved were under a Project License and Personal Licenses from the British Home Office, United Kingdom, under the Animals
(Scientific Procedures) Act 1986. Male C57BL/6 mice (6 weeks old) were purchased from Harlan UK Ltd (Wyton, UK) and kept in Central Biomedical Services, Imperial College London.

The living environment of all animals was monitored and controlled with respect to temperature, humidity, and day/night cycle. The animals’ general conditions were monitored daily.

2.1.3 Human iPSC-MSCs

The iPSC-MSCs were kindly provided by Dr. Lian Q and Professor Tse HF from the Cardiology Division, Department of Medicine, LKS Faculty of Medicine, HKU. The protocol to generate iPSC-MSCs includes two steps: inducing iPSCs from somatic cells, and generating iPSC-MSCs from the iPSCs. Firstly, iMR90 fibroblast cells (CCL-186, American Type Culture Collection, Manassas, VA, USA) were transduced with human OCT4, SOX2, NANOG and LIN28 genes through lentiviral vectors (Plasmid 16577-80, Addgene, Cambridge, MA, USA) overnight. The transduction gene mixture was then replaced with human embryonic stem cell culture medium and cells transferred to inactivated mouse embryonic fibroblast (MEF) feeder. 20 days later, cell colonies with human embryonic stem cell morphology were identified as iPSC-MSCs and propagated. The resulting iPSC lines were differentiated into iPSC-MSCs as previously described (153). iPSCs were harvested and placed on a gelatinized 10-cm dish containing knockout Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% serum replacement medium.
(Invitrogen), 10 ng/mL basic fibroblast growth factor (bFGF) (Invitrogen), 10 ng/mL platelet-derived growth factor AB (Peprotech, Rocky Hill, NH, USA), and 10 ng/mL epidermal growth factor (EGF) (Peprotech) to induce differentiation. Cells were left to differentiate for 7 days after which they were harvested and incubated with CD24-phycoerythrin and CD105-FITC (BD PharMingen, San Diego, CA, USA). The CD24^CD105^+ population were separated by fluorescence-activated cell sorting (FACS). The CD24^CD105^+ cells were diluted and seeded in 96-well plates to select wells containing a single cell. Upon reaching 70% confluence, the single cell-derived clones were serially sub-cultured to confluence in a 175-cm^2 tissue culture flask. They were then trypinised, aliquoted and frozen as stocks in liquid nitrogen for future experiments.

2.1.4 Human bone marrow – derived MSCs (BM-MSCs)

Human BM-MSCs were obtained from a commercial supplier (Cat. No. PT-2501, Cambrex Bioscience, Rockland, ME, USA). Both iPSC-MSCs and BM-MSCs were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 5 ng/mL bFGF (R&D Systems, Minneapolis, MN, USA), 10 ng/mL EGF (R&D Systems), 55 μM 2-mercaptoethanol (Invitrogen), 20 units/ml penicillin and 20 μg/ml streptomycin.

2.1.5 BEAS-2B cells

The BEAS-2B cells were virus-transformed immortalized human bronchial
epithelial cells from autopsy of non-cancerous individuals. The BEAS-2B cells in this study were from a commercial supplier (American Type Culture Collection, Rockville, MD, USA). They were cultured in keratinocyte serum free media K-SFM (Invitrogen) supplemented with 5 ng/ml EGF and 50 mg/ml bovine pituitary extract. Passages 42-50 were used for experiments.

2.1.6 Primary human airway smooth muscle cells (ASMCs)

ASMCs were isolated from biopsies of bronchi or tracheas of healthy transplant donor lungs as previously described (174). Briefly, biopsies were cut into small pieces (<1 mm²) and placed in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 20 units/ml penicillin, 20 μg/ml streptomycin and 2.5 μg/ml amphotericin B. ASMCs were confirmed by the characteristic “hill and valley” morphology under light microscopy. Cells were sub-cultured to reach confluence in 175-cm² tissue culture flasks and then frozen as stock. Passages 3 to 7 were used for experiments. When serum-free medium was required in the experiments, it was prepared as phenol-free DMEM supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, 1:100 non-essential amino acids, 0.1% BSA, 20 units/ml penicillin, 20 μg/ml streptomycin and 2.5 μg/ml amphotericin B.

All of the above cell types were maintained in a humidified incubator of 5% CO₂ and 37°C and fed with medium every 2-3 days.
2.2 Methods

2.2.1 MSCs treatment to cigarette smoke-exposed rats

Male Sprague-Dawley rats were exposed to 4% (v/v) CS for one hour daily for 56 days (Figure 2.1) using a same protocol as described in a previous study (52). Briefly, a maximum of 8 rats were placed into a ventilated 20 liter-chamber. 800 ml of CS was injected into the chamber through a syringe to raise the initial CS concentration to 4%. The filters of the cigarettes were removed beforehand. The chamber was then ventilated with a steady inflow of 4% CS at 1 L/min – a gas mixture of 100% CS (40 ml/min) and normal air (960 ml/min) from two separate peristaltic pumps (Masterflex, Cole-Parmer Instruments Co, Niles, IL, USA) (Figure 2.2). The control group was exposed to sham air (SA) using the same protocol. The exposure was performed in a chemical fume hood at the same time of each day. At day 29 and day 43, CS-exposed rats were intravenously injected with plain PBS, 3x10^6 human BM-MSCs or 3x10^6 human iPSC-MSCs into tail vein (Figure 2.1). The SA group was injected with PBS. Daily check of general conditions showed no obvious adverse reactions towards the injections.

Rats were sacrificed 24 hours after the last CS or air exposure by intraperitoneal injection of over-dosed pentobarbitone (100 mg/kg body weight). The larger lobe of left lungs was firstly inflated with 1 ml formalin and then fixed, dehydrated and paraffin embedded. The paraffin block was cut into 5 μm sections. The rest of the lung tissue was frozen and stored at -80°C.
Figure 2. 1 Schematic diagram of BM-/iPSC-MSCs treatments in a cigarette smoke (CS)-exposed rat model. Male Sprague-Dawley rats were exposed to 4% CS for 1 hour daily for 56 days. At Days 29 and 43, $3 \times 10^6$ human iPSC-MSCs ($n = 8$) or BM-MSCs ($n = 8$) were injected intravenously through tail veins. A sham air (SA) group was included in which the rats were exposed to fresh air. At Day 57, the rats were sacrificed, and the larger lobe of the left lungs were fixed and sectioned for histological studies.
Figure 2.2 The set-up of the ventilated smoking chamber. In the right chamber, the fresh air and CS were mixed in ratio of 96:4 to generate constant flow of 4% CS at 1000 ml/min. In the left chamber, only fresh air was pumped. The whole set-up was placed in a chemical fume hood.

2.2.2 Histology and morphometric analysis of rat lungs

The paraffin sections were always deparaffinised and dehydrated before staining. The slides were incubated at 40°C in an oven for one hour to melt the paraffin, followed by incubation twice in xylene for 15 minutes. A serial rehydration was then applied by incubation in 100%, 95%, 75% ethanol and double distilled water (ddH₂O) successively, each for 10 minutes, twice.

Mean linear intercept (L_m) was measured to evaluate airspace enlargement based on a modified method (62). Briefly, the paraffin sections from the largest lobes of rat
lungs were rehydrated and stained with hematoxylin and eosin. The slides were then mounted in a xylene-based mounting medium, observed using a brightfield microscope at 10x magnification and analysed using AxioVision (Zeiss, Germany). A 100 μm-length interval was inserted in the field of a randomly selected region of the lung sections. The interval passed through several alveoli, resulting in multiple alveolar intercepts. $L_m$ was calculated as 100 μm divided by the number of alveolar intercepts. 10 fields per section, 8 rats per group were analysed.

Masson's trichrome staining was performed to measure the pulmonary fibrosis according to the standard protocol. The pulmonary fibrosis was assessed by semi-quantitative Ashcroft score, which gave each field a score ranging from 0 (normal lung), 1 (minimal fibrous thickening of alveolar or bronchiolar walls), 3 (moderate thickening of walls without obvious damage to lung architecture), 5 (increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses), 7 (severe distortion of structure and large fibrous areas) to 8 (total fibrous obliteration of the field) (12). Scores from 5 fields per section, total 6 rats, were assessed under 20x magnification.

2.2.3 iPSC-MSCs treatment to ozone-exposed mice

Male C57BL/6 mice were put into a chamber ventilated with gas mixture of normal air and ozone produced by an ozone generator (model 500 Ozoniser, Sander, Wuppertal, Germany). The concentration of ozone in the chamber was monitored by an ozone detector and maintained at 3 ppm by tuning the ozone generator (Figure 2.3).
Control groups were exposed to normal air. The exposure lasted for 3 hours.

iPSC-MSCs or PBS were administrated to mice by intravenous injection through tail vein. The administration was performed either 24 hours prior or 6 hours after the exposure (Figure 2.4). There were 5 groups of mice in total: Air, Air with iPSC-MSCs administrated 24 hours prior (-24 hr), Ozone, Ozone with iPSC-MSCs administrated 24 hours prior-exposure (-24 hr) and Ozone with iPSC-MSCs administrated 6 hour post-exposure (+6 hr). The Mice from all groups were anaesthetised for AHR measurements 21 hours after the exposure. They were sacrificed immediately after the AHR measurements by intraperitoneal injection of over-dosed pentobarbitone (100 mg/kg body weight). Bronchoalveolar lavage (BAL) was collected before the collection of the lungs. The right lobes were frozen for mitochondria extraction while left lobes were inflated with and fixed in paraformaldehyde. The larger lobe of the fixed lungs were later embedded into paraffin blocks and sectioned into 5 μm-sections.

Figure 2. 3 The set-up of the ventilated chamber for ozone exposure. Ozone generated from an ozone generator and normal air from an air pump was ventilated into the chamber. The ozone concentration was constantly monitored by an ozone detector and maintained at 3 ppm by tuning the ozone generator.
Figure 2. 4 Schematic diagram of iPSC-MSCs treatments in an ozone-exposed mouse model. Male C57BL/6 mice were exposed to ozone (3 ppm) for 3 hours. Control groups were exposed to fresh air. $10^6$ iPSC-MSCs were intravenously injected into the tail vein either 24 hours prior to the ozone exposure, or 6 hours after the exposure. Airway hyper-responsiveness (AHR) was measured 21 hours after the exposure. The mice were then sacrificed and BAL and lung tissues collected.
2.2.4 Measurement of airways hyper-responsiveness (AHR) of mice

The AHR was measured as previously described (272). Before the measurement of AHR, mice were anaesthetised by intraperitoneal injection of ketamine/xyazine/saline mixture. The trachea was then opened and inserted with a catheter through which the mouse was ventilated (MiniVent type 845, Hugo Sach Electronic, Germany) at 250 breaths/minute and a tidal volume of 250 μl. The animal was continuously monitored in a whole body plethysmograph with a pneumotachograph connected to a transducer (EMMS, Hants, UK). Another transducer was connected to an esophageal catheter to evaluate the intrapleural pressure. The transpulmonary pressure was assessed based on the pressure measurements from the two transducers. Increasing concentrations (4-256 mg/ml) of acetylcholine in PBS were then administered into airway via a nebulizer (Aeroneb Lab Micropump Nebulizer, EMMS) and the pulmonary resistance (RL) was recorded for 3 minute periods during each dose. Baseline RL was defined as the RL with nebulised PBS. A concentration-response curve was plotted for each animal and the concentration of acetylcholine that induced 100% elevation of RL from baseline was derived (PC100). The value of -log PC100 was taken as a measurement of AHR.

2.2.5 Measurement of cells and cytokines in BAL of mice

Bronchoalveolar lavage (BAL) was collected by rinsing the lungs three times with 0.8 ml cold PBS through an endotracheal tube. After centrifugation at 3000 rpm, 4°C for 5 min, the supernatant was collected as BAL fluid (BALF) while the cell
pellets were resuspended in 200 μl PBS and counted on a haemocytometer for total cell number. BAL cell samples were centrifuged again onto glass slides by a cytopsin device at 30g for 6 minutes (Shandon Cytospin 4; Thermo Electron Corporation, Waltham, MA, US). The resulting cell slides were air-dried overnight and stained using Diff-Quick kit (Reagena, Toivala, Finland) according to manufacturer’s instructions. Differential count of white blood cells (neutrophil, macrophage, lymphocyte and eosinophil) was performed under an optical microscope (Olympus BH2, Olympus).

The cytokine levels in BALF of mice were determined using a MAGPIX Luminex multiplexing analyser (Merck Millipore, Darmstadt, Germany), which simultaneously measured levels of multiple analytes, including MCP-1, Eotaxin, IL-6, IL-5 and macrophage inflammatory proteins (MIP)-1α.

2.2.6 Isolation of intact mitochondria from mouse lungs

Intact mitochondria were isolated from mouse lungs using a Mitochondria Isolation Kit for Tissue (Thermo Fisher Scientific) and Dounce tissue grinder set (Sigma) following the manufacturer’s instructions. In brief, approximately 20 mg of lung tissue was washed with PBS supplemented with EDTA-free protease inhibitors (Roche). The tissue was homogenized in corresponding reagent with 5 strokes of Dounce A followed by 20 strokes of Dounce B. The homogenates were centrifuged firstly at 700xg for 10 minutes at 4°C, to sediment large organelle fractions containing nucleus, plasma membrane, endoplasmic reticulum, Golgi and so on. The
supernatants were centrifuged again at 12,000xg for 5 minutes. The supernatants and pellets were collected as cytosolic fractions and mitochondrial fractions, respectively.

2.2.7 Generation of cigarette smoke medium (CSM)

CSM was prepared as previously described (143). Briefly, cigarette smoke from two filter-removed cigarettes was bubbled into 20 ml of serum free medium. The medium was then filtered through a 0.22 μm filter. The optical density (OD) at wavelength 320 nm was measured.

For the study with BEAS-2B cells which was performed at Hong Kong university, the cigarettes smoke was from Camel cigarettes (11 mg TAR and 0.8 mg nicotine), and pumped into serum free medium manually by syringe. 100% CSM was generated by adjusting the OD at 320 nm with serum free medium to 3.5.

For the study of ASMCs which was performed at Imperial College London, the cigarette smoke was from Marlboro Red cigarettes (10 mg TAR and 0.8 mg nicotine) and pumped into serum free medium through a peristaltic pump, which resulted in a lower OD read-out than the manual method. Thus the 100% CSM was generated by adjusting the OD at 320 nm with serum free medium to 1.1. The advantage of peristaltic pump is a very consistent output for each batch of CSM.

After preparation, the 100% CSM was aliquoted and stored at -80℃.

2.2.8 Co-culture of BEAS-2B cells with MSCs.

BEAS-2B cells were pre-stained with CellTrace Violet (Invitrogen) which
fluorescently labelled the cells for several generations while iPSC-MSCs or BM-MSCs were pre-stained with MitoTracker Green (Invitrogen), which specifically stained the mitochondria in the cells. In brief, BEAS-2B cells were harvested, counted, incubated with 5 μM Celltrace Violet for 20 minutes, while MSCs were harvested, counted and incubated with 200 nM Mitotracker Green for 30 minutes. They were centrifuged at 1000 rpm for 5 min and incubated in complete medium for 10 min after the staining to wash away the remaining dye. After another centrifugation, they were re-suspended in 1:1 mixed medium and co-cultured at a 1:1 ratio in a 24-well plate. Approximately 5 hours later, when the cells attached to the plates, 100% CSM was added into the medium to make the final concentration 2%. After 24 hours, cells were visualized by motorized inverted microscope (IX81-ZDC2; Olympus, Shinjuku, Tokyo City, Tokyo, Japan) or analyzed by flow cytometry with a BD FACS Aria I Cell Sorter (BD Biosciences, Oxford, UK).

2.2.9 Determination of ATP content in BEAS-2B cells following co-culture

BEAS-2B cells in the co-culture were sorted as CellTrace positive cells by a BD FACS Aria I Cell Sorter. ATP was extracted with boiling distilled water according to a one-step method previously described (284). The ATP content was measured by a luciferase-catalysed luminescent assay using an ATP Determination Kit (Invitrogen) according to the manufacturer’s instruction.
2.2.10 BM-MSC and iPSC-MSC conditioned medium treatment of CSM-treated BEAS-2B cells

The conditioned medium (CdM) was prepared as previously described (294). Briefly, BM-MSCs and iPSC-MSCs were re-fed with DMEM without serum and supplements. After 24 hr, the medium was collected and concentrated via centrifugation through ultrafiltration conical tubes (Amicon Ultra-15 with membranes selective for 5 kDa). The final concentration was adjusted to 20 times that of the collected CdM.

Alternatively, to investigate the effects of stem cell factor (SCF), SCF was depleted from iPSC-MSCs-CdM as previously described (139). Briefly, 0.5 μg of anti-SCF (AF-255-NA; R&D) or normal human IgG control antibody (1-001-A; R&D) were mixed with protein G-agarose beads in PBS at 4°C for 1 hour with intermittent shaking. After centrifugation, beads were washed three times and used for immunodepletion of SCF. iPSC-MSCs-CdM was incubated with protein G-agarose beads immobilized with anti-SCF antibodies or human IgG control antibody for 1 hour at 4°C. The beads with immune complexes attached were precipitated by centrifugation. The supernatants collected were either SCF-depleted iPSC-MSCs-CdM or control iPSC-MSCs-CdM.

The BEAS-2B cells were cultured on top of coverslips in 24-well plates. The medium was replaced by keratinocyte medium with no supplements 24 hr before the treatment. They were then treated with 2% (v/v) CSM. BM-MSCs-CdM or iPSC-MSCs-CdM containing 3 μg of total protein was added at the same time. After
24 hours, the supernatant was removed and the cells fixed for immunohistochemical tests or terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. To investigate role of SCF, the cells were treated with either SCF-depleted iPSC-MSCs-CdM or recombinant SCF (255-SC, R&D) with 2% CSM.

2.2.11 Immunohistochemistry

To detect expression and localization of various proteins in lung sections and cell culture, standard immunohistochemistry procedures were applied. Briefly, lung sections were serially rehydrated and antigen retrieved by boiling for 5 minutes at 120°C in Sodium Citrate Buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). They were then blocked by 5% bovine serum albumin (BSA) for 30 minutes at room temperature followed by overnight incubation in primary antibodies at 4°C. The primary antibodies included human nuclear antigen (HNA) (MAB1281, Chemicon), human/rat Clara cell secretory protein 10 (CC10) (SC-9772, Santa Cruz, CA, USA), human/rat/mouse Ki-67 (ab15580, Abcam, Cambridge, UK), human/rat Complex I (SC-271387, Santa Cruz), human COX4 (SC-133478, Santa Cruz), rat/mouse CD68 (SC-7084, Santa Cruz), mouse/rat/human C-kit (SC-1494, Santa Cruz). After 3 washes with PBS, the sections were incubated in secondary antibodies including horseradish peroxidase-conjugated or fluorescent-conjugated anti-mouse IgG, anti-rat IgG and anti-rabbit IgG for 1 hour at room temperature. For HRP-conjugated secondary antibodies, the targets were recognized by an intense brown colour after developing with 3,3’-diaminobenzidine (DAB) substrate solution. For fluorescent-conjugated
secondary antibodies, the sections were mounted with mounting medium (ProLong Gold antifade reagent with DAPI, Invitrogen) containing 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. At least five fields per section were randomly captured under a conventional fluorescent microscope (IX81-ZDC2; Olympus) at the desired magnification and analyzed using AxioVision (Zeiss, Oberkochen, Germany).

For cell culture, the cells were seeded and treated on coverslips in 24-well dishes. They were fixed with 4% paraformaldehyde in PBS for 15 min, blocked with 5% BSA for 30 min and permeabilized with 0.1% Triton-100 in PBS for 3 min, all at room temperature. The cells were incubated in primary antibodies including C-kit (SC-1494, Santa Cruz) and cytochrome c (SC-13156, Santa Cruz) at room temperature for 1 hr and then in fluorescent-conjugated secondary antibodies. The coverslips were taken out from the wells, flipped and put on a drop of mounting medium containing DAPI on glass slides. Images of 5 fields for each slide were captured randomly by a motorized inverted microscope (Olympus) and analyzed using AxioVision (Zeiss). The ratio of positively stained cells in the total cell population was calculated as the rate of cytochrome c translocation, apoptosis or proliferation.

2.2.12 Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL assay detects cell apoptosis by labelling the terminal end of nucleic acids
which increase in abundance in cells only if the DNA becomes fragmented. Apoptosis of BEAS-2B cells and mouse lung sections were determined via TUNEL assay using the *In Situ* Cell Death Detection Kit, POD (Roche Applied Science, Mannheim, Germany).

For analysis of the lung sections, they were rehydrated as per the immunohistochemistry protocol, and heated in citrate buffer in a microwave oven for 5 minutes. They were then washed with PBS and incubated with TUNEL reaction mixture for 1 hour. Nucleus of apoptotic cells were stained green by the reaction.

For the BEAS-2B cells, they were fixed with 4% paraformaldehyde in PBS, blocked with 3% H₂O₂ in methanol, permeabilized with 0.1% Triton-100 in 0.1% sodium citrate and washed with PBS. TUNEL reaction mixture was then applied for a one-hour incubation at 37 °C.

All samples were mounted in mounting medium containing DAPI. Images of 5 fields for each slide were captured randomly by a motorized inverted microscope (Olympus) and analysed using AxioVision (Zeiss).

### 2.2.13 Total protein extraction from cell culture

To extract total protein from cultured cells, cells in 6-well plates were first washed with ice-cold PBS and then frozen at -80°C. The cells were then scraped with approximately 50 μl/well T-PER Cell Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific) and 100 mM PMSF. The cell/reagent mixture were vortexed, put on ice for
30 min and centrifuged at 16000xg at 4°C for 30 minutes. The supernatant was collected as cell lysates and stored at -80°C. Protein concentrations were determined by Bradford protein assay using bovine BSA as standard.

2.2.14 Western blot

Cell lysates containing the comparable amounts of total protein (20-40 μg) were denatured and reduced by boiling at 95°C for 5 minutes in Laemmli buffer with sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. The proteins in the samples were separated by size by running through an SDS–polyacrylamide gel. They were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) which was then blocked in 5% skimmed milk in Tris-buffered saline (pH 7.4) with 0.1% Tween-20. The membranes were incubated with diluted anti-C-kit (1:500) (SC-1494, Santa Cruz) or anti-β-actin (1:5000) antibodies at 4°C overnight, washed three times, and then incubated with horseradish peroxide-conjugated anti-goat antibody (1:2000) or anti-mouse antibody (1:2000) (Dako, Danmark) for 1 h at room temperature. After three further washes and a one-minute incubation with enhanced chemiluminescence (ECL), the membranes were exposed to x-ray films (Fujifilm, Tokyo, Japan) on which the target proteins were developed as dark bands.

2.2.15 Enzyme-linked immunosorbent assay (ELISA)

The concentration of SCF in CdM was measured by Human SCF DuoSet ELISA Kit (R&D) according to manufacturer’s instructions. In general, samples and serially
diluted standards were added into 96-well plates coated with capture antibody. After adequate washing, the captured target proteins were further incubated with detecting antibody and streptavidin conjugated with horseradish peroxidase (HRP), consecutively. HRP catalysed a colorimetric reaction of tetramethylbenzidine (TMB) substrates. The reaction was stopped by adding 2 N H₂SO₄, and the OD was measured at wavelength 450/570 nm in a microplate reader (FLUOstar Optima, BMG Labtech). A standard curve was generated according to OD values of the standards, and SCF levels of CdM were calculated from the standard curve.

2.2.16 Treatment of CSM or H₂O₂ to ASMCs or iPSC-MSCs

ASMCs were cultured in 6-well plates or 96-well plates. On reaching 90% confluence, the medium was replaced with serum-free medium for 24 hours. The cells were then treated with different concentrations of H₂O₂ (50, 100 and 200 μM) or CSM (10, 25, 50 and 75%) for 2, 4 and 24 hours. A cessation group was also included, in which after 4 hours of treatment H₂O₂ or CSM was replaced with fresh serum-free medium for the next 20 hours. The iPSC-MSCs were similarly treated for 4 hours and 24 hours. The cell viability, mitochondria membrane potential and mitochondrial ROS levels were measured by methylthiazolyldiphenyl-tetrazoliumbromide (MTT) assay, JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanineiodide) and MitoSOX staining, respectively.
2.2.17 Cell viability assay

MTT assay is a commonly used cell viability assay. In brief, MTT (Sigma-Aldrich) was dissolved in 37 °C serum free medium at 1 mg/ml. ASMCs or iPSC-MSCs cultured in 96-well plates were incubated with 100 μl MTT solution at 37 °C after treatments. After 15 min, the cells were checked every 5 minutes using microscope to observe formation of purple color within. When the color is visible but not too intense, the MTT solution was replaced with 50 μl DMSO. After shaking for 5 minutes, the OD at wavelength 550 nm was measured.

2.2.18 Measurement of mitochondrial ROS

Mitochondrial ROS levels were assessed by staining with MitoSOX Red Mitochondrial Superoxide Indicator (Invitrogen). 50 μg MitoSOX powder was dissolved in 13 μl DMSO to make a 5 mM stock solution and further diluted to 5 μM with relevant buffers to make working solutions.

For the in vitro study of ASMCs, cells in 6-well plates were washed with modified Hank’s Balanced Salt Solution (HBSS) (containing Ca/Mg) (Sigma-Aldrich), and incubated with 5 μM MitoSOX diluted in modified HBSS for 30 minutes. The cells were then washed with HBSS (Sigma-Aldrich) and incubated with 500 μl/well accutase solution (Sigma-Aldrich) for up to 5 min at 37 °C and 5% CO₂. One ml/well DMEM with 4 mM L-glutamine and 10% FBS was then added to the detached cells. After centrifugation and resuspension in HBSS, the intensity of red fluorescence was measured at 510/580 nm by a FACSCanto II flow cytometer (BD Biosciences).
For the *in vivo* study of ozone-exposed mice, the intact mitochondria were isolated from lungs, and then incubated with 5 μM MitoSOX in 96-well plates for 30 minutes at 37°C. The intensity of red fluorescence was measured at 510/580 nm using a fluorescence plate reader.

2.2.19 Mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was determined by staining with JC-1 dye (Invitrogen). The dye exists as monomers with green fluorescence, but when they enter live mitochondria they form J-aggregates which exhibit red fluorescence. The accumulation of the dye in mitochondria is potential-dependent, making the ratio of red to green fluorescence as an indicator of ΔΨm. ASMCs or intact mitochondria from mouse lungs were incubated with 2 μM of JC-1 for 30 minutes at 37°C. The fluorescent signal was determined either using a FACSCanto II flow cytometer (BD Biosciences) for the living cells, or a fluorescence plate reader (Synergy HT Biotek, Winooski, VT, USA) for the isolated mouse mitochondria. The green fluorescence from JC-1 monomers was measured at excitation/emission ratios of 485/535 nm and the red fluorescence from J-aggregates at 560/595 nm.

2.2.20 Annexin V staining

The apoptosis of ASMCs/iPSC-MSCs co-culture was detected by Annexin V staining through flow cytometry. Annexin V detects apoptosis by binding to the externalized phosphatidylserine in apoptotic cells. Briefly, cells were harvested and
resuspended at a density of $10^6$ cells/ml in assay buffer. 5 μl of FITC-conjugated Annexin V (Invitrogen) was added into 100 μl of cell suspension. After 15 minutes-incubation at room temperature, the intensity of Annexin V was measured by a FACSCanto II flow cytometer (BD Biosciences).

2.2.21 Direct co-culture of ASMCs and iPSC-MSCs

To study the effects of direct interaction between ASMCs and iPSC-MSCs on mitochondrial ROS, ΔΨm and apoptosis, ASMCs and iPSC-MSCs were co-cultured in using two different procedures, one as a prophylactic study and the other as therapeutic (Figure 2.5).

For the prophylactic study, the ASMCs and iPSC-MSCs were cultured in T150 flasks separately before the experiments. The ASMCs were trypsinized, resuspended and counted. They were washed with HBSS by centrifugation and resuspension, followed by 20 minutes-incubation with CellTrace Violet (1:1000 in HBSS, 1 ml/10^6 cells). After centrifugation, they were incubated with complete medium for 10 minutes to wash and neutralize the dye. Meanwhile, the iPSC-MSCs were trypsinized, resuspended and counted. The two cell types were mixed and seeded in 6-well plates at a density of $10^5$ ASMCs plus 1.5x$10^5$ iPSC-MSCs per well. Single cell culture groups were included as comparison, in which only $10^5$ ASMCs or 1.5x$10^5$ iPSC-MSCs were seeded into each well. After 20 hours, the medium was replaced with 10% or 25% CSM for 4 hours. The mitochondrial ROS, ΔΨm and apoptosis were then assessed by MitoSOX, JC-1 and Annexin V staining through flow
For the therapeutic study, $10^5$ ASMCs were first seeded in each well of 6-well plates. 20 hours later, they were incubated with CellTrace Violet for 20 minutes followed by treatment with 10% or 25% CSM for 4 hours. The CSM were then removed and $1.5 \times 10^5$ iPSC-MSCs were added into each well. After another 24 hours, the mitochondrial ROS, mitochondrial membrane potential and apoptosis were measured through flow cytometry.

### 2.2.22 Detection of mitochondrial transfer in co-culture of ASMCs and iPSC-MSCs

To detect mitochondrial transfer in the co-culture of ASMCs and iPSC-MSCs, ASMCs pre-stained with CellTrace Violet and iPSC-MSCs pre-stained with MitoTracker Red (for fluorescent microscope) or MitoTracker Green (for flow cytometry) were mixed and seeded into 6-well plates at a density of $10^5$ ASMCs plus $1.5 \times 10^5$ iPSC-MSCs per well. 20 hours later, they were treated with 10% or 25% CSM and analyzed by flow cytometry. The percentage of ASMCs that were MitoTracker positive was subsequently calculated. For fluorescent microscopy, the cells were fixed in 3.7% formaldehyde (Sigma-Aldrich) for 10 minutes, permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) for 5 minutes and stained by Alexa Fluor 488 phalloidin (Invitrogen) in PBS with 1% BSA. Phalloidin is a probe that labels filamentous actin selectively. The cells were then visualized by a motorized inverted fluorescent microscope.
Figure 2. 5 Protocols of direct co-culture of iPSC-MSCs and ASMCs. (A) Prophylactic co-culture with iPSC-MSCs. 1.5x 10⁵ ASMCs were pre-stained with CellTrace Violet and seeded in 6-well plates either with or without 10⁵ iPSC-MSCs. 20 hours later they were treated with CSM for 4 hours. MitoSOX, JC-1 and Annexin V staining were applied to measure mitochondrial ROS, ΔΨm and apoptosis. (B) Therapeutic co-culture with iPSC-MSCs. 1.5x 10⁵ ASMCs were labeled with CellTrace Violet and treated with CSM for 4 hours. CSM was removed and either 10⁵ iPSC-MSCs or serum free medium was added. After 20 hours of co-culture, the mitochondrial ROS and membrane potential were measured.
2.2.23 iPSC-MSCs-conditioned medium treatment to ASMCs

The iPSC-MSCs-CdM was prepared as described above. The ASMCs were pretreated with 20-fold diluted iPSC-MSCs-CdM for 4 hours. The CSM were then added on top of the iPSC-MSCs-CdM to make 10% or 25% CSM. Serum free medium was added to the control group or 10% CSM group to keep the total volume of each well the same. 4 hours later, the mitochondrial ROS, \( \Delta \Psi_m \) and apoptosis were measured.

2.2.24 Transwell co-culture between ASMCs and iPSC-MSCs

To study the effects of paracrine crosstalk between ASMCs and iPSC-MSCs, they were co-cultured in a transwell co-culture system. In brief, \( 10^5 \) ASMCs were seeded in 6-well plates while \( 1.5 \times 10^5 \) iPSC-MSCs were seeded in a cell-culture insert with pores sized 0.4 \( \mu \)m (Falcon, Corning, NY, USA). They were incubated overnight and then combined. Single-culture groups were included with empty inserts or empty wells. After 20 hours’ co-culture, they were treated with 10% or 25% CSM for 4 hours, and assessed for the mitochondrial ROS, \( \Delta \Psi_m \) and apoptosis.

2.2.25 Statistical analysis

Numerical data are presented as mean±standard error of mean (SEM). The statistical analysis was performed using software Prism 5.0 (Graphpad, San Diego, CA, USA). Differences between groups were evaluated by student \( t \)-test or one-way ANOVA followed by Newman-Keuls test when appropriate. Significant difference was defined as when \( p \)-value < 0.05. The number of animals required for the study
was determined by power calculations (78) using data from previous studies conducted in the department. In particular, for CS-exposed rats, the $L_m$ were 31.28±1.75 μm vs. 19.79±0.81 μm for CS and SA groups (n=9) in a previous report (52). Based on this change, the maximal number of animals to attain statistical significance of $p<0.01$ with a 95% probability equated to 6. Considering possible loss of animals during experimental period, n=8 was chosen for the rat experiments. For the ozone-exposed mice, -logPC$_{100}$ was reported to be 1.059±0.127 vs. 2.088±0.136 for ozone and air groups (n=8) respectively (275). Based on these data, the maximal number of animals to attain statistical significance of $p<0.01$ with a 95% probability equated to 5. n=5-6 was chosen for the mouse study.
Chapter 3. iPSC-MSCs Attenuate CS-induced Damage in Airway Epithelial Cells through Mitochondrial Transfer and Paracrine Effect
3.1. Introduction

Cigarette smoke (CS) is the primary cause of chronic obstructive pulmonary disease (COPD) (23, 39). In the first part of this study a rat model of chronic exposure to CS was used to study the effects of BM-MSCs and iPSC-MSCs on CS-induced alterations of lung regarding emphysema, fibrosis, inflammation, apoptosis and proliferation. Male SD rats were exposed to 4% CS for 1 hour/day for 56 consecutive days. $3 \times 10^6$ of BM-MSCs or iPSC-MSCs were intravenously administrated at day 29 and 43. A control group exposed to sham air (SA) was included.

Despite numerous reports of MSCs’ effectiveness in diverse disease models, the mechanism of MSCs’ action remains unresolved (270). A major breakthrough was the report of mitochondrial transfer from BM-MSCs to pulmonary alveoli which protected mice against acute lung injury (118). In this study I hypothesized that a similar phenomenon also existed in the CS-exposed model. The results demonstrated mitochondrial transfer in vivo from BM-MSCs or iPSC-MSCs to the local cells of CS-exposed lung tissue. The in vivo findings were followed by in vitro studies on such activity in a co-culture of MSCs and bronchial epithelial cells (BEAS-2B) treated with CSM. The mitochondrial transfer was determined through fluorescent microscope and flow cytometry, and the effect on ATP depletion was also examined.

Although mitochondrial transfer may be a potent way to rescue damaged cells, such action requires direct cell contact and is therefore restricted to small regions around the MSCs. On the other hand, MSCs have been reported to induce paracrine effects via release of various immunomodulators, such as TGF-β1, hepatic growth
factor, prostaglandin E$_2$, interleukin (IL)-10 and nitric oxide (2, 235, 270). The paracrine effect can be effective in a large radius from MSCs as it does not rely on direct cell-cell contact. Therefore I further investigated the effects of the paracrine factors released from MSCs in an *in vitro* model using bronchial epithelial cells. In particular, the role of stem cell factor (SCF) secreted from MSCs, a growth factor that can mediate cell survival, migration, and proliferation was examined (146). BEAS-2B cells were treated with conditioned medium (CdM) from BM-MSCs or iPSC-MSCs to test a general paracrine effect on apoptosis and proliferation. SCF was then depleted from CdM for a more specific investigation on the role of SCF in the CdM.

The aims of the study include:

1. To examine the effect of iPSC-MSCs on CS-induced lung damage in a chronic CS-exposed rat model compared with BM-MSCs.

2. To determine whether there is mitochondrial transfer from iPSC-MSCs to CS-damaged airway epithelial cells both *in vivo* and *in vitro*.

3. To investigate the involvement of paracrine regulation on the protective effects of iPSC-MSCs on CSM-induced apoptosis in bronchial epithelial cells by using conditioned medium (CdM), and to specifically determine the role of a potential functional molecule, namely stem cell factor (SCF).
3.2. Effects of iPSC-MSCs on CS-induced lung damage in rats.

3.2.1. iPSC-MSCs attenuated CS-induced airspace enlargement, fibrosis and inflammation in rat lung

Emphysema is one of the major characteristics of COPD. In this study, airspace enlargement in the CS-exposed rat lung was assessed by histological examination after haematoxylin and eosin staining. CS exposure caused obvious alveolar destruction (Figure 3.1A), leading to a much longer mean linear intercept ($L_m$) (60 ± 4.6 μm) compared to the SA group (31.0±2.3 μm, $p<0.001$) (Figure 3.1B). The airspace enlargement was attenuated by the BM-MSCs treatment (Figure 3.1A), with a significantly reduced $L_m$ compared with the CS group (48.0±4.0 μm, $p<0.01$) (Figure 3.1B). Treatment with iPSC-MSCs also attenuated alveolar wall destruction (Figure 3.1A), leading to significant reduction in $L_m$ compared to the CS group (40.0±2 μm, $p<0.05$). Moreover, the treatment with iPSC-MSCs was more effective than BM-MSCs, as indicated by a shorter $L_m$ value ($p < 0.05$) (Figure 3.1B).

CS also induced airway fibrosis compared to the SA group, as shown by the Masson's Trichrome staining (Figure 3.2) (5±0.13% vs. 0.98±0.09%, $p < 0.05$). Fibrosis was reduced by both BM-MSCs (3.85±0.17%, $p < 0.05$) and iPSC-MSCs treatment (2.37±0.12%, $p < 0.05$), and iPSC-MSCs demonstrated superior effect compared to BM-MSCs ($p < 0.05$) (Figure 3.2).

The inflammation induced by CS in lung tissues was illustrated by immunohistochemistry staining against CD68, a transmembrane glycoprotein highly
expressed by monocytes and tissue macrophages (26) (Figure 3.3A). CS exposure significantly elevated the number of CD68-positive cells compared to SA group (21.3±1.9 vs. 2.0±0.41%; p<0.01) (Figure 3.3B). Both treatments of iPSC-MSCs and BM-MSCs significantly reduced the number of CD68-positive cells compared to the CS group (4.3±0.8% for iPSC-MSCs and 12.3±1.3% for BM-MSCs respectively; p<0.01) with a superior effect seen iPSC-MSCs (p<0.01) (Figure 3.3B).
Figure 3.1 Human iPSC-MSCs and BM-MSCs alleviated CS-induced airspace enlargement in rats. (A) Histological assessment of lung sections stained with haematoxylin and eosin at 10x magnification. Scale bar = 100 μm. (B) Morphometric analysis of mean linear intercept (Lm) (n=8). *p<0.05 compared with SA group; #p<0.05 compared with CS group; †p<0.05 compared with BM-MSCs group.
Figure 3.2 BM-MSCs and iPSC-MSCs attenuated CS-induced lung fibrosis in rats. (A) Masson's trichrome staining of lung sections. Blue staining indicates site of fibrosis. 40x magnification. Scale bar = 50 μm. (B) Percentage of bronchioles with fibrosis (n=8). *p<0.05 compared to SA group; #p<0.05 compared to CS group; †p<0.05 compared to BM-MSCs group.
Figure 3.3 BM-MSCs and iPSC-MSCs ameliorated CS-induced inflammation in rat lungs. (A) Illustration of monocytes/macrophages as CD68 positive cells with brown staining. (B) Number of CD68 positive cells in lung sections (n=6-8). **p<0.01 compared to SA group; ###p<0.01 compared to CS group; ††p<0.01 compared to BM-MSCs group.
3.2.2. iPSC-MSCs attenuated CS-induced apoptosis/proliferation imbalance in rat lung epithelium.

To illustrate apoptosis in the epithelium, lung sections were co-stained with the TUNEL assay which labels apoptotic cells, and Clara cell 10-kDa protein (CC-10) which is a marker of epithelium (Figure 3.4A). CS exposure induced increased apoptosis in lung epithelium (5.2±0.7% vs. 0.4±0.2%; \( p<0.01 \)), which was attenuated by both BM-MSCs (3.4±0.5%; \( p<0.01 \) in comparison to CS group) and iPSC-MSCs (2.0±0.6%; \( p<0.01 \) in comparison to CS group) (Figure 3.4B). The treatment of iPSC-MSCs was more effective than BM-MSCs group (\( p<0.01 \)) (Figure 3.4B).

CS exposure also led to reduction of proliferation in the epithelium, which was shown by co-staining against CC-10 and Ki-67, a marker of cell proliferation (221) (Figure 3.4A). CS exposure significantly reduced the number of proliferating cells in the epithelium (5.5±0.5% vs. 25.8±2.8%; \( p<0.01 \)), which was improved by the treatment with either BM-MSCs (11.2±0.8%; \( p<0.01 \) in comparison to CS group) or iPSC-MSCs (15.2±2.3%; \( p<0.01 \) in comparison to CS group) (Figure 3.4B). The iPSC-MSCs group demonstrated higher proliferation activity than the BM-MSCs group (\( p<0.01 \)) (Figure 3.4B).
3.2.3. Mitochondrial transfer from MSCs to adjacent lung cells

The epithelium in the rat lung was stained with CC-10, and mitochondria in both MSCs and host cells were labeled with an antibody against human/rat Complex I. Meanwhile, human mitochondria were detected by staining using a human specific Cox-4 antibody (Figure 3.5A&B). For both BM-MSCs and iPSC-MSCs treatments, human mitochondria were detected inside some of the CC-10-expressing cells (Figure 3.5A&B), indicating events of mitochondrial adoption by the bronchial epithelium from the foreign human MSCs. With the same number of cells injected, rats treated with iPSC-MSCs demonstrated a higher number of cells receiving MSC-derived mitochondria than those treated with BM-MSCs (number/40x field: 8.4 ± 1.6 vs. 4.6 ± 0.9, p< 0.05; Figure 3.5C).
Figure 3.4 BM-MSCs and iPSC-MSCs attenuated CS-induced increase in apoptosis and reduction of proliferation in rat lung epithelium. (A) Apoptotic cells in airway epithelium. Red (CC-10): airway epithelium; green (TUNEL): apoptotic cells; blue (DAPI): nuclei. (B) Proliferative cells in airway epithelium. Green (CC-10): airway epithelium, Red (Ki-67): proliferative cells; Blue (DAPI): nuclei. (C) Number of apoptotic cells in airway epithelium (n=6-8). (D) Number of proliferating cells in airway epithelium. **p<0.01 compared to SA; ##p<0.01 compared to CS; ††p<0.01 compared to BM-MSCs.
Figure 3.5 Mitochondria transfer from MSCs to lung epithelium in rats. Lung sections treated with (A) BM-MSCs and (B) iPSC-MSCs were co-stained against CC-10 (red) marking lung epithelium, human/rat Complex I (green) labeling human/rat mitochondria and human Cox-4 (violet) detecting human mitochondria specifically. Co-localization of three signals indicates receiving of human mitochondria by lung epithelium of rats. Scale bar = 50 μm. (C) Number of cells receiving foreign mitochondria in 40x fields. *$p$<0.05 compared between iPSC-MSCs and BM-MSCs groups.
3.3. Mitochondrial Transfer from MSCs to BEAS-2B cells \textit{in vitro}

3.3.1. Mitochondrial transfer from MSCs to BEAS-2B cells

Mitochondrial transfer was further investigated in an \textit{in vitro} co-culture model in the context of CSM treatment. According to a previous report, treatment of 2% CSM for 24 hours induces a significant inflammatory response and oxidative stress without loss of viability in human bronchial epithelial BEAS-2B cells (143). This condition was adopted in the co-culture study. BEAS-2B cells were pre-labeled with CellTrace Violet while mitochondria of BM-MSCs or iPSC-MSCs were pre-labeled with MitoTracker Green. BEAS-2B cells were then co-cultured with either BM-MSCs or iPSC-MSCs in a 1:1 ratio and treated with 2% CSM for 24 hours. The MitoTracker-labeled mitochondria which originated from the MSCs were observed in neighboring BEAS-2B cells, indicating that mitochondria transferred from MSCs to BEAS-2B cells within 24 hours (Figure 3.6A&B). Moreover, formation of tunneling nanotube (TNT)-like structures between the MSCs and BEAS-2B cells was identified after 24 hours (Figure 3.6A&B). The formation of TNTs was reported to be blocked by Cytochalasin B at nanomolar concentrations without affecting endocytosis and phagocytosis (40). In this study, when 300 nM of cytochalasin B was added to the co-culture together with the CSM, the formation of TNTs was highly inhibited and few MSCs-derived mitochondria were observed in BEAS-2B cells (Figure 3.6 C&D), indicating that mitochondrial transfer may be mediated through TNTs.
Figure 3.6 Mitochondrial transfer from BM-MSCs and iPSC-MSCs to BEAS-2B cells in co-culture. (A) BM-MSCs or (B) iPSC-MSCs pre-stained with MitoTracker Green and BEAS-2B cells pre-labeled by CellTrace Violet were co-cultured in a 1:1 ratio and treated with 2% CSM for 24 hours. The observation of MitoTracker Green in the BEAS-2B cells (Blue) indicated the adoption of MSCs-derived mitochondria by BEAS-2B cells. Connection between the cells through TNT-like structures was observed. Treatment of Cytochalasin B suppressed the formation of TNT-like structures and mitochondrial transfer in both (C) BM-MSCs and (D) iPSC-MSCs group. Scale bar = 20 μm. B2B: BEAS-2B cells; DIC: differential interference contrast. A representative image from three independent experiments is shown.
3.3.2. **CSM promoted mitochondrial transfer from MSCs to BEAS-2B cells**

In addition to fluorescent microscopy, mitochondrial transfer in the co-culture was further evidenced by flow cytometry at different time-points. The percentage of CellTrace/MitoTracker double positive cells in the whole CellTrace positive population was defined as the transfer rate (Figure 3.7A). There were three findings from this experiment. First, the transfer rate gradually increased along the co-culture period in both BM-MSCs and iPSC-MSCs groups. Second, after 24 hours, the iPSC-MSCs group demonstrated a higher transfer rate compared with the BM-MSCs group (38.8±2% vs. 27.8±1.6%, \( p<0.05 \)), suggesting a higher potential for mitochondrial transfer between iPSC-MSCs and BEAS-2B cells. Lastly, at the 24 hour time-point, CSM treatment significantly increased the transfer rate compared to the untreated group, both for the BM-MSCs co-culture (27.8±1.6% vs. 14.4±1%, \( p<0.05 \)) and iPSC-MSCs co-culture (38.8±2% vs. 18±1.9%, \( p<0.05 \)), suggesting that mitochondrial transfer is promoted in the presence of CSM.

3.3.3. **Co-culture with iPSC-MSCs elevated ATP levels in BEAS-2B cells**

Given the observation of mitochondrial transfer in the co-culture, the effect of co-culture with BM-MSCs and iPSC-MSCs on intracellular ATP levels in BEAS-2B cells was investigated. BEAS-2B cells were identified from the co-culture followed by determination of intracellular ATP levels. The 2% CSM treatment for 24 hours significantly reduced the intracellular ATP level (percentage of control: 76.5 ±3.5% vs. 100 ±10.1%, \( p<0.05 \)) (Figure 3.8). The drop of ATP level was restored by co-culture
with iPSC-MSCs (percentage of control: 97.8 ±9.1% vs. 76.5 ±3.5% for iPSC-MSCs and CSM group respectively, \( p<0.05 \)), but not the BM-MSCs (percentage of control: 80.7 ± 7.1%) (Figure 3.8).

**Figure 3.7 CSM promoted mitochondrial transfer.** (A) Representative flow cytometry outcome of single culture of MSCs (left), BEAS-2B cells (middle) and co-culture (right). BM-MSCs or iPSC-MSCs pre-labeled with MitoTracker Green and BEAS-2B cells pre-labeled with CellTrace Violet were co-cultured at a 1:1 ratio in the presence of 2% CSM. (B) Effects of time, CSM and cell type on mitochondrial transfer rate (n=3). Transfer rate was defined as the percentage of BEAS-2B cells containing MSCs-derived mitochondria in the total BEAS-2B population. W/O stands for CSM untreated group while other groups were treated with 2% CSM for 24 hours. *\( p<0.05 \) represents statistical difference as indicated.
Figure 3.8 iPSC-MSCs restored intracellular ATP levels of BEAS-2B cells. BEAS-2B cells were co-cultured with MSCs with 2% CSM for 24 hours before sorted out through flow cytometry and determination of intracellular ATP levels (n=3). *\(p<0.05\) compared to control group; \(^{#}p<0.05\) compared to CSM group; \(^{†}p<0.05\) compared to BM-MSCs group.

3.4. iPSC-MSCs Attenuated CSM-induced Apoptosis and Proliferation Imbalance in BEAS-2B Cells by Paracrine Effect

3.4.1. iPSC-MSCs-CdM attenuates CSM-induced apoptosis in BEAS-2B cells

Conditioned medium (CdM) from BM-MSCs and iPSC-MSCs contains paracrine factors released from the MSCs accumulated over 24 hours. Thus the effects of MSCs-CdM on BEAS-2B cells were investigated in order to study the paracrine effect. BEAS-2B cells were treated with 2% CSM and CdM for 24 hr. The release of cytochrome \(c\) from mitochondria and its translocation to nucleus (Figure 3.9A) is a major event in triggering the onset of apoptosis (184, 211). CSM elevated the percentage of cells with cytochrome \(c\) translocation compared to the untreated group.
(73.4±6.8% vs. 1.88±1%; p<0.01), which was attenuated by both BM-MSCs-CdM (41.2±6.3%; p<0.01 in comparison to CSM group) and iPSC-MSCs-CdM (16.6±5%; p<0.01 in comparison to CSM group) (Figure 3.9D). iPSC-MSCs-CdM exhibited a superior effect compared with to BM-MSCs-CdM (p<0.01) (Fig. 3.9D). In addition, cellular apoptosis was identified by TUNEL staining (Figure 3.9B). CSM elevated the percentage of apoptotic cells (16.2±2% vs. 1.4±0.4%; p<0.01), which was attenuated by both BM-MSCs-CdM (9.3±0.7%; p<0.01 in comparison to CSM group) and iPSC-MSCs-CdM (5.5±0.5%; p<0.01 in comparison to CSM group) (Figure 3.9E). There were fewer apoptotic cells in the iPSC-MSCs-CdM group compared to BM-MSCs-CdM group (p<0.01), suggesting a better anti-apoptotic activity of iPSC-MSCs-CdM.

While apoptosis was enhanced, the percentage of proliferating cells was reduced by the 2% CSM (3.4±0.5% vs. 19.2±0.8%; p<0.01) as determined by staining against Ki-67 (Figure 3.9C&F). This reduction was improved by both BM-MSCs-CdM (8.2±0.6%; p<0.01 in comparison to CSM group) and iPSC-MSCs-CdM (13.4±0.5%; p<0.01 in comparison to CSM group) (Figure 3.9F), in which iPSC-MSCs-CdM demonstrated a more potent effect (p<0.1) (Figure 3.9F), indicating a higher ability to promote cell proliferation.
Figure 3.9 CdM from BM-MSCs and iPSC-MSCs ameliorated CSM-induced apoptosis/proliferation imbalance in BEAS-2B cells. BEAS-2B cells were treated with 2% CSM and BM-MSCs-CdM or iPSC-MSCs-CdM for 24hr. (A) Cytochrome c translocation in BEAS-2B cells. Green: cytochrome c; blue: DAPI. Cells with cytochrome c translocation to nuclei are marked by red arrows. (B) Apoptotic BEAS-2B cells as stained by TUNEL. Green: TUNEL; blue: DAPI. (C) Proliferative BEAS-2B cells as marked by Ki-67. Red: Ki-67; blue: DAPI. (D) Percentage of BEAS-2B cells with cytochrome c translocation (n=3). (E) Percentage of apoptotic BEAS-2B cells (n=3). (F) Percentage of proliferative BEAS-2B cells. **p<0.01 compared to control group; ## p<0.01 compared to CSM group; †† p<0.01 compared to BM-MSCs-CdM group.
3.4.2. Effects of iPSC-MSCs-CdM on apoptosis and proliferation of BEAS-2B cells were SCF-dependent

Given the superior effects of iPSC-MSCs-CdM over BM-MSCs-CdM regarding apoptosis and proliferation, the active components responsible for such effects were further investigated. In particular, the role of SCF was examined in my model.

First of all, the levels of SCF in CdM were measured, showing that iPSC-MSCs-CdM contains a much higher level of SCF than BM-MSCs-CdM (518±57 vs. 113±13 pg/mg protein; p<0.01) (Figure 3.10A). The expression of SCF receptor, C-kit, by BEAS-2B cells was confirmed by both immunohistochemistry staining and Western blotting (Figure 3.10B) which showed that the level of C-kit was not changed by CSM treatment (Figure 3.10B).

To investigate the role of SCF, SCF-deprived iPSC-MSCs-CdM was generated by incubation with immobilized anti-SCF antibody. Effects of iPSC-MSCs-CdM, SCF-deprived iPSC-MSCs-CdM and recombinant SCF were evaluated on BEAS-2B cells treated with 2% CSM for 24 hours. All three treatments attenuated CSM-induced morphological change (Figure 3.11A), cytochrome c translocation (22.0±2.1%, 42.4±2.5% and 41.8±1.9% respectively, p<0.01 in comparison to CSM group, 74.8±2.9%) (Figure 3.11BE), apoptosis (5.6±0.5%, 10.4±0.5% and 10.2±1.0% respectively, p<0.01 in comparison to CSM group, 20±1.7%) (Figure 3.11CF) and reduction of proliferation (15.4±0.5%, 11.0±0.5% and 11.0±0.9% respectively, p<0.01 in comparison to the CSM group, 5.8±0.4%) (Figure 3.11DG). Compared to the normal iPSC-MSCs-CdM treatment, depletion of SCF in the CdM resulted in
significantly weakened activity, as shown by more cytochrome c translocation (p<0.01), more apoptosis (p<0.01) and less proliferation (p<0.01). This finding, in combination with the result that recombinant SCF was able to induce considerable effects by itself, indicated that SCF contributed to the activity of iPSC-MSCs-CdM. Similarly, the recombinant SCF were less effective than iPSC-MSCs-CdM regarding cytochrome c translocation (p<0.01), apoptosis (p<0.01) and proliferation (p<0.01). Given that the deprived iPSC-MSCs-CdM still retained some capacity to restore all three parameters, this finding indicated that in addition to SCF, there were other components promoting similar effects. In summary, the data suggested that the action of iPSC-MSCs-CdM to alleviate CSM-induced apoptosis/proliferation imbalance was partly through SCF.
Figure 3.10 Secretion of SCF by BM-MSCs and iPSC-MSCs and expression of C-kit by BEAS-2B cells. (A) SCF levels in CdM from BM-MSCs or iPSC-MSCs as measured by ELISA (n=3). (B) Expression of C-kit in BEAS-2B cells. C-kit expression was identified by immunohistostaining (i) with or (ii) without 2% CSM treatment for 24 hours, and by (iii) Western blotting. Red: C-kit; blue: DAPI. **p<0.01 compared to BM-MSCs-CdM.
Figure 3.11 The anti-apoptotic and pro-proliferation activity of iPSC-MSCs-CdM was partly through SCF. Immobilized anti-SCF antibody was used to scavenge SCF from iPSC-MSCs-CdM. BEAS-2B cells were treated with 2% CSM and iPSC-MSCs-CdM for 24 hours before fixation and TUNEL staining or immunostaining against cytochrome c and Ki-67 (n=3). (A) The morphological change of the BEAS-2B cells. DIC: differential interference contrast. (B) Cytochrome c translocation in BEAS-2B cells. Green: cytochrome c; blue: DAPI. Cells with cytochrome c translocation to nuclei are marked by red arrows. (C) Apoptotic BEAS-2B cells as stained by TUNEL. Green: TUNEL; blue: DAPI. (D) Proliferative BEAS-2B cells as marked by Ki-67. Red: Ki-67; blue: DAPI. (E) The percentage of BEAS-2B cells with cytochrome c translocation to nuclei (n=3). (F) The apoptotic rate of BEAS-2B cells (n=3). (G) Percentage of proliferative BEAS-2B cells (n=3). **p<0.01 compared to control group; ***p<0.01 compared to CSM group; ††p<0.01 compared to normal iPSC-MSCs-CdM group.
3.5. Discussion

This study elucidated that iPSC-MSCs are more effective than BM-MSCs in attenuating CS-induced alterations in rat lungs. The higher efficacy may be explained by a higher activity of mitochondrial transfer, which was evidenced *in vivo* and further confirmed by *in vitro* co-culture study. Meanwhile, the higher efficacy may also be attributed to more potent paracrine effects, partly through SCF.

MSCs isolated from BM (94, 112), amniotic fluid (151) and adipose tissue (222) have been reported to attenuate CS-induced emphysema in murine models. These MSCs are isolated directly from adult tissue, hence relevant patient tissues such as bone marrow and adipose tissue need to be obtained to collect them. The technique to differentiate iPSCs into MSCs provides a different source of MSCs, which has several advantages over classic MSCs. Firstly, large quantities of iPSC-MSCs can be generated from a small number of somatic cells which can be of a number cell types such as fibroblasts. In other words, the supply of iPSC-MSCs is unlimited and personalised. Secondly, iPSC-MSCs have a strong capacity of proliferation and differentiation, with differentiation potential maintained for over 120 population doublings (152), while normal MSCs may reach senescence after 20 doublings (69). Thirdly, impaired survival and differentiation ability may be associated with MSCs isolated from aged subject or patients with ageing-related disorders (87, 100, 267, 294). However, iPSC-MSCs may hold hope to overcome these issues because when deriving iPSC-MSCs, somatic cells are firstly reprogrammed to an embryonic stem cell-like state, which may provide the cells with a rejuvenated phenotype. Therefore
iPSC-MSCs are valuable candidates for the development of cell therapy against COPD whose prevalence, morbidity, and mortality are all highly associated with age (38, 126, 137). Regarding airway disease models, iPSC-MSCs have been reported to attenuate allergic airway inflammation in mice (244).

The first part of this study evaluated a general effect of iPSC-MSCs on CS-induced lung alterations in rats. The parameters included emphysema, fibrosis, inflammation, apoptosis and proliferation. My results show that, iPSC-MSCs demonstrated a better effect regarding each of the parameters in comparison to those elicited in response to treatment with BM-MSCs. In fact, as far as iPSC-MSCs can produce comparable effects to BM-MSCs, due to iPSC-MSCs’ advantages regarding the resource and ageing issue, they would at the outset be a better candidate than the BM-MSCs. The superior efficacy over BM-MSCs further defined iPSC-MSCs as a promising candidate. Moreover, for the BM-MSCs, a placebo-controlled, randomized clinical trial in COPD subjects has already been reported (271). The trial observed no toxicity caused by the treatment. However, there were also no effects regarding both pulmonary function and indicators of quality-of-life. It did reveal some potential anti-inflammatory activity though, as the treatment decreased C-reactive protein (CRP) levels at an early stage. There were some limitations of the study including the determination of doses, but it still pointed out to the possibility that BM-MSCs might not work in the clinical setting, despite the clear efficacy demonstrated in animal models. From our study, iPSC-MSCs are much more effective than BM-MSCs in the rat model. Therefore the possible failure of BM-MSCs in the clinical context will not
necessarily indicate a failure of iPSC-MSCs. Instead, it will call for improved MSCs therapies, which makes study of iPSC-MSCs even more worthwhile and important. However, on the other hand, the clinical study also revealed the difference between animal models and diseased subjects, thus the effects of iPSC-MSCs in the clinical context are not predictable.

Despite numerous reports of MSCs’ effectiveness in diverse disease models, the mechanism of MSCs’ action remains unresolved (270). A major breakthrough is the report of that in vivo mitochondrial transfer from BM-MSCs into pulmonary alveoli protect the lung against lipopolysaccharide-induced acute lung injury (118), illustrating a new mechanism of MSCs’ action. However, mitochondrial transfer in the context of CS-induced lung damage was unknown. This study demonstrated that BM-MSCs and iPSC-MSCs could effectively transfer mitochondria to CS-damaged lung epithelial cells, similar to sending in a “Trojan horse”. This is one of the very early demonstrations of such activity following the first report in the acute lung injury model mentioned above. Although the signaling that drives the MSCs to transfer mitochondria for rescue of lung epithelial cell was unknown, in vitro formation of TNTs was observed between MSCs and bronchial epithelial cells. The interruption of TNT formation by cytochalasin B almost completely blocked mitochondrial transfer, suggesting that mitochondria might be transferred via TNTs in vitro. Such TNT-mediated mitochondrial transfer between MSCs and damaged lung epithelial cells might be an important mechanism through which the exhausted epithelial cellular bioenergetics can be restored and damaged lung epithelium be rejuvenated.
Bronchial epithelial cells were chosen for the *in vitro* study because they act as the first barrier against CS exposure. The inflammation and oxidative stress in COPD was initiated by the bronchial epithelial cells’ response to CS-exposure such as expression of cell injury–associated endogenous molecules and secretion of inflammatory mediators including TNF-α and IL-8 (190). Mitochondria are mostly known as the powerhouse of the cell. In patients with COPD, decreased energy metabolism is associated with declining lung function (285). Given the observation of mitochondrial transfer from MSCs to bronchial epithelial cells, the effect of MSCs on the cellular bioenergetics was studied by measuring the ATP level. The CSM treatment reduced ATP levels of bronchial epithelial cells, which was alleviated by co-culture with iPSC-MSCs, indicating improvement in bioenergetics. Such activity was coupled with CSM-induced enhancement of mitochondrial transfer from iPSC-MSCs to bronchial epithelial cells, which may be the source of the restored bioenergetics. Moreover, iPSC-MSCs have demonstrated a more active role in mitochondrial transfer in both *in vivo* and *in vitro* experiments, which may contribute to the higher efficacy of iPSC-MSCs in attenuating CS-induced lung alterations.

The limitation of mitochondrial transfer is its working radius. Relying on direct contact between iPSC-MSCs and pulmonary cells, the primary effects of mitochondrial transfer might be only effective within a limited distance from the iPSC-MSCs. Although the retention of the iPSC-MSCs were identified even two weeks after injection based on our previous study (150), it is unlikely that all the cells in the lungs would have the opportunity to directly adopt mitochondria from them.
The full activity of the MSCs may be better explained with a complementary mechanism that overcomes the restriction imposed by the distance between cells, namely, the paracrine effect. The paracrine effect of MSCs has been documented in various studies, with different active factors identified, such as vascular endothelial growth factor, TGF-β, hepatic growth factor, bFGF and platelet derived growth factor (2, 235, 270). In this study the potential of paracrine effects was demonstrated by treating bronchial epithelial cells with CdM in vitro. The treatment of CSM led to increased apoptosis and reduced proliferation in bronchial epithelial cells in vitro, resulting in an imbalance between apoptosis and proliferation. CdM restored the imbalance by reducing the apoptotic rate and promoting proliferation. The iPSC-MSCs-CdM was superior than BM-MSC-CdM regarding all the observed effects. From the in vivo data discussed earlier, iPSC-MSCs have shown superior effects than BM-MSCs in attenuating apoptosis and promoting proliferation in rat lung epithelium. The in vivo and in vitro findings together suggested a better anti-apoptotic and pro-proliferation capacity of iPSC-MSCs through paracrine action.

There are two reasons for studying the effects on apoptosis. Firstly, most studies on paracrine effects have focused on the immunoregulatory role (e.g. suppression of inflammation) of MSCs, which is therefore well demonstrated by previous reports (2, 235, 270). In contrast, the modulation of apoptosis by the CdM is less well investigated. Secondly, there is mounting data suggesting that the gradual alveolar destruction in COPD pathogenesis may be partly driven by imbalance between apoptosis and proliferation (67), although classically it is explained by the imbalance
of protease and anti-protease activity (68, 76, 77, 189). Apoptosis and proliferation are well equilibrated in healthy tissue. The induction of apoptosis by CS has been reported in various cell types of lung, such as endothelial cells, alveolar epithelial cells, fibroblasts and immune cells (16, 103, 116, 127, 166, 223). Excess apoptosis in alveolar cells without compensation from proliferation led to the gradual destruction of alveolar walls in lung (67). Apoptosis of alveolar wall and endothelial cells was reported to induce emphysema even without infiltration of inflammatory cells in an animal model (67). Therefore the anti-apoptotic and pro-proliferation potential might identify a key capacity of iPSC-MSCs as a promising candidate for cell therapy development against COPD.

Given the effects of CdM on apoptosis and inflammation, the active component of CdM was the next research question. SCF, a dimeric protein, triggers homodimerization and autophosphorylation of its receptor c-Kit upon binding, which further activates diverse pathways involving cell survival, migration, and proliferation (146). In addition to its ability to induce proliferation, SCF/c-Kit may also affect apoptosis through down-stream activation of the serine/threonine kinase Akt (35). Akt subsequently phosphorylates and inhibits pro-apoptotic protein Bad which regulates the cytochrome c release from the mitochondria through Bcl-2/Bax signalling (35, 265, 283). Despite of a lack of understanding regarding the mechanism, the SCF/c-Kit signalling has been proved essential to maintain normal alveolar structure in mouse lung. Deficiency in SCF/c-Kit signalling was reported to cause spontaneous alveolar destruction, increased ex vivo lung compliance and enlarged residual volume in mice
Given its important role in proliferation/apoptosis regulation and maintaining alveolar structure, this study targeted SCF as a potential active component in CdM. SCF alone was able to attenuate CSM-induced cytochrome c translocation, apoptosis and reduction in proliferation, indicating its anti-apoptotic and pro-proliferative activity. Meanwhile, SCF depletion led to a weakened ability of iPSC-MSCs-CdM to attenuate CSM-induced cytochrome c translocation, apoptosis and reduction in proliferation, indicating that SCF played an important role in the action of iPSC-MSCs-CdM. However, the data also showed that SCF alone was less effective than iPSC-MSCs-CdM, and that SCF depletion only weakened but did not eliminate the efficacy of iPSC-MSCs-CdM, suggesting that the action of iPSC-MSC-CdM was only partly dependent on SCF. Moreover, iPSC-MSCs secreted much higher levels of SCF than BM-MSCs, which may explain its superior anti-apoptotic and pro-proliferation effects through paracrine action.

From the above discussion, this study has demonstrated two possible mechanisms through which the iPSC-MSCs protect lung epithelial cells against CS-induced damage. However, the relative importance of each mechanism was not evaluated in the study. As mitochondrial transfer was limited by distance, the total therapeutic effects of the treatment strongly rely on the number of MSCs retained in the lung tissue. However, the ability of the MSCs to be retained in the lung varies between different reports. From our previous study in the CS-exposed model, clusters of MSCs were identified even two weeks after injection. Similarly, adipose stem cells have been reported to be retained in lung tissue in CS-exposed model after 7 days or 21
days (222). However, in another study using BM cells in CS-exposed female rats, fluorescent in situ hybridization (FISH) only found 2-13 male BM cells per entire lung from 1 day to 1 month (112). The factors that determine this retention ability remain unknown, and such factors would also be key to give mitochondrial transfer higher importance in therapeutic action of MSCs. The paracrine effect, on the other hand, may be more effective when the retained MSCs are less abundant, as the effects might be initiated by low level secretion of relevant paracrine factors but amplified by signalling pathways in the responding cells. The MSCs in the circulation might also possess systemic effects through release of molecules into the circulation. However, regarding acute responses, the direct delivery of mitochondria seemed to be a more straightforward and immediate rescue, which may be why the mitochondrial transfer has been identified as such an important mechanism in the context of acute lung injury models where bioenergetics is rapidly impaired.

There are nonetheless several limitations of this study, and more future research questions can be raised from current findings.

First of all, for the general advantages discussed earlier, although the iPSC-MSCs may be able to overcome the age-related impairment of normal MSCs, its phenotype when derived from patients with genetic mutations/inherited diseases remains unclear. About 1-3% of COPD patients are associated with a genetic alteration which leads to deficiency of serine protease α-1 antitrypsin (241). There are also other genes suspected to be associated, such as TGF-β₁ (47).

The mitochondrial transfer was proposed as an important mechanism, but the
TNT may also facilitate the transfer of other organelles, proteins and compounds. For example, whether the ATP was directly transferred from MSCs to bronchial epithelial cells was not determined. In addition, other mechanisms for the transfer of material between cells may also play important roles, such as gap junction and microvesicles. Lastly, despite the identification of mitochondrial transfer via TNTs in vitro, the route of mitochondrial transfer in vivo was not demonstrated and requires further investigation.

Regarding the paracrine effects, the CdM was collected from normal culture of BM-MSCs or iPSC-MSCs without any interactions with BEAS-2B cells. However, it is possible that CSM can induce BEAS-2B cells to release cytokines, which may act on MSCs and modulate secretory functions of MSCs. A trans-well co-culture with both cells in the same system may overcome this limitation in vitro.

SCF has been identified to contribute to the anti-apoptosis and pro-proliferation action of iPSC-MSCs-CdM, but the depletion of SCF did not eliminate the effects. Therefore more active molecules remain to be determined.

In summary, this study demonstrated that iPSC-MSCs attenuate CS-induced lung damage and observed evidence of mitochondrial transfer from iPSC-MSCs to lung epithelial cells. The higher activity to transfer mitochondria to CS-damaged bronchial epithelial cells in vitro and in vivo, in line with the higher anti-apoptosis and pro-proliferation capacity by paracrine effect partly due to more abundant SCF secretion, together explained the superior effects of iPSC-MSCs over BM-MSCs. This
study defines iPSC-MSCs as a promising candidate for the development of cellular therapy against COPD.
Chapter 4. iPSC-MSCs attenuated CSM-induced Mitochondrial Dysfunction in Human Airway Smooth Muscle Cells
4.1. Introduction

There is an increasing interest in the role of mitochondria in the pathogenesis of airway diseases. Mitochondria, most well-known as the powerhouse of the cells, also play crucial roles in other cellular functions in addition to bioenergetic regulation, such as calcium metabolism, reactive oxygen species (ROS) modulation and onset of apoptosis (45, 90, 95, 212). As the site of oxidative phosphorylation, mitochondria are an important intracellular source of ROS. Defective oxidative phosphorylation may lead to overwhelming generation of ROS in mitochondria. Increased external and internal ROS production, without sufficient protection from anti-oxidants, leads to cellular oxidative stress, which is a key player in the pathogenesis of COPD (57). The effectiveness of MSCs has been demonstrated in multiple airway disease models including emphysema and allergic airway inflammation, but the underlining mechanisms remain unresolved (270). Given that mitochondria are a potential therapeutic target for airway diseases, investigating the effects of MSCs on oxidative stress-induced mitochondrial damage in airway cells may be of value. In this study I hypothesized that interaction with iPSC-MSCs can attenuate cigarette smoke medium (CSM)-induced mitochondrial damage in ASMCs.

Recently, mitochondrial dysfunction has been reported in airway smooth muscle cells (ASMCs) isolated from patients with COPD (273), indicating that mitochondria may be a promising therapeutic target for COPD. Airway smooth muscle plays an important role in airway remodelling and inflammation (56). In addition to their contractile properties, ASMCs are also involved in airway inflammation by releasing
pro-inflammatory cytokines (10). The first part of this chapter aims to evaluate changes in mitochondrial function in normal ASMCs in response to oxidative stress. Cigarette smoke (CS), the primary cause of COPD, is a significant source of oxidants. Cigarette smoke extract was reported to alter the morphology of mitochondria in vitro in both airway epithelial cells (105) and ASMCs (10), leading to mitochondrial fragmentation. Therefore CSM was used in this study to induce mitochondrial damage. In addition, H₂O₂ was used as a direct source of oxidants. Mitochondrial ROS levels and ΔΨm were examined to evaluate changes in mitochondrial function.

Mitochondria play a key role in the regulation of cell fate. The depolarization of mitochondria is regarded as an early event triggering mitochondrial apoptosis (90). Therefore, in the second part of this chapter I investigated the effects of co-culture with iPSC-MSCs on CSM-induced mitochondrial dysfunction and apoptosis in ASMCs.

Paracrine regulation by MSCs is a widely reported mechanism through which MSCs can suppress inflammation (2, 235, 270) or attenuate apoptosis, as suggested in the previous chapter. The third part of this chapter aims to determine whether the effects of iPSC-MSCs on CSM-induced mitochondrial alterations were through secretion of paracrine factors. To investigate this, the effects of iPSC-MSCs-conditioned medium (iPSC-MSCs-CdM) on CSM-induced mitochondrial alterations were firstly examined. The contents of the iPSC-MSCs-CdM represented the paracrine factors secreted by iPSC-MSCs under normal conditions. However, in a real interaction, ASMCs may affect the iPSC-MSCs
and modulate the latter’s synthetic and secretory functions. CSM may also affect the paracrine effects of iPSC-MSCs as well as the interaction between iPSC-MSCs and ASMCs. Therefore a study using a trans-well co-culture system was carried out in which the two cell types are separated by a membrane that is permeable to the paracrine factors but prevents direct cell-cell contact.

Another recently reported mechanism of the action of MSCs is through mitochondrial transfer from MSCs to recipient cells, which was firstly described in vivo in an acute lung injury rodent model (118). In the previous chapter, mitochondrial transfer from iPSC-MSCs to airway epithelial cells was observed in CS-exposed rat lungs and CSM-treated bronchial epithelial cells. However, the effect of mitochondrial transfer on markers of mitochondrial damage was not studied. In the final part of this chapter, I studied mitochondrial transfer between ASMCs and iPSC-MSCs using fluorescent microscopy and flow cytometry.

The aims of this chapter were:

1. To evaluate oxidative stress-induced mitochondrial dysfunction in human primary ASMCs
2. To examine the capacity of iPSC-MSCs to reduce CSM-induced mitochondrial dysfunction and apoptosis in ASMCs
3. To evaluate the relative contributions of paracrine effects and cell-cell contact in the protective effects of iPSC-MSC’s on mitochondrial dysfunction and apoptosis in ASMCs
4. To investigate mitochondrial transfer from iPSC-MSCs to ASMCs
4.2. Oxidative Stress-induced Mitochondrial Dysfunction in ASMCs and iPSC-MSCs

4.2.1. Oxidative stress reduced the viability of ASMCs

Normal ASMCs isolated from bronchi or tracheas of healthy transplant donor lungs were treated with \( \text{H}_2\text{O}_2 \) (50, 100 and 200 \( \mu \text{M} \)) or CSM (10, 25, 50 and 75%) for 2, 4 and 24 hours. In the 4/20 hour group cells were stimulated for 4 hours and then incubated with serum-free medium for 20 hours. Cell viability was measured by MTT assay. \( \text{H}_2\text{O}_2 \) led to a concentration-dependent reduction in viability at each time point (Maximum reduction at 200 \( \mu \text{M} \), fold-change vs. controls: 2 hours, 0.70±0.05, \( p<0.001 \); 4 hours, 0.62 ±0.02, \( p<0.001 \); 24 hours, 0.62 ±0.06, \( p<0.01 \); 4/24 hours, 0.59 ±0.06, \( p<0.001 \)) (Figure 4.1A). A similar effect was observed at all time points.

CSM also caused loss of viability in a concentration-dependent manner at 2 hours, 4 hours and 24 hours (Maximum effects at 75% CSM, fold-change vs. controls: 2 hours, 0.88±0.04, \( p<0.01 \); 4 hours, 0.81±0.07, \( p<0.01 \); 24 hours, 0.57±0.13, \( p<0.05 \)) (Figure 4.1B). However, no significant reduction in viability was observed in the 4/20 hours group (fold to control, 75% CSM: 0.93±0.15). Moreover, the 75% CSM treatment led to a significantly lower viability at 24 hours compared with the viability at 2 hours (\( p<0.05 \)) or 4 hours (\( p<0.05 \)), indicating that the effect of 75% CSM was time-dependent.
MTT assay is based on NAD(P)H-dependent reductive reactions catalyzed by oxidoreductases in cells (30-32). Mitochondria are one of the major carriers of such enzymes, thus the reduction of MTT readout may be a reflection of mitochondrial dysfunction.

Figure 4. 1 Effects of H₂O₂ and CSM on the viability of ASMCs. Viability of ASMC was measured by MTT assay after treatment with (A) H₂O₂ (50, 100 and 200 μM) or (B) CSM (10, 25, 50 and 75%) for 2 hours, 4 hours or 24 hours. In the 4/20 hr group ASMCs were stimulated for 4 hours and then incubated with serum-free medium for 20 hours. *p<0.05, **p<0.01 and ***p<0.001 compare corresponding groups with relevant control groups; #p<0.05 compares groups as indicated.
4.2.2. Oxidative stress induced mitochondrial ROS in ASMCs

Mitochondrial ROS levels were measured by staining with MitoSOX, a dye that targets the mitochondria of live cells and emits red fluorescence when oxidized by superoxide. H$_2$O$_2$ treatment increased the mitochondrial ROS levels in a concentration-dependent manner at 2 hours (Maximum induction at 200 µM, fold to control, $1.61\pm0.16, p<0.05$ vs. control) and 4 hours (Maximum induction at 200 µM, fold to control, $1.75\pm0.15, p<0.01$ vs. control). After 24 hours only 200 µM H$_2$O$_2$ increased mitochondrial ROS levels (fold to controls: 24 hours, $1.6\pm0.25, p<0.05$ vs. control) (Figure 4.2A). Treatment for 24 hours did not show a more potent induction of mitochondrial ROS than the 2 hour or 4 hour treatments. The induction of mitochondrial ROS persisted up to 20 hours after the removal of H$_2$O$_2$ (Maximum induction at 200 µM, fold to control, $1.56\pm0.22, p<0.01$ vs. control).

Mitochondrial ROS was highly induced by CSM treatment in a concentration-dependent manner (Max induction at 75% CSM, fold to controls: 2 hours, $8.54\pm1.99, p<0.001$; 4 hours, $8.70\pm1.17, p<0.001$; 24 hours, $11.98\pm2.85, p<0.001$; $p$-value compared to control of each time point) (Figure 4.2B). The increase in mitochondrial ROS persisted up to 20 hour after the removal of CSM (Max induction at 75% CSM, $8.74\pm2.87, p<0.001$).
Figure 4. 2 H$_2$O$_2$ and CSM induced mitochondrial ROS in ASMCs. Mitochondrial ROS in ASMCs was measured by staining with MitoSOX after treatment with (A) H$_2$O$_2$ (50, 100 and 200 μM) or (B) CSM (10, 25, 50 and 75%) for 2, 4 or 24 hours. In the 4/20 hr group ASMCs were stimulated for 4 hours and then incubated with serum-free medium for 20 hours. *p<0.05, **p<0.01 and ***p<0.001 compare corresponding groups with relevant control groups; #p<0.05 compares groups as indicated.
4.2.3. Oxidative stress induced reduction of ΔΨm in ASMCs

Maintenance of the mitochondrial membrane potential (ΔΨm) is very important for the normal function of mitochondria. Mitochondrial depolarization can lead to an interruption in the action of the respiratory chain and triggers apoptosis (90). In this study ΔΨm was measured by staining with JC-1. A decreased ratio of red/green fluorescence indicates loss of ΔΨm.

The ΔΨm was reduced by H₂O₂ treatment in a concentration-dependent manner at each time point (Maximum effects at 200 μM, fold to controls: 2 hours, 0.88±0.02, p<0.05; 4 hours, 0.89±0.01, p<0.05; 24 hours, 0.70±0.10, p<0.01; p-value compared to control of each time point) (Figure 4.3A). The reduction of ΔΨm was observed even 20 hours after the removal of H₂O₂ (Maximum effects at 200 μM, fold to controls: 0.64±0.09, p<0.01).

There was a trend showing a decrease in ΔΨm after 2 hours treatment with CSM, which did not reach statistical significance. Significant reduction in ΔΨm was observed in a concentration-dependent manner at the 4 hour and 24 hour time points (Maximum effects at 75% CSM, fold to controls: 4 hours, 0.62±0.04, p<0.001; 24 hours, 0.39±0.07, p<0.001) (Figure 4.3B). Moreover, 75% CSM treatment led to significantly lower ΔΨm at 24 hours compared to the 2 hours (p<0.001) and 4 hours (p<0.001) time points. The reduction of ΔΨm was still evident even 20 hours after the removal of CSM (Maximum effects at 75% CSM, fold to controls: 0.67±0.07, p<0.01)
Figure 4. 3 H$_2$O$_2$ and CSM reduced $\Delta\Psi_m$ in ASMCs. $\Delta\Psi_m$ in ASMCS was measured by staining with JC-1 after treatment with (A) H$_2$O$_2$ (50, 100 and 200 $\mu$M) or (B) CSM (10, 25, 50 and 75%) for 2, 4 or 24 hours. In the 4/20 hr group ASMCs were stimulated for 4 hours and then incubated with serum-free medium for 20 hours. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compare corresponding groups with relevant control groups; #$p<0.05$, ##$p<0.01$, ###$p<0.001$ compare groups as indicated.
4.2.4. Oxidative stress reduced cell viability of iPSC-MSCs

To examine the effects of oxidative stress on iPSC-MSCs, iPSC-MSCs were treated with H$_2$O$_2$ (50, 100 and 200 μM) or CSM (10, 25, 50 and 75%) for 4 and 24 hours. The effects were compared with those observed in ASMCs.

H$_2$O$_2$ reduced the viability of iPSC-MSCs in a concentration-dependent manner after 4 and 24 hours (Max effect at 200 μM, fold to controls: 4 hours, 0.43 ±0.14, $p<0.05$; 24 hours, 0.44 ±0.05, $p<0.001$; $p$-value compared to control of each time point), in line with the effects seen in ASMCs (Figure 4.4A).

CSM also reduced the viability of iPSC-MSCs in a concentration-dependent manner after 4 and 24 hours. At 4 hours, significant reduction was observed with 50% CSM (fold to controls: 0.60 ±0.12, $p<0.05$ vs. control) and 75% CSM (fold to controls: 0.49 ±0.09, $p<0.01$ vs. controls; Figure 4.4B). At 24 hours, significant reduction was observed with 25% CSM (fold to controls: 0.76 ±0.09, $p<0.05$ vs. control), 50% CSM (0.18 ±0.02, $p<0.001$ vs. control) and 75% CSM (0.16±0.02, $p<0.001$ vs. control). The viability of iPSC-MSCs was significantly lower than ASMCs ($p<0.05$ at each treatment and time point), indicating that iPSC-MSCs are more sensitive to high concentrations of CSM than ASMCs with respect to their viability.
Figure 4. Effects of H$_2$O$_2$ and CSM on viability of iPSC-MSCs. Changes in the viability of iPSC-MSCs were measured by MTT assay after treatment with (A) H$_2$O$_2$ or (B) CSM for 4 hours and 24 hours. Changes in the viability of ASMCs were shown for comparison. *p<0.05, **p<0.01 and ***p<0.001 compare corresponding groups with relevant control groups; #p<0.05 compares groups as indicated.
4.2.5. Oxidative stress induced mitochondrial ROS in iPSC-MSCs

H$_2$O$_2$ increased the mitochondrial ROS levels in iPSC-MSCs in a concentration-dependent manner at both 4 and 24 hours (Maximum effect at 200 μM, fold to controls: 4 hours, 4.12±1.73, $p<0.05$; 24 hours, 4.46±0.81, $p<0.05$; $p$-value compared to control of each time point) (Figure 4.5A). The increase in mitochondrial ROS levels induced by CSM (200 μM) after 24 hours was greater in iPSC-MSCs compared to ASMCs ($p<0.01$.)

CSM also induced mitochondrial ROS in iPSC-MSCs, in a concentration-dependent manner after 4 and 24 hours (Maximum effect at 75% CSM, fold to controls: 4 hours, 6.36±1.94, $p<0.05$; 24 hours, 10.33±3.29, $p<0.001$; $p$-value compared to control of each time point) (Figure 4.5B). No significant difference was observed between the response of iPSC-MSCs and ASMCs.
Figure 4.5 Effects of H\textsubscript{2}O\textsubscript{2} and CSM on mitochondrial ROS of iPSC-MSCs. Mitochondrial ROS levels of iPSC-MSCs were measured by MitoSOX staining after treatment with (A) H\textsubscript{2}O\textsubscript{2} or (B) CSM for 4 and 24 hours. Changes in the mitochondrial ROS levels of ASMCs were shown for comparison. *p<0.05, **p<0.01 and ***p<0.001 compare corresponding groups with relevant control groups; ##p<0.01 compares groups as indicated.
4.2.6. Oxidative stress induced reduction of ΔΨm in iPSC-MSCs

H$_2$O$_2$ reduced the ΔΨm in iPSC-MSCs in a concentration-dependent manner at both 4 hours and 24 hours (Maximum effect at 200 μM, fold to controls: 4 hours, 0.80±0.09, p<0.05; 24 hours, 0.41±0.09, p<0.001; p-value compared to control of each time point) (Figure 4.6A). The reduction in ΔΨm by H$_2$O$_2$ (200 μM) was stronger in iPSC-MSCs compared to ASMCs but it did not reach statistical significance.

CSM also reduced the ΔΨm in iPSC-MSCs in a concentration-dependent manner at both 4 and 24 hours (Maximum effect at 75% CSM, fold to controls: 4 hours, 0.23±0.06, p<0.001; 24 hours, 0.21±0.01, p<0.001; p-value compared to control of each time point) (Figure 4.6B). The reduction in ΔΨm was significantly stronger in iPSC-MSCs compared with ASMCs after treatment with 50% and 75% CSM for 4 hours or with any concentration of CSM for 24 hours.
Figure 4.6 Effects of H$_2$O$_2$ and CSM on ΔΨm of iPSC-MSCs. The ΔΨm of iPSC-MSCs was measured by JC-1 assay after treatment with H$_2$O$_2$ (A) or CSM (B) for 4 hours and 24 hours. Changes in the ΔΨm of ASMCs were shown for comparison. *p<0.05, **p<0.01 and ***p<0.001 compare corresponding groups with relevant control groups; #p<0.05, ##p<0.01 compare groups as indicated.
4.3. Direct Co-culture with iPSC-MSCs Attenuated CSM-induced Mitochondrial Dysfunction and Apoptosis in ASMCs

In order to determine whether iPSC-MSCs are capable of preventing CSM-induced mitochondrial dysfunction in ASMCs, in a prophylactic protocol iPSC-MSCs were directly co-cultured with CellTrace-labeled ASMCs for 20 hours and then treated with CSM (10 and 25%) for 4 hours. As a control, single cultures of ASMCs were stained with Cell Trace, incubated for 20 hrs and then treated with CSM for 4 hours (Figure 2.5 A). In a therapeutic protocol, CellTrace-labeled ASMCs were treated with CSM (10 and 25%) for 4 hours before iPSC-MSCs were added to the culture and incubated for a further 20 hours. As a control, single cultures of ASMCs were stained with Cell Trace, treated with CSM for 4 hours and then incubated in the absence of stimulation for 20 hrs (Figure 2.5B).

4.3.1. iPSC-MSCs prevented and reversed CSM-induced mitochondrial ROS in ASMCs

The two cell types in the co-cultures were distinguished by the CellTrace staining using flow cytometry (Figure 4.7A). The mitochondrial ROS levels were measured in the gated ASMC (CellTrace-positive) population and compared with the ASMCs in the single-culture (Figure 4.7B). 10% CSM significantly increased the levels of mitochondrial ROS in ASMCs in the single culture (fold to control: 3.26±0.33, p<0.01 in comparison to control), which was prevented by co-culture with
iPSC-MSCs (fold to control: $2.12\pm0.17$, $p<0.05$ in comparison to single-culture). 25% CSM further increased the level of mitochondrial ROS in single-culture compared to 10% CSM (fold to control: $6.71\pm0.75$, $p<0.001$), which was also reduced by iPSC-MSCs (fold to control: $4.01\pm0.44$, $p<0.05$ in comparison to single-culture). However, a concentration-dependent increase of mitochondrial ROS levels of ASMCs in the co-culture was still observed, indicating the effects of CSM were only alleviated but not eliminated.

In addition, a therapeutic protocol was also performed, in which the CSM was removed from the ASMCs and replaced by either serum free medium or iPSC-MSCs (Figure 4.7C). Under these conditions only 25% CSM led to a significant increase in mitochondrial ROS (fold to control: $3.42\pm0.46$, $p<0.01$ in comparison to control). A small but significant reduction in mitochondrial ROS was observed in the co-culture group compared to the single culture group treated with 25% CSM (fold to control: $2.70\pm0.48$, $p<0.05$), indicating therapeutic recovery from CSM-induced mitochondrial ROS by iPSC-MSCs. Again the concentration-dependent elevation of mitochondrial ROS was still evident among the co-culture groups, indicating the effects of CSM were not eliminated.
Figure 4. 7 Effects of direct co-culture with iPSC-MSCs on mitochondrial ROS in ASMCs. (A) Flow cytometry histograms showing CellTrace positive and negative populations corresponding to ASMCs and iPSC-MSCs, respectively. (B) Prophylactic effects of iPSC-MSCs on CSM-induced mitochondrial ROS in ASMCs. iPSC-MSCs were co-cultured with CellTrace-labeled ASMCs for 20 hours and then treated with CSM (10 and 25%) for 4 hours. Single ASMCs cultures were used as controls. Mitochondrial ROS levels were determined using MitoSOX staining. (C) Therapeutic effects of iPSC-MSCs on CSM-induced mitochondrial ROS in ASMCs. CellTrace-labeled ASMCs were treated with CSM (10 and 25%) for 4 hours. CSM was then removed and iPSC-MSCs were loaded. Single ASMCs cultures were used as controls. *p<0.05, **p<0.01, ***p<0.001 compare groups as indicated.
4.3.2. iPSC-MSCs prevented but did not reverse CSM-induced reduction in ΔΨm in ASMCs

In the prophylactic protocol, CSM induced reduction of ΔΨm at both 10% (fold to control: 0.80±0.04, \( p<0.01 \)) and 25% concentration (fold to control: 0.52±0.07, \( p<0.001 \)). 25% CSM led to lower ΔΨm than 10% CSM (\( p<0.001 \)). Co-culture with iPSC-MSCs improved the ΔΨm under both 10% CSM treatment (fold to control: 0.95±0.04, \( p<0.01 \)) and 25% CSM treatment (fold to control: 0.78±0.10, \( p=0.059 \)) (Figure 4.8A). CSM still led to a trend showing a reduction in ΔΨm in ASMCs in the co-culture, but the difference was not statistically-significant.

In the therapeutic protocol, after recovery for 20 hours, single-cultures of ASMCs still showed significantly reduced ΔΨm in both 10% CSM-treated group (fold to control: 0.84±0.07, \( p<0.01 \)) and 25% CSM-treated group (fold to control: 0.75±0.59, \( p<0.001 \)) (Figure 4.8B). There is a trend showing an increase in mitochondrial potential in the co-culture groups compared to the single culture at both 10% (fold to control: 0.94±0.04) and 25% CSM treatments (fold to control: 0.87±0.11), which did not reach statistical significance.
Figure 4. 8 Effects of direct co-culture with iPSC-MSCs on ΔΨm in ASMCs. (A) Prophylactic effects of iPSC-MSCs on CSM-induced reduction in ΔΨm in ASMCs. iPSC-MSCs were co-cultured with CellTrace-labeled ASMCs for 20 hours and then treated with CSM (10 and 25%) for 4 hours. Single ASMCs cultures were used as controls. ΔΨm was determined using JC-1 staining. (B) Therapeutic effects of iPSC-MSCs on CSM-induced reduction in ΔΨm in ASMCs. CellTrace-labeled ASMCs were treated with CSM (10 and 25%) for 4 hours. CSM was then removed and iPSC-MSCs were loaded. Single ASMCs cultures were used as controls. *p<0.05, **p<0.01, ***p<0.001 compares groups as indicated.
4.3.3. iPSC-MSCs prevented CSM-induced apoptosis of ASMCs

Based on the observations of a protective role of iPSC-MSCs against CSM-induced mitochondrial dysfunction in ASMCs, further investigations were carried out on whether such mitochondrial improvement led to improved function of the cells. In particular apoptosis, as a possible outcome of severe mitochondrial damage, was investigated in the co-culture using the prophylactic protocol (Figure 4.9). Results from Annexin V staining confirmed induction of apoptosis by CSM in single-cultured ASMCs at 25% CSM (46.6±1.3% vs. 17.4±2.9%, \( p<0.001 \)) and 50% CSM (86.8±3.8% vs. 17.4±2.9%, \( p<0.001 \)). In the absence of stimulation, the iPSC-MSCs-treated ASMCs demonstrated higher percentage of apoptosis than those in the single-culture (30.7±1.4% vs. 17.4±2.9%, \( p<0.05 \)), which indicates that introducing iPSC-MSCs to the system may induce stress to the ASMCs at baseline. However, iPSC-MSCs led to a significant reduction in apoptosis induced by 25% (34.7±1.5% vs. 46.6±1.3%, \( p<0.05 \)) and 50% CSM (49.5±4.2% vs. 86.8±3.8%, \( p<0.001 \)), indicating that interaction with iPSC-MSCs can protect ASMCs from CSM-induced mitochondrial dysfunction and apoptosis.
Figure 4. 9 Prophylactic effects of iPSC-MSCs on CSM-induced apoptosis in ASMCs. iPSC-MSCs were co-cultured with CellTrace-labeled ASMCs for 20 hours and then treated with CSM (10 and 25%) for 4 hours. Single ASMCs cultures were used as controls. Apoptosis was detected by Annexin V staining. *p<0.05, **p<0.01, ***p<0.001 compare groups as indicated.
4.4. Paracrine Effects are only Partly Involved in the Protective Effects of iPSC-MSCs on CSM-induced Mitochondrial Dysfunction and Apoptosis in ASMCs

4.4.1. Effects of conditioned medium from iPSC-MSCs on CSM-induced mitochondrial dysfunction in ASMCs

In order to examine the role of paracrine effects in the protective effects of iPSC-MSCs on CSM-induced mitochondrial damage in ASMCs, the effects of conditioned medium from iPSC-MSCs (iPSC-MSCs-CdM) on CSM-induced mitochondrial damage in ASMCs were investigated. CdM was a concentrate of all paracrine factors released over a 24 hour period from confluent cultures of iPSC-MSCs. The ASMCs were pre-treated with iPSC-MSCs-CdM for 4 hours followed by CSM treatment for a further 4 hours in the presence of CdM. The evaluation of mitochondrial ROS revealed very similar results with the direct co-culture system (Figure 4.10A). CSM treatments increased mitochondrial ROS in a concentration-dependent manner in both untreated ASMCs (fold to controls: 10% CSM, 2.96±0.34, \( p<0.05 \); 25% CSM, 7.76±0.06, \( p<0.001 \); \( p \)-value compared to relevant controls) and ASMCs treated with iPSC-MSCs-CdM (fold to controls: 10% CSM, 2.56±0.29, \( p<0.01 \); 25% CSM, 5.81±0.49, \( p<0.001 \); \( p \)-value compared to relevant controls). However, compared to untreated ASMCs, ASMCs treated with iPSC-MSCs-CdM demonstrated significantly lower levels of mitochondrial ROS in both 10% CSM group (\( p<0.05 \)) and 25% CSM group (\( p<0.05 \)).

The reduction of mitochondrial ROS by iPSC-MSCs-CdM was not associated
with prevention of ΔΨm reduction. CSM treatments reduced ΔΨm in the untreated ASMCs (fold to controls: 10% CSM, 0.84±0.03, p<0.01; 25% CSM, 0.59±0.05, p<0.001; p-value compared to relevant controls) as well as in the ASMCs treated with iPSC-MSCs-CdM (fold to controls: 10% CSM, 0.87±0.02, p<0.05; 25% CSM, 0.59±0.05, p<0.001; p-value compared to relevant controls; Figure 4.10B). The effect of CSM on ΔΨm was similar in untreated and iPSC-MSCs-CdM-treated ASMCs. These findings suggest that paracrine factors are only partly involved in the capacity of iPSC-MSCs to protect ASMCs from CSM-induced mitochondrial damage

4.4.2. Effect of iPSC-MSCs-CdM on CSM-induce apoptosis of ASMCs

The effect of iPSC-MSCs-CdM on CSM-induced ASMC apoptosis was also determined. CSM increased the percentage of apoptotic ASMCs in a concentration-dependent manner (Maximum induction by 50% CSM: 64.8±9.2% vs. 12.6±1.4%, p<0.001; p-value compared to control). Within the iPSC-MSCs-CdM treated groups, a similar induction was observed (Maximum induction by 50% CSM: 67.8±9.0% vs. 13.8±1.3%, p<0.001; p-value compared to control) (Figure 4.11). Treatment with iPSC-MSCs-CdM did not affect the baseline or CSM-induced apoptosis.
Figure 4. Effects of iPSC-MSCs-CdM on mitochondrial ROS and ΔΨm in ASMCs. ASMCs were pre-treated with iPSC-MSCs-CdM for 4 hours before stimulating with CSM for 4 hours. (A) Mitochondrial ROS was determined by MitoSOX staining and (B) ΔΨm by JC-1 staining. *p<0.05, **p<0.01, ***p<0.001 compare groups as indicated.
**Figure 4.** Effects of iPSC-MSCs-CdM on CSM-induced apoptosis in ASMCs. ASMCs were pre-treated with iPSC-MSCs-CdM for 4 hours before stimulating with CSM for 4 hours. Apoptosis was determined by Annexin V staining. **p<0.01, ***p<0.001 compare groups as indicated.
4.4.3. Effects of trans-well co-culture with iPSC-MSCs on CSM-induced mitochondrial alterations in ASMCs

To better replicate the crosstalk between the ASMCs and iPSC-MSCs through paracrine effects, trans-well co-culture was performed using cell culture inserts with 0.4 μm pores. ASMCs in 6-well plates were incubated with either blank inserts or inserts containing iPSC-MSCs for 20 hours before being treated with CSM (10 or 25%) for 4 hours (Figure 4.12A). Mitochondrial ROS levels were increased by 10% CSM (fold to control: 3.55±0.59, *p*<0.01) and 25% CSM (fold to control: 8.35±0.83, *p*<0.001) in the ASMCs treated with blank inserts. In the trans-well co-culture group, mitochondrial ROS levels in ASMCs were also elevated by 10% CSM (fold to control: 3.12±0.57, *p*<0.05) and 25% CSM (fold to control: 7.15±0.84, *p*<0.001), but the mitochondrial ROS induced by 25% CSM was significant reduced compared to the blank insert group (*p*<0.05) (Figure 4.12B).

Significant reduction of ΔΨm was induced by 25% CSM at both blank insert group (fold to control: 0.39±0.06, *p*<0.01) and iPSC-MSCs-treated group (fold to control: 0.49±0.12, *p*<0.01). No significant difference was observed between the iPSC-MSCs treatment and blank insert (Figure 4.12C). These results are in line with the findings from the iPSC-MSCs-CdM experiment.
Figure 4. 12 Effects of co-culture with iPSC-MSCs using the trans-well system on mitochondrial ROS and ΔΨm in ASMCs. (A) Illustration of the trans-well setup. Inserts with or without iPSC-MSCs were placed into the wells of 6-well tissue culture plates, containing ASMCs, for 20 hours. The whole system was treated with CSM for 4 hours. In the ASMCs mitochondrial ROS was determined by (B) MitoSOX staining and (C) ΔΨm by JC-1 staining. *p<0.05, **p<0.01, ***p<0.001 compare groups as indicated.
4.4.4. Effect of trans-well co-culture with iPSC-MSCs on CSM-induced apoptosis in ASMCs

The effect of co-culture using a trans-well system on ASMC apoptosis was also examined. 25% CSM induced a significant increase in ASMC apoptosis in both the blank insert group (48.6±10.9% vs. 11.7±2.6%, \(p<0.05\)) and the iPSC-MSC-treated group (54.6±8.3% vs. 16.7±3.3%, \(p<0.01\)) (Figure 4.13). No difference was observed in the iPSC-MSCs-treated group in comparison with the blank group.

![Figure 4.13](image_url)

**Figure 4.13** Effects of trans-well co-culture with iPSC-MSCs on apoptosis in ASMCs. Inserts with or without iPSC-MSCs were placed into the wells of 6-well tissue culture plates, containing ASMCs, for 20 hours. The whole system was treated with CSM for 4 hours. In the ASMCs apoptosis was determined by Annexin V staining. \(*p<0.05, **p<0.01\) compares corresponding groups with relevant controls.
4.5. Mitochondrial Transfer from iPSC-MSCs to ASMCs

4.5.1. Mitochondrial transfer from iPSC-MSCs to ASMCs

A possible mechanism underlining the protective effects of iPSC-MSCs against CSM-induced mitochondrial dysfunction in ASMCs is mitochondrial transfer. To investigate whether mitochondrial transfer from iPSC-MSCs to ASMCs takes place, ASMCs were pre-stained with CellTrace Violet while iPSC-MSCs were pre-stained with MitoTracker Red, a mitochondrial-targeted fluorescent dye. They were co-cultured for 20 hours followed by 4 hours stimulation with 25% CSM. The cells were fixed and stained with phalloidin which selectively labels F-actin. Using fluorescent microscopy, iPSC-MSCs-derived MitoTracker-labeled mitochondria were observed in CellTrace-labeled ASMCs, indicating transfer of mitochondria from iPSC-MSCs to ASMCs (Figure 4.14). In addition, tunneling nanotube (TNT)-like structures, containing iPSC-MSC mitochondria, were observed connecting iPSC-MSCs and ASMCs. Actin filaments were identified in the TNTs by phalloidin staining confirming a connection between the cytoskeleton systems of iPSC-MSCs and ASMCs, which may provide a path through which the mitochondria are transferred.

4.5.2. CSM increased mitochondrial transfer from iPSC-MSCs to ASMCs

Mitochondrial transfer was quantified by determining the percentage of MitoTracker-positive cells in the ASMC population using flow cytometry. Mitochondrial transfer was detected after co-culture for 24 hours without CSM.
stimulation or after stimulation with 10% or 25% CSM during the last 4 hours of the co-culture period. 10% CSM enhanced mitochondrial transfer, as shown by a significant elevation of the transfer rate (48.9±6.8% vs. 18.8±4.1%, p<0.05). 25% CSM further increased the transfer rate compared to 10% CSM (75.5±8.5% vs. 48.9±6.8%, p<0.05), suggesting that CSM promoted mitochondrial transfer from iPSC-MSCs to ASMCs in a concentration-dependent manner (Figure 4.15).
Figure 4. Mitochondrial transfer from iPSC-MSCs to ASMCs. ASMCs were pre-stained with CellTrace and iPSC-MSCs with MitoTracker. Cells were co-cultured for 20 hours and then treated with 25% CSM for 4 hours. Phalloidin staining was applied after fixation. The presence of MitoTracker-labeled mitochondria was observed in CellTrace-labeled cells, indicating transfer of iPSC-MSC-derived mitochondria to ASMCs. Tunneling nanotube–like structures between iPSC-MSCs and ASMCs can be observed. Scale bar=25 μm.
Figure 4. 15 Effect of CSM on the rate of mitochondrial transfer from iPSC-MSCs to ASMCs. ASMCs were pre-stained with CellTrace and iPSC-MSCs with MitoTracker. Cells were co-cultured for 20 hours and then treated with 25% CSM for 4 hours. (A) Mitochondrial transfer from iPSC-MSCs (MitoTracker-positive) to ASMCs (CellTrace-positive) was determined by flow cytometry. The ASMC population is indicated by the gate in the plots of co-culture. (B) The rate of mitochondrial transfer was evaluated by the percentage of MitoTracker-positive ASMCs in total ASMC population. *p<0.05, ***p<0.001 compare groups as indicated.
4.6. Discussion

This study demonstrated increased mitochondrial ROS and reduced ΔΨm in healthy ASMCs, in response to H₂O₂ or CSM. Direct contact with iPSC-MSCs protected the ASMCs from CSM-induced mitochondrial ROS and reduction of ΔΨm, which may explain the attenuation of CSM-induced apoptosis. When the interaction between the two cell types was restricted to paracrine effects, only CSM-induced mitochondrial ROS but not CSM-induced ΔΨm loss and apoptosis in ASMCs were ameliorated, indicating that the paracrine effects can only partly explain the protective effects of iPSC-MSCs. Under direct interaction, mitochondria were transferred from iPSC-MSCs to ASMCs, which was enhanced by CSM treatment. Formation of TNTs between iPSC-MSCs and ASMCs was observed in the co-culture. The prophylactic effects of iPSC-MSCs on CSM-induced mitochondrial damage in ASMCs were lost when the co-culture was carried out using a trans-well system. As the trans-well inserts would block nanotube formation, whilst allowing only paracrine communication, this suggests an important role of mitochondrial transfer in the protective effects of iPSC-MSCs. These findings highlight that the full protective capacity of iPSC-MSCs may rely on direct contact with target cells through which mitochondrial transfer can take place. This study suggested that iPSC-MSCs may be promising candidates for cell-based treatments targeting mitochondrial damage in patients with COPD.

Targeting mitochondrial damage provides a new promising strategy for developing treatments for COPD. Mitochondrial dysfunction was recently
demonstrated in ASMCs from patients with COPD, as characterized by reduced ATP levels, mitochondrial complex protein expression and ΔΨm, as well as increased mitochondrial ROS (273). The study also demonstrated mitochondrial damage, as reflected by the same parameters, in an ozone-induced mouse model of airway inflammation and hyper-responsiveness. Mitochondrial damage as well as airway inflammation and hyper-responsiveness were attenuated by the mitochondrial-targeted antioxidant MitoQ (273). In an earlier report, swelling and fragmented mitochondria with depleted cristae were observed in airway epithelial cells from patients with stage 4 COPD (105). In the present study, oxidative stress-induced mitochondrial damage was identified by increased mitochondrial ROS and loss of ΔΨm in ASMCs, which provided a model in which to study the effects of iPSC-MSCs.

CS, the major cause of COPD, contains numerous oxidants. One puff of cigarette smoke contains approximately $10^{15}$ oxidants in the gas phase and $10^{18}$ oxidants in the tar phase, together with 3000 ppm of nitrite oxide (57, 58). Acute exposure to CS is reported to impair energy metabolism and mitochondrial protein expression in mouse lungs, leading to switch of glucose metabolism to pentose phosphate pathway and reduction of substrate supply to mitochondrial respiration (4). Cigarette smoke medium was reported to alter the morphology of mitochondria in vitro, in both airway epithelial cells (105) and ASMCs (10), inducing mitochondrial fragmentation in both cell types and mitochondrial ROS elevation in ASMCs. However, the effect on ΔΨm was not examined in these studies. In the present study CSM was demonstrated to
induce mitochondrial ROS, ΔΨm loss and apoptosis in normal ASMCs from healthy subjects. Although inhalation of CS primarily affects the epithelium, CS-derived chemicals could also affect airway smooth muscle directly via the circulation (205) or indirectly through inducing the production of epithelial cell-derived inflammatory mediators such as IL-1β, IFN-γ and TNF-α which affect ASMC function (70, 231). Moreover, chronic exposure to CS can lead to a defective epithelial barrier function (102, 226), which may result in a direct exposure of CS to ASMCs. Hence the in vitro model of CSM-induced ASMC dysfunction is physiologically relevant. On the other hand, it is to my interest to establish a model of oxidative stress-induced mitochondrial damage, through which mitochondrial-targeted therapeutic approaches can be tested. The concentrations of CSM chosen for the co-culture experiments were based on the findings of concentration response experiments performed on single cultures of ASMCs, as it would be impossible to determine physiologically-relevant concentrations of cigarette smoke constituents. CSM-induced apoptosis was observed in association with mitochondrial dysfunction. Induction of apoptosis by CS has also been demonstrated in other cell types in the lung including endothelial cells, alveolar epithelial cells, fibroblasts and immune cells (16, 103, 116, 127, 166, 223). The anti-apoptotic role of iPSC-MSCs demonstrated in the ASMC model may also be important in other cell types in the lung.

MSCs have been reported to attenuate lung emphysema and allergic airway inflammation in murine models (94, 150, 151, 244). Although MSCs are considered as putative cell-based therapies for COPD, their potential mechanisms of their action
remain unclear. It is possible that their effects are at least partly mediated by improving mitochondrial function in COPD lungs. Indeed, MSCs have been shown to rescue defective mitochondria and impaired bioenergetics of mitochondria in an animal model of lipopolysaccharide-induced acute lung injury (118), as well as in an *in vitro* model of mitochondrial DNA mutation (238). In my study, the effects of iPSC-MSCs on CSM-induced mitochondrial damage in ASMCs were examined. Using a prophylactic protocol, I have shown that interaction with iPSC-MSCs prevents CSM-induced mitochondrial ROS and reduction in ΔΨm in ASMCs. CSM-induced apoptosis in ASMCs was also attenuated by iPSC-MSCs, which may be a result of improved mitochondrial function. Using the therapeutic protocol, I observed significant attenuation of CSM-induced mitochondrial ROS but not reversal of CSM-induced ΔΨm reduction. The therapeutic effects of iPSC-MSCs appear to be less pronounced, possibly due to the partial recovery of mitochondrial function in absence of CSM stimulation, or the progression of mitochondrial damage to an irreversible stage. Therefore the prophylactic protocol was chosen for later experiments. These findings support the hypothesis that iPSC-MSCs can attenuate CSM-induced mitochondrial damage, suggesting their potential capacity to repair mitochondrial dysfunction in patients with COPD.

MSCs have been reported to induce paracrine effects by releasing immunoregulatory cytokines such as VEGF, TGF-β₁, HGF, bFGF and platelet derived growth factor (2, 235, 270). In the previous chapter iPSC-MSCs-CdM was found to attenuate CSM-induced apoptosis in bronchial epithelial cells *in vitro*. However, in
the present study, iPSC-MSCs-CdM was only able to ameliorate CSM-induced mitochondrial ROS in ASMCs without any effectiveness on ΔΨm or apoptosis. As iPSC-MSCs were not in close proximity to ASMCs or exposed to CSM treatment before the collection of CdM, the lack of effect may indicate that the protective effects of iPSC-MSCs on ASMCs may be induced by the CSM or mediators produced by the ASMCs themselves. However, in a trans-well co-culture system where there is paracrine crosstalk between the two cell types, just as in the direct co-culture system, the iPSC-MSCs prevented CSM-induced mitochondrial ROS production but not the reduction of ΔΨm or apoptosis in ASMCs.

Therefore, the protective role of iPSC-MSCs against CSM-induced mitochondrial depolarization and apoptosis in ASMCs seems to be dependent on the direct contact between the two cell types, which may also involve mitochondrial transfer. Mitochondrial transfer was firstly identified in co-cultures of BM-MSCs and lung epithelial cells with defective mitochondria (238). Since then, mitochondrial transfer from BM-MSCs to epithelial cells, endothelial cells, cardiomyocytes and osteosarcoma cells has been demonstrated (55, 192, 204, 238). Mitochondrial transfer from MSCs to epithelial cells was also observed in vivo in rodent models of acute lung injury (118) and CS-induced emphysema (150). In this study mitochondrial transfer from iPSC-MSCs to ASMCs was demonstrated. Moreover, CSM significantly enhanced the transfer in a concentration-dependent manner. A previous study reported that CS exposure can lead to a reduction in the supply of substrates to the electron transport chain, leading to increased mitochondrial workload to maintain normal
energy supply (4). The transfer of healthy mitochondria may alleviate such burden and thus relieve the mitochondrial stress as indicated by the reversal of mitochondrial depolarization. In this study the formation of TNTs connecting the cytoskeleton systems of the two cells were evident by staining the actin filaments. TNTs are highly sensitive nanotubular structures between cells which can facilitate the selective transfer of membrane vesicles and organelles (215). TNTs have been reported to mediate mitochondrial transfer from BM-MSCs to epithelial cells, from endothelial cells to cancer cells and from vascular smooth muscle cells to MSCs (196, 238, 258). ASMCs are also reported to form TNTs with CD4+ T cells in vitro, leading to enhanced T cell survival (7). The formation of TNTs between iPSC-MSCs and ASMCs may provide a path through which mitochondria can be transferred, but the mechanisms underlining the regulation of TNT formation by the two cell types or by CSM remain unknown. The mechanism by which cells regulate mitochondrial transfer through the TNTs is also unclear. A recent study demonstrated that mitochondria are carried by a mitochondrial Rho-GTPase, Miro1, which can move along the actin filament in the TNTs between two cell types (6). Possible mechanisms of regulation of Miro1 by CS should be further investigated in the future.

Despite the evidence indicating iPSC-MSC-mediated protection of ASMCs from CSM-induced mitochondrial damage, some of my findings need to be considered carefully. Firstly, although MSCs have been reported to rescue injured cells in various models of inflammation and oxidative stress, their own response to oxidative stress is often neglected. In this study I have demonstrated that iPSC-MSCs are not more
resistant to oxidative stress than ASMCs. Therefore caution is required in the
development of MSC-based therapies as MSCs may also show mitochondrial damage
under oxidative stress. Secondly, in the absence of CSM treatment, the co-culture with
iPSC-MSCs increased the apoptosis of ASMCs suggesting that iPSC-MSCs may
induce stress in the target cells at baseline. Such data imply that the safety of
iPSC-MSCs should be carefully examined if they are to be used therapeutically in
man.

There are several limitations to this study and therefore further work is required
to better understand the protective effects of iPSC-MSCs and the mechanisms
mediating these effects. Firstly, the ASMCs used in my study were isolated from
healthy subjects. A better understanding of the therapeutic potential of iPSC-MSCs in
COPD would result from a study of ASMCs from COPD patients, in which
mitochondria are known to be already impaired (273). Secondly, although the
paracrine effects were not shown to mediate the full protective effect of iPSC-MSCs,
protection from CSM-induced mitochondrial ROS was still evident. The molecules
mediating these effects require further investigation. Thirdly, the formation of TNTs
may also facilitate the exchange of other cellular content, which may also protect the
ASMCs from mitochondrial damage. Lastly, the mechanism of CSM’s effect on
promotion of mitochondrial transfer need to be further addressed.

In summary, this study demonstrated that the capacity of iPSC-MSCs to attenuate
CSM-induced mitochondrial damage in ASMCs relies highly on direct cell-cell
contact. Mitochondrial transfer through the TNTs may play an important role in this
process. I elucidated the potential of iPSC-MSCs to target mitochondrial dysfunction in patients with COPD, thus further highlighting iPSC-MSCs as a promising candidate for development of cell-based therapies in COPD.
Chapter 5. Effects of iPSC-MSCs on Ozone-induced Airway Hyper-responsiveness, Lung Inflammation, Apoptosis and Mitochondrial Dysfunction in Mice
5.1. Introduction

Ozone (O$_3$) is a potent oxidizing pollutant. High concentration of ozone in the environment have been reported to be associated with worsening of symptoms in patients with COPD and asthma (18, 74, 84, 173, 197, 239, 257). In mouse models, exposure to ozone is reported to induce airway oxidative stress, airway hyper-responsiveness (AHR) and inflammation (54, 253, 272, 274, 275). Repeated exposure to ozone can destroy alveolar structures in mice, finally leading to emphysema (203, 253).

Beneficial effects of iPSC-MSCs have been reported in rodent models of allergic airway inflammation and CS-exposed emphysema (150, 244). However, their effects on AHR have not been investigated yet. In this study, a previously reported mouse model of acute ozone-exposure was used (274, 275) in order to examine the effects of iPSC-MSCs on oxidative stress-induced AHR and airway inflammation.

Although in the last chapter the capacity of iPSC-MSCs to prevent oxidative stress-induced mitochondrial dysfunction in ASMCs was demonstrated in vitro, the capacity of iPSC-MSCs to modulate oxidative stress-induced mitochondrial dysfunction in the lung in vivo remains unknown. Mitochondrial dysfunction in lung tissue has been documented in the ozone-exposed mouse model (273). Therefore the other aim of the study was to examine the effects of iPSC-MSCs on oxidative stress-induced mitochondrial dysfunction in the ozone-exposed mouse model. The hypothesis of the study is that intravenous administration of iPSC-MSCs can prophylactically prevent and/or therapeutically reverse ozone-induced AHR, airway
inflammation, apoptosis and mitochondrial dysfunction in the lung.

The aims of this chapter were:

1. To evaluate the effects of iPSC-MSCs on AHR and lung inflammation in an acute ozone-exposed mouse model.

2. To examine effects of iPSC-MSCs on mitochondrial dysfunction and apoptosis in the lung, in an acute ozone-exposed mouse model.

5.2. Results

5.2.1. iPSC-MSCs prevented ozone-induced AHR

Male C57BL/6 mice were exposed to normal air or ozone (3 ppm) for 3 hours. 1x10^6 iPSC-MSCs or PBS were administrated to mice through intravenous injection either 24 hours prior or 6 hours after the exposure. There were 5 groups of mice in total: the Air/saline (n=5), the Air with iPSC-MSCs administrated 24 hours prior-exposure (Air/-24hr) (n=5), the Ozone/saline (n=6), the Ozone with iPSC-MSCs administrated 24 hours prior-exposure (Ozone/-24hr) (n=6) and the Ozone with iPSC-MSCs administrated 6 hour post-exposure (Ozone/+6hr) (n=6).

Pulmonary resistance (R_L) to increasing concentration of acetylcholine was measured 24 hours after exposure (Figure 5.1A). The injection of iPSC-MSCs 24 hours prior to air exposure did not show significant effect on AHR compared to the Air/saline group, as indicated by -log PC_{100} (2.01±0.06 vs. 2.15±0.06, p>0.05) Figure 5.1B). Ozone/saline group demonstrated significantly increased AHR compared with Air/saline group, as indicated by significantly reduced -log PC_{100} (1.68±0.06 vs.
The treatment of iPSC-MSCs 24 hours prior-exposure significantly reduced ozone-induced AHR as compared with Ozone/saline group (-log PC\textsubscript{100}: 1.99±0.08 vs.1.68±0.06, \( p < 0.05 \)). The Ozone/+6hr group, on the other hand, did not exhibit a difference in AHR compared to the Ozone group (-log PC\textsubscript{100}: 1.75±0.07 vs.1.68±0.06, \( p > 0.05 \)). The data suggest that intravenously injected iPSC-MSCs can prevent rather than reverse ozone-induced AHR in mice.

**Figure 5. 1 Effect of iPSC-MSCs on ozone-induced AHR.** Mice were exposed to ozone (3 ppm) or air for 3 hours. iPSC-MSCs were intravenously injected 24 hrs before or 6 hours post exposure. (A) \( R_L \) in response to increasing concentration of acetylcholine. (B) -log PC\textsubscript{100} derived from plot. **\( p < 0.01 \) compared to air/saline; †\( p < 0.05 \) compared to ozone/saline.
5.2.2. Effect of iPSC-MSCs on ozone-induced infiltration of inflammatory cells in the lung

The total cell number in bronchoalveolar lavage (BAL) was determined as indication of inflammatory cell infiltration in the lung (Figure 5.2A). The Ozone/saline group demonstrated significantly higher total cell numbers in the BAL compared to the Air/saline group (51.5±6.7x10^5 vs. 13.8±4.8x10^5, p<0.01). The Ozone/-24hr group also demonstrated higher total cell numbers in the BAL compared to Air/-24hr group (31.9±4.3x10^5 vs. 12.4±2.1x10^5, p<0.01). However, the total cell numbers in Ozone/-24hr group was significantly lower compared to Ozone/saline group (p<0.05). The Ozone/+6hr group did not show any significant difference in total cell numbers compared to the Ozone/Saline group.

Diff-Quik staining was performed on the BAL cells after cytospin. The numbers of neutrophils, macrophages, eosinophils and lymphocytes were determined. In the saline groups, ozone exposure significantly increased the neutrophil number in the BAL compared to air exposure (20.6±3.5x10^5 vs. 0.44±0.20x10^5, p<0.05). Treatment with iPSC-MSCs 24 hours before the ozone exposure significantly reduced the neutrophil number compared to the ozone/saline group (7.6±1.3x10^5 vs. 20.6±3.5x10^5, p<0.01), but the level was still higher than the air/-24hr group (7.6±1.3x10^5 vs. 0.26±0.07x10^5, p<0.05). The Ozone/+6hr group did not show any significant difference compared to Ozone/Saline group. Macrophage number was also elevated in Ozone/saline group compared to Air/saline (21.6±1.7x10^5 vs. 1.58±0.67x10^5, p<0.01). There was a trend of decrease in Ozone/-24hr compared to Ozone/saline but not
reaching significance (14.6±2.8x10^5 vs. 21.6±1.7x10^5). The Ozone/+6hr group did not show trend of difference compared to Ozone/Saline group (21.2±3.1x10^5 vs. 21.6±1.7x10^5). Similarly, eosinophil number in the BAL was increased in Ozone/saline group compared with Air/saline (2.58±0.63x10^5 vs. 0.16±0.09x10^5, p<0.01). There was a trend of decreased eosinophil number in Ozone/-24hr group compared with Ozone/saline though not reaching significance (0.98±0.27x10^5 vs. 2.58±0.63x10^5). Similar trend existed in Ozone/+6hr group as compared with Ozone/Saline group, also not reaching significance (1.31±0.52x10^5 vs. 2.58±0.63x10^5). As for number of lymphocytes, the induction by ozone is significant (5.76±1.21x10^5 vs. 1.06±0.41x10^5, p<0.05). There were trends of decrease in both Ozone/-24hr (4.44±0.86x10^5) and Ozone/+6hr groups (4.13±1.21x10^5), neither of which reached significance.

The results suggested that iPSC-MSCs were able to prevent but not reverse ozone-induced recruitment of inflammatory cells into mouse lung.
Figure 5. 2 Effects of iPSC-MSCs on total and differential cell counts in BAL from ozone-exposed mice. BAL was collected 24 hours after ozone exposure. (A) The number of total cells was determined. (B) Differential counts of cells in BAL. BAL cells were subjected to a cytopsin protocol and stained by Diff-Quik. Neutrophils, macrophages, eosinophils and lymphocytes were identified, based on their color and morphology, and counted. *p<0.05, **p<0.01 compared to air/saline; #p<0.05, ##p<0.01 compared to air/-24hr, †p<0.05, ††p<0.01 compared to ozone/saline.
5.2.3. Effect of iPSC-MSCs on cytokine contents of BALF

The BAL fluid (BALF) was collected as the non-cellular compartment of the BAL. Levels of the inflammatory mediators MCP-1, eotaxin, IL-6, IL-5 and MIP-1α in BALF were measured by MAGPIX Luminex multiplexing analyzer. Compared with the Air/saline group, ozone exposure significantly elevated the levels of MCP-1 (181.5±13.3 pg/ml vs. 151.9±1.6 pg/ml, p<0.05), eotaxin (48.6±2.2 pg/ml vs. 30.2±2.7 pg/ml, p<0.01), IL-6 (76.1±9.8 pg/ml vs. 37.4±2.7 pg/ml, p<0.01), IL-5 (11.9±2.6 pg/ml vs. 1.9±0.07 pg/ml, p<0.05) and MIP-1α (9.2±1.6 pg/ml vs. 2.2±0.1 pg/ml, p<0.05). Prophylactic administration of iPSC-MSCs significantly reduced IL-6 (45.1±3.2 pg/ml, p<0.05) and IL-5 (4.66±0.60 pg/ml, p<0.01) levels compared with the Ozone/saline group. Trends of reduction in eotaxin (40.8±2.3 pg/ml, p=0.053) and MIP-1α (5.4±0.8 pg/ml, p=0.055) in the BALF were also demonstrated in the Ozone/-24hr group compared with Ozone/saline group. Therapeutic treatment with iPSC-MSCs reversed the ozone-induced increase in eotaxin (36.8±2.5 pg/ml, p<0.01), IL-6 (47.8±3.6 pg/ml, p<0.05), IL-5 (4.6±0.9 pg/ml, p<0.05) and MIP-1α (4.8±0.6 pg/ml, p<0.05) in the BALF. Both prophylactic and therapeutic treatments induced a trend of reduction in MCP-1 levels which did not reach statistical significance (Ozone/-24hr, 160.0±1.0 pg/ml; Ozone/+6hr, 157.9±2.8 pg/ml).

The results indicated that iPSC-MCSs prevented and reversed ozone-induced elevation in inflammatory mediator levels in BALF, including eotaxin, IL-6, IL-5 and MIP-1α.
Figure 5. 3 Effects of iPSC-MSCs on inflammatory mediator levels in BALF after ozone exposure. Levels of MCP-1, eotaxin, IL-6, IL-5 and MIP-1α in BALF were measured using a MAGPIX Luminex multiplexing analyser. *p<0.05, **p<0.01 compares to air/saline; #p<0.05, ##p<0.01 compared to air/-24hr, †p<0.05, ††p<0.01 compares to ozone/saline.
5.2.4. Effects of iPSC-MSCs on apoptosis and proliferation in ozone-exposed lung tissue

Apoptosis in lung sections was detected using TUNEL staining (Figure 5.4A). Apoptotic cells were labeled green and the nuclei were labeled blue. The Ozone/saline group demonstrated significantly increased number of apoptotic cells in lung sections in comparison with the air/saline group (number/20x field: 21.7±2.9 vs. 3.7±0.4, \(p<0.05\)) (Figure 5.4B). Both Ozone/-24hr and Ozone/+6hr groups showed significantly reduced number of apoptotic cells compared with Ozone/saline group (number/20x field: 9.0±1.2, \(p<0.01\) and 9.3±1.3, \(p<0.01\) respectively) (Figure 5.4B).

Proliferative cells in lung sections were identified by immunofluorescent staining against Ki-67, a marker of proliferation (Figure 5.5A). Ozone exposure significantly reduced the number of proliferating cells in lung sections (number/20x field: 54.6±5.5 vs. 77.2±5.9, \(p<0.05\)) (Figure 5.5B). The Ozone/-24hr group did not show a significant difference in proliferating cell number compared to Air/-24hr group (number/20x field: 66.4±7.9, vs.71.5±6.4), indicating that negative effect of ozone on proliferation might be prevented by treatment with iPSC-MSCs (Figure 5.4B). However, the Ozone/-24hr group only showed a trend of increased proliferating cell number compared to Ozone/saline group which did not reach statistical significance. The Ozone/+6hr group also did not show a significant difference in the number of proliferating cells compared with the Ozone/saline group.
Figure 5. 4 Effects of iPSC-MSCs on ozone-induced apoptosis in mouse lungs. (A) Apoptotic cells were identified by TUNEL staining (green) indicated by the arrows. The nuclei were visualized by DAPI staining (blue). (B) Number of apoptotic cells in lung sections. Apoptotic cells were counted in five randomly selected 20x fields for each mouse using a fluorescent microscope. *p<0.05 compared to air/saline; ††p<0.01 compares to ozone/saline.
Figure 5. Effects of ozone and iPSC-MSCs on cellular proliferation in mouse lungs. (A) Detection of proliferating cells by Ki-67 staining (red). The nuclei were visualized by DAPI staining (blue). (B) Number of proliferative cells in lung sections. Proliferative cells were counted in five randomly selected 20x fields for each mouse using a fluorescent microscope. *p<0.05 compared to air/saline.
5.2.5. Effect of iPSC-MSCs on ozone-induced cellular ROS and mitochondrial dysfunction in mouse lungs

Intact mitochondria and cytoplasmic fractions were extracted from lung tissue. The ROS levels in the cytoplasm fractions were measured by DCF assay (Figure 5.6A). Ozone exposure significantly elevated the cytoplasmic ROS levels (RFU/mg protein: 3131±269.8 vs. 2061±123.9, p<0.01). When treated with iPSC-MSCs 24 hours prior to ozone exposure, the cytoplasmic ROS levels were still significantly higher compared with the Air/-24hr group (RFU/mg protein: 2787±171.9 vs. 2030±178.9, p<0.05). The Ozone/+6hr group also showed significantly higher level of cytoplasmic ROS compared with Air/saline group (RFU/mg protein: 2909±194.4 vs. 2061±123.9, p<0.05). No significant reduction was observed in both Ozone/-24hr and Ozone/+6 groups in comparison with the Ozone/saline group.

The mitochondrial ROS levels in the intact mitochondria were measured by MitoSOX staining (Figure 5.6B). Mitochondrial ROS level was significantly elevated in the Ozone/saline group compared with the Air/Saline group (RFU/mg protein: 166.4±9.5 vs. 119.9±8.8, p<0.05). Both Ozone/-24hr and Ozone/+6hr groups demonstrated significantly reduced mitochondrial ROS levels compared to Ozone/saline group (RFU/mg protein: 122.8±10.0, p<0.05 and 62.9±10.3, p<0.01 respectively). In addition, the mitochondrial ROS level in Ozone/+6hr group is significantly lower than the Ozone/-24hr group (p<0.01).

Mitochondrial membrane potential (ΔΨm) of the intact mitochondria was accessed by JC-1 staining (Figure 5.6C). Exposure to ozone without treatment of
iPSC-MSCs reduced the ΔΨm in comparison to the Air/saline group (fold to Air/saline: 0.692±0.057 vs. 1.00±0.041, \( p<0.01 \)). The treatment of iPSC-MSCs 24 hours prior to the ozone-exposure significantly improved the ΔΨm compared with the Ozone/saline group (fold to Air/saline: 1.004±0.076 vs. 0.692±0.057, \( p<0.01 \)). However, the Ozone/+6hr group didn’t show significant difference as compared with Ozone/saline group, and the ΔΨm was still significantly lower than the Air/saline group (fold to Air/saline: 0.778±0.060 vs. 1.00±0.041, \( p<0.01 \)).
Figure 5. 6 Effects of iPSC-MSCs on ozone-induced ROS and mitochondrial dysfunction in mouse lungs. Intact mitochondria and cytoplasmic fractions were isolated from mouse lungs. (A) Cytoplasmic ROS was measured by DCF staining. (B) Mitochondrial ROS was measured in intact mitochondria by MitoSOX staining. (C) ΔΨm was determined in intact mitochondria by JC-1 staining. *p<0.05, **p<0.01 compared to air/saline; #p<0.05 compared to air/-24hr, †p<0.05, ††p<0.01 compared to ozone/saline; §§p<0.01 compared to ozone/-24hr.
5.3. Discussion

This study demonstrated that intravenously administrated iPSC-MSCs were able to prevent ozone-induced AHR, airway inflammation, apoptosis, mitochondrial ROS and reduction in ΔΨm. The therapeutic administration of iPSC-MSCs failed to reverse ozone-induced AHR, airway inflammation and reduction in ΔΨm, but the ozone-induced apoptosis and mitochondrial ROS were reversed. Although the data also showed lack of effects on ozone-induced cytoplasmic ROS and reduction in proliferation, the findings suggested a protective role of iPSC-MSCs on ozone-induced lung damage.

Ozone is a potent oxidant. High concentration of environmental ozone was reported to be associated with worsening of symptoms in patients with COPD and asthma (18, 74, 84, 173, 197, 239, 257). In mouse models, exposure to ozone is reported to induce airway oxidative stress, AHR and inflammation (54, 253, 272, 274, 275). Oxidative stress-associated airway inflammation is believed to be a key component of the pathogenesis of COPD (65). Indeed, repeated exposure to ozone can lead to airway inflammation accompanied by destruction of alveolar structure, leading to an emphysema-like phenotype (203, 253). In the present study mice were exposed to ozone (3 ppm) for a single period of 3 hours. The recruitment of inflammatory cells to the lungs was robust after 24 hours, as indicated by increased cell counts in the BAL. Increased levels of inflammatory mediators including MCP-1, eotaxin, IL-6, IL-5 and MIP-1α were also observed in the BALF. These findings confirmed the induction of inflammation by ozone in the model. The prophylactic injection of
iPSC-MSCs prevented ozone-induced inflammation, as indicated by the reduction of both total cell counts in the BAL and levels of inflammatory mediators including eotaxin, IL-6, IL-5 and MIP-1α, in the BALF. On the other hand, administration of iPSC-MSCs after ozone exposure did not reverse the recruitment of inflammatory cells. However, it did reverse the induction of several inflammatory mediators in the lungs, including eotaxin, IL-6, IL-5 and MIP-1α, which suggested that iPSC-MSCs still had an anti-inflammatory effect. Failure to reverse inflammatory cell infiltration 6 hours after exposure may be due to a stronger inflammatory response or due to the shorter length of treatment time in the therapeutic protocol (18 hours) compared to the prophylactic protocol (48 hours). These findings are in agreement with my results showing lack of therapeutic effects of iPSC-MSCs on AHR. Although, the anti-inflammatory role of iPSC-MSCs has been demonstrated by previous studies in allergic (244) and CS-induced airway inflammation models (as shown in previous chapters), the ability of iPSC-MSCs to ameliorate AHR has never been reported. AHR is a major clinical feature of asthma, however it is also observed in some patients with COPD (291). A previous study in an acute ozone-exposed mouse model demonstrated that ozone-induced AHR results from the activation of p38 mitogen-activated protein kinase (147). The exact mechanism of the action of iPSC-MSCs in this model requires further investigation.

Excessive protease activity associated with oxidative stress and chronic inflammation is believed to lead to alveolar destruction in patients with COPD (68, 76, 77, 189). However, accumulating evidence suggest that the imbalance between
apoptosis and proliferation may also contribute to the gradual alveolar destruction (67). In this study increased lung apoptosis and reduced proliferation was observed after ozone exposure, which is consistent with the imbalance between apoptosis and proliferation in the chronic CS-exposed model described in Chapter 3. Treatment with iPSC-MSCs, either before or after ozone exposure, reduced ozone-induced apoptosis, but the ozone-induced reduction in proliferation was not alleviated. These findings indicate a possibly higher anti-apoptotic ability of iPSC-MSCs than pro-proliferative ability in a context of oxidative stress.

Mitochondrial dysfunction has been characterized in the ozone-exposed mouse model in a previous report (273). Ozone exposure can lead to reduced ATP levels, mitochondrial complex proteins expression and ΔΨm as well as increased mitochondrial ROS. The mitochondria-targeted antioxidant, MitoQ, was able to attenuate mitochondrial dysfunction in the model, as well as the ozone-induced AHR and inflammation, indicating the AHR and inflammation may be partly driven by mitochondrial dysfunction (273). Moreover, mitochondrial dysfunction can trigger the cellular apoptosis (90). Therefore, the effects of iPSC-MSCs on AHR, inflammation and apoptosis may all relate to its effects on ozone-induced mitochondrial dysfunction in lung. Indeed, the prophylactic administration of iPSC-MSCs reduced ozone-induced mitochondrial ROS and reduction in ΔΨm, in line with the effects on ozone-induced inflammation, AHR and apoptosis. The administration after ozone exposure only reversed the ozone-induced mitochondrial-ROS level, but not the reduction of ΔΨm. The findings are in agreement with the results of our co-culture
experiments described in the Chapter 4. There I show that iPSC-MSCs can prevent and reverse CSM-induced mitochondrial ROS, whereas they can prevent but not reverse CSM-induced ΔΨm loss.

Despite the reduction in ozone-induced mitochondrial ROS by iPSC-MSCs, ozone-induced cytoplasmic ROS levels were not modulated by iPSC-MSCs. Such result may indicate a lack of anti-oxidant effects by iPSC-MSCs in the model. Further investigation on oxidative stress and anti-oxidant mechanisms in the lung should better address this issue. On the other hand, the data also suggested that mitochondrial and cytoplasmic ROS are differentially regulated in cells. Although the mechanisms by which iPSC-MSCs modulate mitochondrial ROS are unclear, the prevention of ΔΨm loss and the lack of effect on cytoplasmic ROS suggest that this possibly occurs through mechanisms that specifically target the mitochondria, such as mitochondrial transfer.

This study has several limitations and further investigations are required. Firstly, although the acute exposure of ozone can induce AHR and inflammation, a model involving multiple ozone exposures would be more relevant to COPD (253). Investigation of effects of iPSC-MSCs on ozone-induced emphysema, AHR, inflammation and mitochondrial dysfunction in such model would be an important experiment to perform. Secondly, the mechanisms by which iPSC-MSCs prevent ozone-induced mitochondrial dysfunction and the associated inflammation, apoptosis and AHR remain unclear. Lastly, the effects of iPSC-MSCs on the oxidant and anti-oxidant balance in the lungs can be further investigated.
In summary, iPSC-MSCs prevented ozone-induced mitochondrial dysfunction as well as lung inflammation and apoptosis, and AHR. In the therapeutic protocol, iPSC-MSCs showed no effect on AHR and airway inflammation but reversed apoptosis and mitochondrial dysfunction in response to ozone. I demonstrate for the first time the capacity of iPSC-MSCs to prevent mitochondrial dysfunction and AHR in the lungs, highlighting iPSC-MSCs as promising candidates for the development of cell-based therapies for COPD.
Chapter 6. General Discussion
6.1. General Discussion

In this study, I demonstrated a superior efficacy of iPSC-MSCs to attenuate chronic CS-exposure-induced lung emphysema, inflammation and apoptosis in rats compared to BM-MSCs, which was associated with a higher capacity for mitochondrial transfer from iPSC-MSCs to lung epithelium. *In vitro* studies also demonstrated a higher ability of iPSC-MSCs to transfer mitochondria to and attenuate CSM-induced ATP depletion in CSM-treated bronchial epithelial cells. In addition, iPSC-MSCs-CdM demonstrated better efficacy in attenuating CSM-induced apoptosis and reducing proliferation in bronchial epithelial cells compared to BM-MSCs, which was partly mediated by SCF. Therefore, iPSC-MSCs demonstrated higher capacity of both mitochondrial transfer and paracrine release of anti-apoptotic cytokines to bronchial epithelial cells. I then investigated whether such activities were able to restore mitochondrial health under oxidative stress. This study was carried out *in vitro* first in a CSM-treated model of ASMCs. Direct co-culture with iPSC-MSCs protected ASMCs from CSM-induced reduction in ΔΨm and elevation of mitochondrial ROS and apoptosis. However, when direct cell-cell contact was prevented, iPSC-MSCs only reduced CSM-induced mitochondrial ROS but not reduction of ΔΨm and increase of apoptosis in ASMCs. Mitochondrial transfer from iPSC-MSCs to ASMCs was observed in direct co-culture which was enhanced by CSM treatment. In line with the *in vitro* findings, in a mouse model of acute ozone exposure, intravenous administration of iPSC-MSCs protected lung tissue from ozone-induced mitochondrial dysfunction and apoptosis. Ozone-induced AHR and inflammation was
also reduced by iPSC-MSCs. In summary, I demonstrated that mitochondrial transfer activity from iPSC-MSCs was able to alleviate oxidative-stress-induced ATP depletion, mitochondrial dysfunction and apoptosis in target cells. The full capacity of iPSC-MSCs to achieve these effects may rely on combined mechanisms of both direct cell-cell contact and release of paracrine factors (Figure 6.1). The capacity of iPSC-MSCs to transfer their mitochondria to and protect airway cells from oxidative stress-induced damage is superior to that of BM-MSCs.

The study was initially motivated by three observations regarding current status of respiratory medicine and stem cells. First of all, current medications for COPD can only reduce symptoms, exacerbations, and improve health status and exercise tolerance, and no current medication is able to attenuate the progressive decline of lung function in COPD (263). As a disease predicted to be the 4th leading cause of death by 2030 (172) associated with enormous economic and social burden, seeking new treatments for COPD is an urgent and important task in respiratory medicine. Secondly, there have been numerous reports on the efficacy of MSCs in animal models of lung disease (269, 270). Despite a lack of knowledge of the underlining mechanisms, MSCs from BM, adipose or cord blood have been demonstrated to attenuate emphysema induced by CS, elastase or VEGF deficiency (94, 112, 151, 222, 269, 270). However, in a clinical trial, intravenously infused BM-MSCs failed to modulate quality of life and lung function in patients with COPD (271). Although there are multiple variables that may contribute to the lack of efficacy such as dose and timing, the results still point out the potential gap between the pre-clinical models
and patients, and the possibility that current findings of therapeutic effects of MSCs in COPD-related models may not be necessarily translated into treatments for patients with COPD. Therefore, despite current studies already having demonstrated adequate efficacy of MSCs in pre-clinical models, the search for improved MSC therapies with more profound effects in pre-clinical models should not stop. After all, the clinical trial demonstrated extraordinary tolerance and safety of intravenously infused BM-MSCs in patients with severe COPD, leaving the door open for future trials. Thirdly, the *in vitro* derivation of iPSC-MSCs from iPSCs has provided a new opportunity to develop improved MSC therapy against COPD. Compared to BM-MSCs, iPSC-MSCs are more readily available in abundance and possess a higher ability of proliferation without loss of differentiation potential and telomerase activity (153). They may hold the hope of overcoming ageing-associated dysfunction of BM-MSCs by the induction of pluripotency during their generation. As age is an important risk factor for COPD, iPSC-MSCs may be a good candidate for getting one own source of MSCs. Moreover, the process of inducing somatic cells into iPSCs and differentiating iPSCs into iPSC-MSCs provided an opportunity to genetically manipulate the cells at different stages. Using MSCs as a vehicle for gene therapy may also be a future option to develop improved therapy against COPD (122). In summary, iPSC-MSCs may represent a novel and improved candidate for cell therapy of COPD. From the data presented in Chapter 3, my study demonstrated better efficacy of iPSC-MSCs than BM-MSCs in attenuating CS-induced lung emphysema, inflammation and apoptosis, possibly via higher activity of mitochondrial transfer and
paracrine secretion of cytokines such as SCF. My study has demonstrated the potential of iPSC-MSCs as an improved MSC therapy, hence providing a new option that may lead to a better treatment for COPD.

One striking observation from the study of CS-exposed rats is the mitochondrial transfer from iPSC-MSCs to epithelial cells, which led to focusing my attention to the other theme of the thesis, namely the oxidative stress-induced mitochondrial dysfunction in pulmonary cells. Developing new treatments for COPD relies on the understanding of the pathogenesis of COPD. One of the recent advances is the demonstration of mitochondrial dysfunction associated with COPD. Primary bronchial epithelial cells from ex-smokers with severe COPD have been shown to exhibit swelling and fragmented mitochondrial morphology which is similar to CSM-treated BEAS-2B cells (105). Primary ASMCs from patients with COPD were shown to have elevated mitochondrial ROS levels, and reduced ΔΨm, complex protein levels and respiratory function (273). Moreover, the mitochondria-targeted anti-oxidant MitoQ was able to reduce chronic ozone exposure-induced mitochondrial dysfunction, inflammation and AHR in mice, highlighting mitochondria as a potential target for COPD treatment (273). Meanwhile, despite a large number of reports on the efficacy of MSCs in lung disease models, their mechanisms of action remain largely unknown. One of the latest advances is the in vivo observation of mitochondrial transfer from BM-MSCs to pulmonary epithelial cells in an acute lung injury model (118). In other words, mitochondria are a novel target for COPD treatment as well as a novel player in the action of MSCs. Therefore, the second half of my study aimed to
investigate whether mitochondria for lung cells are damaged by oxidative stress, whether this damage can be prevented or reversed by iPSC-MSCs, and whether iPSC-MSCs exert such protective actions through mitochondrial transfer. The results demonstrated that CSM led to reduced ATP contents in BEAS-2B cells, and increased mitochondrial ROS, apoptosis and reduced ΔΨm in primary ASMCs in vitro. Acute ozone exposure also led to increased mitochondrial ROS and reduced ΔΨm in mouse lungs in vivo. This oxidative stress-induced mitochondrial dysfunction was alleviated by iPSC-MSCs either through direct co-culture in vitro, or through intravenous administration in vivo. Mitochondrial transfer was demonstrated from iPSC-MSCs to airway epithelium in the CS-exposed model in vivo, and from iPSC-MSCs to BEAS-2B cells and ASMCs in vitro. CSM enhanced mitochondrial transfer from iPSC-MSCs to both BEAS-2B cells and ASMCs. The enhanced mitochondria transfer may be driven by the need for repair, and may be responsible for the alleviation of CSM-induced mitochondrial dysfunction. Therefore this study demonstrated that oxidative stress can induce mitochondrial dysfunction of pulmonary cells in vitro and in vivo, and iPSC-MSCs are able to transfer mitochondria to these damaged cells in response to the oxidative stress-induced damage and alleviate the mitochondrial dysfunction.

Improved mitochondrial function may bring other potential benefits to the cell. Mitochondria serve as the powerhouse of the cells. It has been reported that CS exposure led to altered lung energy metabolism, with a switch of glucose metabolism toward pentose phosphate pathway and a reduced supply of substrate to mitochondria.
The transfer of mitochondria into the lung cells may help maintain the energy supply (4). In addition, as described in Chapter 1.1.4.7, mitochondria play an important role in regulating ROS generation and cell fate. Oxidant/anti-oxidant imbalance plays an important role in the pathogenesis of COPD, with the process of oxidative phosphorylation in mitochondria being a major source of intracellular ROS (149, 165). Severe mitochondrial damage may lead to depolarization of mitochondria and release of cytochrome c, and to mitochondrial pathways of cell apoptosis (90). Mitochondrial damage can also mediate autophagy and senescence, processes that could contribute to the development of COPD (11).

In general, mitochondrial dysfunction can be targeted using three different strategies: repair via scavenging excess ROS, reprogramming via regulatory pathways, and replacement by exogenous functional mitochondria (5). Mitochondria-targeted anti-oxidant such as MitoQ is a direct way to reduce mitochondrial ROS, which may subsequently improve other mitochondrial functions such as ΔΨm and respiration. This has been demonstrated in the chronic ozone-exposed mouse model (273). Nutrient overload and metabolic changes associated with obesity are common causes of mitochondrial stress (5) and improvement of mitochondrial function by exercise, diet and possibly pharmacotherapy such as metformin has been reported (5). Clinical trials on COPD have demonstrated the potential of metformin to reduce dyspnea and increase exercise capacity, but more comprehensive studies are awaited to confirm its efficacy (225). Both repair and reprogramming relies on the recovery of the current defective mitochondria, which may not be easy for cells of severely-damaged
mitochondria. Also, if mitochondrial DNA has been damaged, such strategies may not work. In such a situation, replacement of damaged mitochondria with fresh mitochondria may be a better strategy. Mitochondrial replacement can be achieved by microinjection, incubation with mitocytoplasts or intact purified mitochondria, and the cell-cell contact-dependent mitochondrial transfer from MSCs (225). The latter method used in my thesis, namely mitochondrial transfer, is the only way that does not involve any disturbance of the cells or involve isolating mitochondria and injecting the cells but involves a natural method of transferring organelles such as mitochondria between cells. It provides a perfect approach to deliver exogenous mitochondria into damaged cells. My study confirmed that mitochondrial transfer can occur in vivo following its first report in 2012 (118). Moreover, as iPSC-MSCs have a higher expanding ability than BM-MSCs, the approach we used therefore provided a more abundant source of mitochondrial donor.

Although mitochondrial transfer was demonstrated both in vivo and in vitro in this study, it should be noted that the recipient cells were human bronchial epithelial or ASMCs in the in vitro experiments, while in the in vivo study mitochondria were transferred from human MSCs into rodent lung cells. The functional compatibility between the human mitochondria and rodent lung cells was not tested in the current study. Previous reports have demonstrated the transfer of human mitochondria from human BM-MSCs to rat cardiomyocytes in vitro (204) and to AEC2s in mouse lungs (118), but the functional compatibility was not discussed. In a further study, despite reporting efficient mitochondrial fusion between mouse and human mitochondria
within 4 hours (288), the study demonstrated low compatibility between mouse nuclear and human mitochondrial genomes. Therefore the functional significance of the mitochondrial transfer observed in vivo in this thesis is largely unknown.

Two animal models have been used in this thesis to study the rescuing effect of MSCs. The first one is a rat model exposed to CS for 1 hour/day for 56 days, while the second one is a mouse model with a single ozone exposure for 3 hours. The two models are related in some aspects but also different. Both CS and ozone are potent sources of oxidants. Therefore, both models can be referred to as oxidative stress-induced lung damage, which makes them distinct from some other COPD-related models, such as elastase-induced emphysema in which oxidative stress is not the direct driving force. Cigarette smoking is the major risk factor of COPD, and chronic CS-exposure is a classic way to induce COPD-like phenotype such as airspace enlargement and lung inflammation. Although ozone is not a major cause of COPD, elevated ozone levels have been reported to increase hospital admission due to COPD (173). Exposure to ozone twice per week for 6 weeks can induce COPD-like phenotype in mice including emphysema, chronic inflammation, cell infiltration and steroid resistance (253, 272). Moreover, the ozone-exposed mouse model has been reported to exhibit a gene expression signature similar to that seen in human COPD (273). The major difference between the two models in the present study is that the CS-exposed model is a chronic model with multiple exposures, while the ozone-exposed model is an acute model with a single exposure period. The acute exposure of ozone cannot induce airspace enlargement, but infiltration of neutrophils
and other inflammatory cells was demonstrated and, as such, could be taken as a model mimicking an exacerbation of COPD which is a frequent manifestation of advanced COPD. In general, acute exposure indicates that the animals were responding to the stimulation based on a normal physiological condition, whereas in chronic models, along the progression of the damage, the animals respond to the stimulation based on a damaged, abnormal condition. While the efficacy of iPSC-MSCs in the chronic CS-exposed model specifically defines iPSC-MSCs as a promising candidate for MSC therapy of COPD, the efficacy of iPSC-MSCs in the acute ozone-induced model may provide a more general implication on an acute oxidative stress insult such as during an exacerbation. Although the acute ozone-exposed model did not exhibit airspace enlargement, this model has been used to investigate some parameters that have not been measured in the CS-exposed model, including AHR, cytosolic ROS, mitochondrial ROS and ΔΨm. Nevertheless, both models in the study have demonstrated inflammation and apoptosis in the lung. Using the two different models, I demonstrated the capacity of iPSC-MSCs to alleviate oxidative stress-induced lung damage in both acute and chronic setups.

The *in vitro* study involved two different airway cell types. Although both cell types were treated with CSM, it should be noted that the treatments were optimized based on different parameters. In the study on bronchial epithelial BEAS-2B cells, the concentration (2%) and length of treatment (24 hours) was adapted from a previous study, with an induction of IL-8 release without reduction of viability. As demonstrated in Chapter 3, such condition was sufficient to induce apoptosis and
reduce ATP content. In the study of primary ASMCs, the concentration of CS (10% or 25%) and length of treatment (4 hours) was determined by the induction of mitochondrial dysfunction as measured by mitochondrial ROS and ΔΨm. The CSM stimulation was more acute and strong in the ASMCs study, leading to a more acute induction of apoptosis. Such difference may explain why the released cytokines from iPSC-MSCs (iPSC-MSCs-CdM) were able to attenuate CSM-induced apoptosis in BEAS-2B cells but not in ASMCs in the study. On the other hand, enhanced mitochondrial transfer has been detected from iPSC-MSCs to both BEAS-2B cells and ASMCs under the corresponding CSM treatments. Mitochondrial transfer and paracrine effects may serve as two different mechanisms of iPSC-MSCs’ action. By using apoptosis as a functional output, it is clear that the relative contribution of mitochondrial transfer and paracrine effects varies among different target cell types and treatments. In the 24 hour-treated BEAS-2B cells, paracrine effects from iPSC-MSCs were sufficient to reduce apoptosis. By contrast, in the 4 hour-treated ASMCs, the anti-apoptotic efficacy fully relied on cell-cell contact that facilitated the mitochondrial transfer which is a more direct mechanism to rescue mitochondrial dysfunction. Such differences demonstrated the complexity and the dynamics of mechanisms of action of iPSC-MSCs.

The in vivo and in vitro data from different chapters complement each other very well in this study. The mitochondrial transfer from iPSC-MSCs to damaged cells in vivo in the CS-exposed model was also observed in vitro for both BEAS-2B cells and ASMCs. The anti-apoptotic effect of iPSC-MSCs in both CS- and ozone-exposed
animal models was also demonstrated in both BEAS-2B cells and ASMCs. The attenuation of mitochondrial dysfunction by iPSC-MSCs was demonstrated *in vivo* in ozone-exposed mice as well as *in vitro* in ASMCs. Moreover, both studies demonstrated time-dependent effects. In the CSM-treated ASMCs, iPSC-MSCs prevented but not reversed the CSM-induced mitochondrial dysfunction. Similarly, in the ozone-exposed mice, iPSC-MSCs prevented but not reversed the ozone-induced inflammation, AHR and reduction of ΔΨm in the lung. This may be explained by the fact that ASMCs or mice were treated with iPSC-MSCs for more time in the preventive protocols, or that the oxidative stress-induced damage has progressed to a stage that cannot be reversed by iPSC-MSCs. Interestingly, in the ozone-exposed mice, although the preventive treatment did not attenuate ozone-induced reduction of ΔΨm, it attenuated ozone-induced mitochondrial ROS in the lung. This is very similar to the effects of iPSC-MSCs-CdM or iPSC-MSCs in trans-well inserts *in vitro*, which also demonstrated reduction of CSM-induced mitochondrial ROS but no change in ΔΨm in ASMCs. In theory, excess mitochondrial ROS can lead to defective electron transport chain which is responsible for proton transfer across the inner mitochondrial membrane and defective permeability transition pore which controls the permeability of inner membrane (170). Both effects lead to ΔΨm reduction. The reduction of mitochondrial ROS without effects on ΔΨm in the study may be because the reduced mitochondrial ROS levels were still high enough to induce a reduction in ΔΨm, or because there are other mechanisms regulating ΔΨm which were less targeted by iPSC-MSCs, such as cytosolic ROS levels.
The most direct clinical implication of the study is that intravenously-infused iPSC-MSCs may exert a stronger effect in patients with COPD compared to BM-MSCs which have not been shown to be effective in the clinical trials. However, it is of concern that the genetic reprogramming may also change the safety profile of iPSC-MSCs compared to BM-MSCs. In a more general perspective, iPSC-MSCs may be effective in other oxidative stress-mediated airway diseases such as asthma. In addition, the higher mitochondria transfer capacity and abundant resource also suggests that iPSC-MSCs may serve as a useful mitochondrial donor to target mitochondrial dysfunction in various diseases.

There are several limitations of the work in my thesis. Firstly, due to time limitations, not all parameters were investigated in all models. As mitochondrial dysfunction was not the initial focus of the study, mitochondrial ROS and $\Delta\Psi_m$ were not measured in the CS-exposed rat model and CSM-treated BEAS-2B cells. Secondly, although inflammation was investigated in the in vivo models, there was no direct measurement of inflammation in the in vitro models. A common inflammatory measurement used in cell culture studies is measurement of released inflammatory mediators. However, as both cell types in the co-culture are from human, the secretory function of one particular cell type is difficult to ascertain. Thirdly, there are other potential mechanisms involved in iPSC-MSCs action which may also contribute to the efficacy observed in the study. For example, formation of TNTs may also facilitate transfer of other organelles such as lysosomes. iPSC-MSCs may also secrete protective microvesicles to lung cells. Lastly, mitochondrial ROS and $\Delta\Psi_m$ may not
represent all the functions of mitochondria. A measurement of mitochondrial respiration profile would have been ideal to address the issue.

6.2. Conclusion

I demonstrated the superior effects of iPSC-MSCs in attenuating CS-induced emphysema, inflammation and apoptosis in rat lung compared to BM-MSCs, which was attributed to higher mitochondrial transfer and paracrine release of cytokines including SCF. iPSC-MSCs can also alleviate oxidative stress-induced AHR, inflammation, mitochondrial dysfunction and apoptosis in mouse lungs. Mitochondrial transfer from iPSC-MSCs was able to alleviate oxidative-stress-induced ATP depletion, mitochondrial dysfunction and apoptosis in target cells. The full capacity of iPSC-MSCs to achieve this efficacy may rely on combined mechanisms of both direct cell-cell contact and release of paracrine factors.

These findings support iPSC-MSCs as a promising candidate for the development of MSCs-based therapy against COPD, which may be driven by multiple mechanisms.
Figure 6.1 Summary of the effects of iPSC-MSCs on oxidative stress-induced lung cell damage. The full capacity of iPSC-MSCs to alleviate oxidative stress-induced mitochondrial dysfunction and apoptosis may rely on combined mechanisms of both direct cell-cell contact and release of paracrine factors. SCF: stem cell factor; Mito-ROS: mitochondrial ROS; iPSC-MSCs: induced pluripotent stem cell-derived mesenchymal stem cells; ASMCs: airway smooth muscle cells; CS: cigarette smoke; AHR: airway hyper-responsiveness.
6.3. Future Studies

Several future investigations can be extended from this study.

In this study, the effects of iPSC-MSCs on mitochondrial dysfunction in animal models with COPD-like phenotypes have not been demonstrated. It would be important to further investigate effects of iPSC-MSCs on mitochondria of the lung in a chronic CS-/ozone-exposed models focusing particularly on the development of emphysema.

In the study I used cytochalasin B or trans-well membrane to block the formation of TNTs between iPSC-MSCs and target cells. However, such methods are not selective blockers of mitochondrial transfer. A recent study has demonstrated that mitochondrial transfer is mediated by Miro-1 (6). Knock-down or over-expression of Miro-1 in BM-MSCs leads to reduced or enhanced mitochondrial transfer activity respectively. This would provide a more selective approach to block mitochondrial transfer without interruption of TNT formation. Regarding my study, the following investigations could be proposed. First, whether Miro-1 is also responsible for the mitochondrial transfer in iPSC-MSCs can be examined. Determination of efficacy of iPSC-MSCs and mitochondrial transfer after Miro-1 knock-down and over-expression would address the question. Second, how does CSM enhance mitochondrial transfer? One hypothesis is that CSM treatment can directly regulate capacity of mitochondrial transfer of iPSC-MSCs. This can be investigated by measurement of Miro-1 expression in response to CSM and identification of the possible pathways involved, such as mitogen-activated protein kinase pathways, using specific inhibitors of these
pathways. On the other hand, the enhanced mitochondrial transfer may be a response to signals from injured target cells. How the recipient cells regulate the mitochondrial transfer will also require further investigation.

Another important follow-up study is to determine whether iPSC-MSCs can improve mitochondrial function of primary ASMCs from patients with COPD. ASMCs from patients with COPD already demonstrated mitochondrial dysfunction compared to normal controls. How this would change the mitochondrial transfer between the ASMCs and iPSC-MSCs should also be investigated.


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