Extracellular vesicle-mediated cellular crosstalk in lung repair, remodelling and regeneration

Tsukasa Kadota[1,2], Yu Fujita[1,2], Jun Araya[1], Takahiro Ochiya[3] and Kazuyoshi Kuwano[1]

[1]Division of Respiratory Diseases, Dept of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan. [2]Dept of Translational Research for Exosomes, The Jikei University School of Medicine, Tokyo, Japan. [3]Institute of Medical Science, Tokyo Medical University, Tokyo, Japan.

Corresponding author: Yu Fujita (yuugot@jikei.ac.jp)

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Extracellular vesicles are important intracellular mediators in lung repair, remodelling and regeneration, and are potential therapeutic agents for lung regenerative medicine
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Abstract

The unperturbed lung is highly quiescent, with a remarkably low level of cell turnover. However, once damaged, the lung shows an extensive regenerative capacity, with resident progenitor cell populations re-entering the cell cycle and differentiating to promote repair. This quick and dramatic repair response requires interactions among more than 40 different cell lineages in the lung, and defects in any of these processes can lead to various lung pathologies. Understanding the mechanisms of interaction in lung injury, repair and regeneration thus has considerable practical and therapeutic implications. Moreover, therapeutic strategies for replacing lung progenitor cells and their progeny through cell therapy have gained increasing attention. In the last decade, extracellular vesicles (EVs), including exosomes, have been recognised as paracrine mediators through the transfer of biological cargo. Recent work has revealed that EVs are involved in lung homeostasis and diseases. In addition, EVs derived from specific cells or tissues have proven to be a promising cell-free modality for the treatment of lung diseases. This review highlights the EV-mediated cellular crosstalk that regulates lung homeostasis and discusses the potential of EV therapeutics for lung regenerative medicine.

Introduction

The lung is a structurally complex organ with a large and highly vascularised epithelial surface area, comprising more than 40 different cell lineages from the trachea to the alveolar spaces [1, 2]. Two major compartments are present in the lungs: the conducting airways, including the trachea, bronchi and bronchioles; and the respiratory airways, including the alveoli [3]. The conducting airways represent the first line of defence and first domain of contact between the external environment and the respiratory system, while the alveoli represent the site of gas exchange between inhaled air and the pulmonary circulation. The lung is continuously exposed to various environmental substances, including dust, smoke and pathogens, which can cause infection and injury. Under normal conditions, the lung is a highly quiescent tissue with a remarkably slow cellular turnover, compared with other organs such as the skin and gastrointestinal tract [4]. However, the lung demonstrates a significant capacity for regeneration and repair following injury. This low cellular turnover under homeostatic conditions and facultative regenerative response after injury require cell-intrinsic factors and dynamic cellular interactions between stem cells and the surrounding environment – the niche in which they reside [5]. Such stem cells receive and respond to various feedback signals originating from the stem cells themselves, neighbouring cells within the same niche, or even other tissues. Other forms of signalling such as contact-dependent Notch signalling, signalling guided by mechanical and physical cues, and signalling from the extracellular matrix through adhesion receptors also provide important signals to stem cells [6, 7]. If the regulatory process is not completed successfully, lung function can be reduced concomitant with chronic inflammation and

pathological remodelling [8]. Understanding the crosstalk between stem cells and surrounding cells in the lung has considerable practical and therapeutic implications.

In the last decade, extracellular vesicles (EVs) including exosomes have been recognised as a new paracrine mediator through the transfer of biological cargos [9, 10]. In addition, EVs derived from specific cells or tissues present a promising cell-free modality for the treatment of lung diseases. In this review, we highlight the roles of EVs derived from each cell type in lung repair, remodelling and regeneration and their therapeutic potential for lung regenerative medicine.

Extracellular vesicles

All cells release not only soluble factors such as cytokines and growth factors, but also EVs into their environment. EVs contain various molecules such as proteins, RNA transcripts, microRNAs and active lipids, enclosed in a phospholipid bilayer derived from either the plasma membrane or endocytic compartments of the cell [10, 11]. These enclosed molecules can be transferred to other cells, triggering a broad range of cellular activities and biological responses [9, 11, 12]. EVs are often categorised as exosomes, microvesicles (MVs) or apoptotic bodies, based on their size, biogenesis and secretory mechanisms [10]. Exosomes are approximately 100 nm in diameter and are generated by the fusion of multivesicular bodies (MVBs) with the plasma membrane. Fusion of the MVB with the plasma membrane is in part regulated by neutral sphingomyelinase 2, endosomal sorting complex required for transport, tetraspanins, Rab proteins, syntenin, programmed cell death 6 interacting protein and phospholipase D2 [13–15]. In contrast, MVs are a few nanometres to a few micrometres in diameter and are generated by outward budding from the plasma membrane. These EVs are rich in several lipids and phosphatidylserine and contain membrane components similar to those of the parental cell membrane [16, 17]. Apoptotic bodies are several micrometres in diameter and are released from the plasma membrane during cell apoptosis *via* indiscriminate blebbing [18]. However, current methods cannot distinguish between these different types of EVs because of overlapping physical characteristics [9, 10]. For instance, the small MVs, exosomes and enveloped viruses share the same biophysical characteristics in terms of size, density and membrane orientation [9]. Most studies therefore rely on regular EV isolation techniques that, even if they use the term “exosomes”, analyse very heterogeneous mixtures of different EVs. Based on the evidence, the International Society for Extracellular Vesicles consensus recommends using the generic term EV for particles naturally released from the cell in the nomenclature [10].

EVs are involved in various cell-to-cell signalling pathways and act as important molecular messengers in diverse biological and pathological processes [12, 19]. As a result, investigations into EV-mediated intercommunication have elucidated other important mechanisms behind normal homeostasis and diseases. More details on EV-mediated intercommunication mechanisms and various EV-specific functions can be found in several recent reviews [9, 10, 13, 19–22]. Importantly, EVs appear to play diverse roles in the subsequent behaviours of various stem/progenitor cells and cells within their niche in a region-dependent manner during regeneration.

EV-mediated lung microenvironment at homeostasis and in response to injury

Different regions of the human lung exhibit different strategies for lung regeneration. The human proximal airways start with the trachea, which then branches to give rise to bronchi, each of which then gives rise to bronchioles, which eventually terminate in tiny air sacs called alveoli. In the proximal region, the airway is lined by a pseudostratified epithelium of basal cells, ciliated cells and secretory cells (club and goblet cells), as well as small numbers of neuroendocrine cells, ionocytes and tuft cells [2, 23–26]. Basal cells act as major progenitor cells that self-renew and, when necessary, give rise to multiple cell types such as secretory, goblet, ciliated and neuroendocrine cells to maintain the epithelial integrity of the proximal airways [27–29]. In addition to basal cells, certain populations of secretory cells can possess both the ability to self-renew and to transform into differentiated cell types for purposes of replacement. In the distal region, alveoli are distinct in structure, comprising tiny air sacs composed of alveolar type 1 (AT1) and alveolar type 1 (AT2) cells. It is well accepted that AT2 cells can act as stem/progenitor cells in the alveolar epithelium during development and injury in the adult lung [24, 30, 31]. Furthermore, there is emerging evidence of the importance of the stromal cells that support the epithelial stem cell niche, including mesenchymal and immune cells in lung regeneration. Here we describe the EV-mediated signals from lung stem/progenitor cells themselves and the neighbouring cells (figure 1).

Airway epithelial cell-derived EVs

Basal cells are cuboidal in shape, secured to the basement membrane and are specifically characterised by the expression of transformation-related protein 63 (Trp63), cytokeratin 5 (Krt5) and nerve growth factor receptor [23]. Basal cells in the mouse lung continuously self-renew at a low rate and the homeostatic

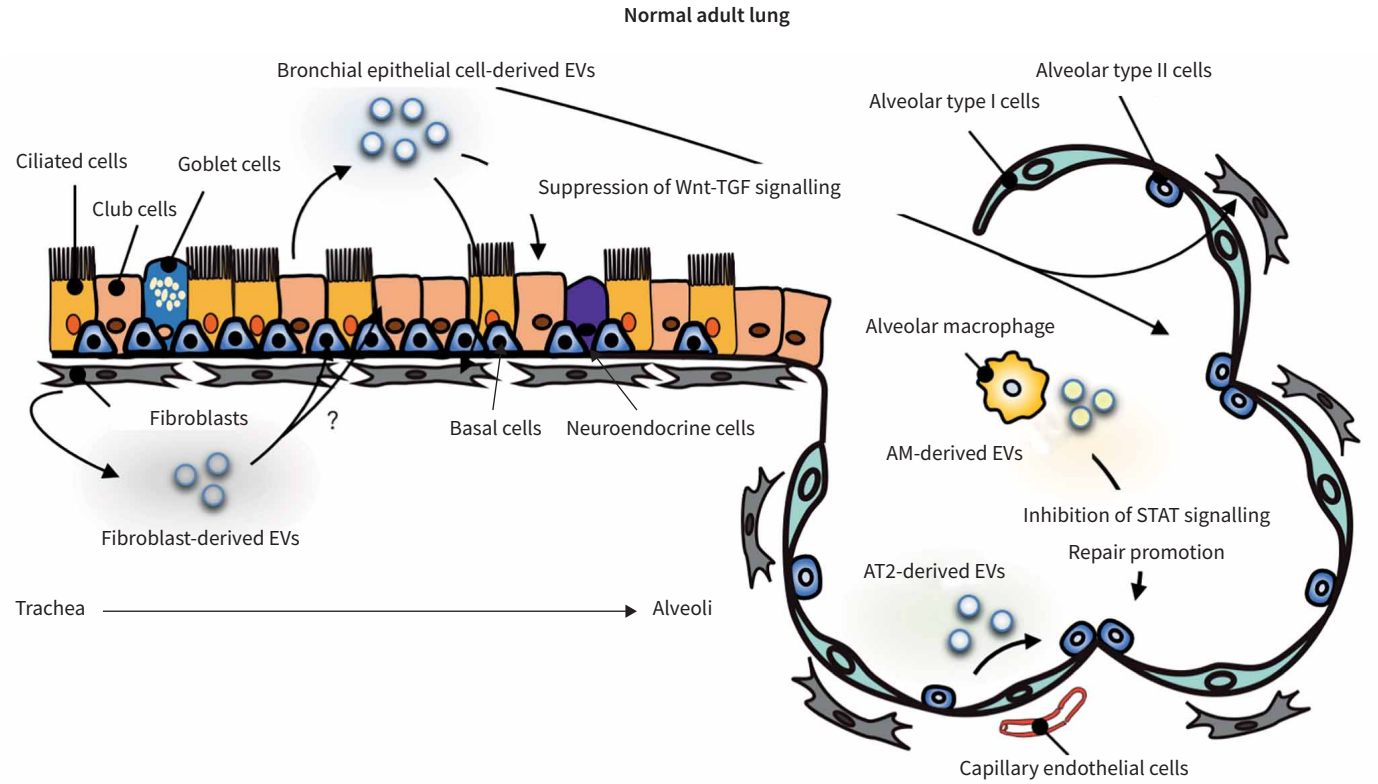

FIGURE 1 Roles of extracellular vesicles (EVs) in lung normal and injured stem cell niches. We propose that EVs derived from epithelial stem cells and the surrounding cells maintain lung quiescence under conditions of lung homeostasis and regulate repair and regeneration after injury. Human bronchial epithelial cell-derived EVs inhibit transforming growth factor-β (TGF-β)-mediated induction of epithelial cell senescence and myofibroblast by attenuating Wnt signalling in the lung. Alveolar macrophage-derived EVs mediate inhibition of signal transducer and activator of transcription (STAT) activation in epithelial cells by delivering vesicular suppressor of cytokine signalling (SOCS) proteins after injury. Alveolar macrophage-derived EVs also promote proliferation of lung epithelial cells *via* shuttling miR-221/222 after injury. AM: alveolar macrophage; AT1: alveolar type 1; AT2: alveolar type 2.

turnover is regulated in part *via* fibroblast growth factor (FGF) receptor 2 and bone morphogenetic protein (BMP) [32, 33]. Notch signalling regulates their preferable differentiation towards the secretory cell lineage in homeostasis and repair [29, 34, 35]. In addition, other signalling types are known to impact cell proliferation and growth in murine injury repair models, including Wnt signalling [36–38], Hippo signalling [39] and p53 [40]. Moreover, basal cells can also proliferate and migrate to the alveolar regions of the lung after severe injury [41]. These basal-like cells also express Trp63 and Krt5, and have been seen to contribute to injury repair [42, 43]. In these cells, the Notch pathway is activated for repair after injury, which can induce differentiation of basal cells into secretory cells [29]. In recent years, signals between stem cells and their daughter cells and stem cell self-signalling loops in homeostasis and repair have been a focus of attention [34, 44]. However, the mechanisms underlying quiescence and response to injury in the lung have yet to be fully elucidated. In particular, interactions between airway epithelial cells and alveolar epithelial cells remain poorly understood.

EVs derived from airway epithelial cells can be involved in epithelial cell homeostasis in airways and alveoli. Gupta *et al.* [45] reported that airway epithelial cells can communicate with each other through EVs. Treatment with EVs derived from different types of human airway cells resulted in differential expression/regulation of certain proteins and microRNAs (miRNAs) in the target airway epithelial cells. Our recent study highlighted the finding that EVs derived from human bronchial epithelial cells, which express Trp63 and Krt5, can be transferred to epithelial cells and fibroblasts in airway and alveoli, and may inhibit epithelial cell senescence and myofibroblast differentiation *via* inhibition of transforming growth factor-β (TGF-β) and Wnt signalling pathways [46] (figure 1). Mechanistically, the EV miRNA cargo is primarily responsible for attenuating both myofibroblast differentiation and cellular senescence by inhibition of Wnt3A, Wnt5A and Wnt10B. Crosstalk between the TGF-β superfamily and Wnt signalling

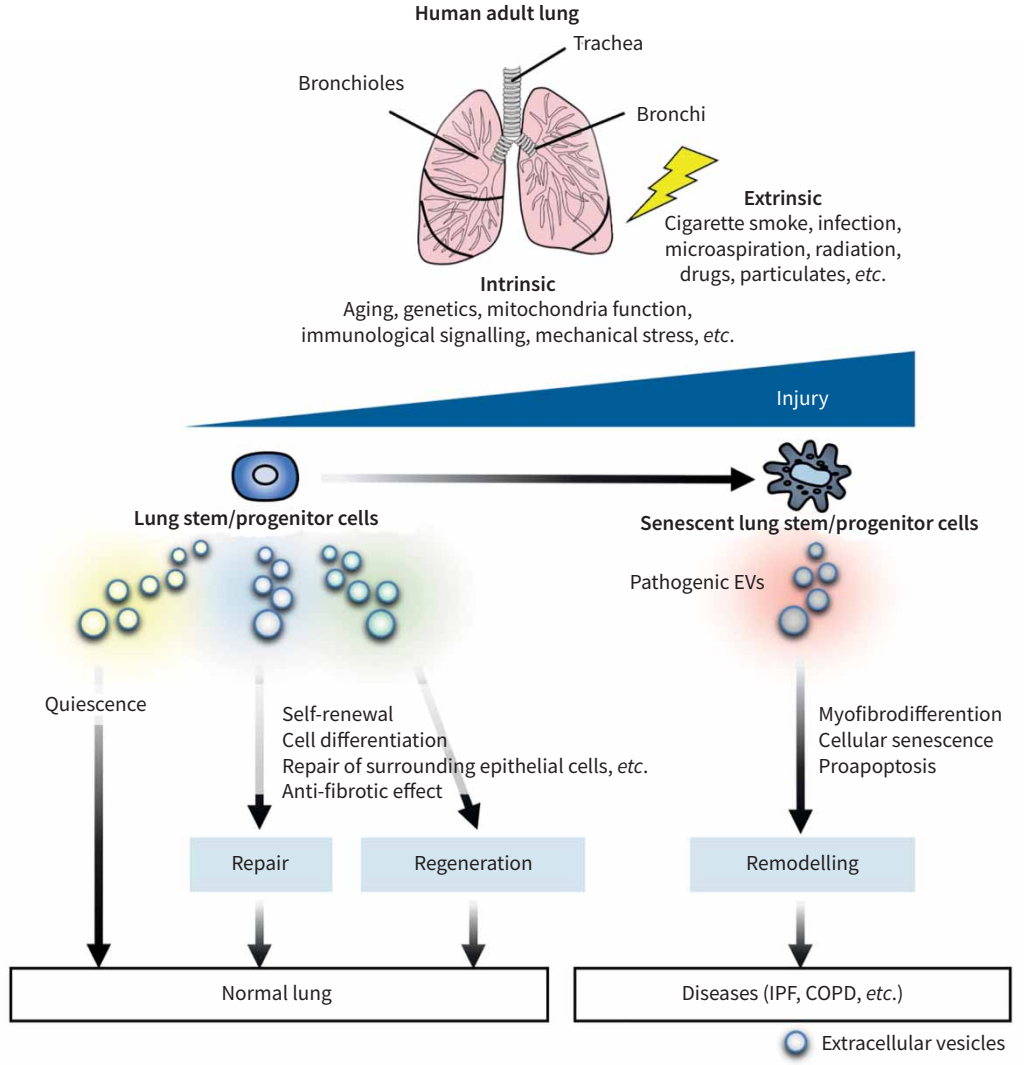

FIGURE 2 Proposed model of the involvement of epithelial cell-derived extracellular vesicles (EVs) in injury-repair responses in the lung. Intrinsic (*e.g.*, ageing, genetics, mitochondria function, immunological signalling, mechanical stress) and extrinsic (*e.g.*, cigarette smoke, infection, microaspiration, radiation, drug) sources of injury can cause epithelial cell death or cellular senescence, leading to dysfunction of resident epithelial stem cells. In the absence of injury, epithelial stem cell-derived EVs maintain lung quiescence. Under injury, epithelial stem cell-derived EVs can preserve lung homeostasis (repair). However, if these are unsuccessful, the EVs can also help to regenerate lung tissue. On the contrary, pathogenic EVs derived from damaged epithelial stem cells (*e.g.*, senescent cells) develop a remodelling, which can be involved in lung pathogenesis such as idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD).

pathways plays an essential role in regulating stem/progenitor cell and mesenchyme functions during quiescence and injury repair [47, 48]. In alveoli, canonical Wnt signalling is activated after injury, while the activity is low in most epithelial cells, including AT2 cells [49]. During alveoli regeneration after injury, active Wnt signalling is essential for the expansion of a Wnt-responsive subpopulation of AT2 cells and the inhibition of differentiation of AT2 to AT1 cells [50]. In airways, basal cells also activate canonical Wnt signalling following tracheal damage [36–38]. Thus, through regulation of TGF-β and Wnt signalling, bronchial epithelial cell-derived EVs may maintain quiescent state and self-renewal, and regulate their repair and regeneration in alveolar and airway (figure 2).

Alveolar epithelial cell-derived EVs

The alveoli are distinct in structure, comprising tiny air sacs composed of AT1 and AT2 cells. Alveolar epithelial cells show a relatively slow rate of cellular turnover under normal conditions. However, after

injuries such as diphtheria toxin-mediated AT2-specific ablation, pneumonectomy, or hyperoxia-induced injury [51–53], AT2 cells show a robust regenerative capacity through extensive cell proliferation and differentiation into AT1 cells in mice. Recent studies have shown several distinct subclassifications of AT2 cells according to single-cell transcriptomic data. For example, two clusters of AT2 cells have been identified in the human lung [2]. One cluster expressed higher levels of some canonical AT2 markers such as surfactant protein C, and other selectively expressed genes involved in Wnt signalling and detoxification. In addition, in response to acute injury, a Wnt-responsive subpopulation of AT2 cells has been identified as major facultative progenitor cells in the distal lung [50, 54]. Currently, in addition to Wnts and their antagonists, a number of other signalling factors such as BMPs, endothelial growth factor (EGF) and vascular EGFs (VEGFs) have been identified as regulators within the alveolar stem cell niche [55, 56]. Inflammatory conditions are also required for epithelial maintenance [57]. However, the cellular and molecular mechanisms underlying alveolar niche construction remain largely unknown.

AT2 cells can communicate with each other for the maintenance or repair of injured alveoli through EVs (figure 1). Quan *et al*. [58] showed that human AT2 cell-derived EVs support AT2 cell-specific proliferation *via* miR-371b-5p transfer in response to lung injury. They showed that miR-371b-5p in EVs derived from the AT2 cell line (A549) promoted AT2 cell-specific proliferation, but not differentiation in pluripotent stem cell-derived cultures. They also observed that bleomycin-treated human-induced pluripotent stem cell-differentiated AT2 cells (hiPSC-AT2) and primary human AT2 cells secrete miR-371b-5p in EVs. These results suggest the possibility that AT2 cell-derived EV miR-371b-5p may serve as alveolar niche signalling in response to lung injury, promoting alveolar re-epithelialisation [58]. Mitchell *et al*. [59] suggested the potential beneficial effects of human AT2 cell-derived EVs on hyperoxia-induced lung injury in a mouse model. They administered EVs derived from hiPSCs and hiPSC-AT2 to mice with hyperoxia-induced lung injury and found that those mice receiving the EVs displayed less damage than mice receiving the control EV. These results imply that AT2 cell-derived EVs may promote repair of surrounding epithelial cells after injury in alveoli (figure 1). Currently, the roles of EVs derived from AT1 cells and subsets of AT2 cells such as the Wnt-responsive subpopulation remain unknown in both normal alveolar homeostasis and response to injury.

Mesenchymal cell-derived EVs

The mesenchymal niche surrounding the airways and alveoli plays essential roles in both maintaining homeostasis and regenerating epithelium. The mesenchyme displays anatomically distinct cell types and region-dependent heterogeneity [60, 61]. These populations include myofibroblasts, lipofibroblasts, adventitial fibroblasts, alveolar fibroblasts and fibromyocytes [2, 62, 63]. In the trachea, mesenchymal lineages promote epithelial repair [60, 62]. Recent work has shown that airway mesenchyme including smooth muscle, which expresses important factors such as FGF10, acts in a paracrine fashion to support the regeneration of basal cells in mice [61, 64]. FGF10 is a potent pro-regenerative growth factor that poses protective and therapeutic effects in the context of various injury models [65]. Evidence indicates that most leucine-rich repeat-containing G-protein coupled receptor 6 (Lgr6)-expressing cells are airway smooth muscle cells, which promote airway differentiation of epithelial progenitors *via* Wnt–FGF10 crosstalk in response to naphthalene injury [60]. In the alveoli, lung fibroblasts (LFs) and AT2 cells have direct and extensive contacts [66]. These alveolar interstitial fibroblasts, especially platelet-derived growth factor receptor alpha (PDGFRα)-expressing fibroblasts (including lipofibroblasts), have a critical role to play in homeostatic alveolar regeneration following injury [51, 62, 67]. Moreover, Lgr5-expressing cells are located in alveolar compartments and appear to be distinct from PDGFRα-expressing cells. This population is sufficient to promote alveolar differentiation of epithelial progenitors through Wnt activation [60]. Furthermore, mesenchymal cells have been suggested to promote differentiation of AT1 cells and suppress AT2 cell proliferation *via* BMP antagonist production [56, 62]. Although considerable research efforts have been undertaken to elucidate mesenchymal-to-epithelial crosstalk, the mechanisms and pathways are not fully understood.

To date, no direct evidence has elucidated the EV-mediated mesenchymal feedback to epithelial stem/progenitor cells during regeneration. However, EVs derived from LFs isolated from the lung tissue of patients can drive abnormal epithelial regeneration. For example, in the airways, Haj-Salem *et al*. [68] showed that bronchial fibroblasts derived from severe eosinophilic asthma patients promote epithelial cell proliferation by delivering lower levels of TGF-β2, which may be involved in an established feature of airway remodelling. Conversely, in alveoli, we have reported that LFs from patients with idiopathic pulmonary fibrosis (IPF) have myofibroblast and senescent phenotypes and secrete EVs, which induce mitochondrial damage and subsequent cellular senescence in lung epithelial cells [69]. Together with the need for co-culture of primary AT2 cells with PDGFRα-positive fibroblasts [70] and the beneficial effects of the secretome from mesenchyme on pathologic alveolar epithelial cells [71], these observations suggest

promising roles of LF-derived EVs in epithelial stem cell homeostasis and response to injury (figure 1). Furthermore, EVs from stimulated LFs affect neighbouring fibroblasts in the lung [72]. Interleukin (IL)-1β-treated fibroblasts produce antifibrotic prostaglandin-containing EVs that can inhibit TGF-β1-induced myofibroblast differentiation of naïve LFs [72]. Overall, further work is needed to define the functional roles of EVs derived from heterogeneous populations of mesenchymal cells in lung homeostasis and regeneration.

Immune cell-derived EVs

The lung contains various populations of resident immune cells with multiple subclasses of dendritic cells, innate lymphoid cells and interstitial and alveolar macrophages. After acute lung injury, immune cells are recruited to the alveolar region and can promote alveolar regeneration by modulating the regenerative niche [73, 74]. Macrophages and monocytes appear to play distinct roles in this process. Resident alveolar macrophages can directly support the growth of AT2 cell proliferation and self-renewal [75, 76]. Recent studies have shown that bone marrow-derived macrophages are recruited in the lung through a C–C motif ligand 2-C-C chemokine receptor 2 axis after partial pneumonectomy and are involved in the compensatory regrowth of AT2 cells [75]. To date, various cytokines and growth factors secreted by immune cells, such as IL-1β, TNFα and IL-6, have been shown to exert direct effects on the airway or alveolar niche [76–78].

Macrophages are thought to be significant producers of EVs in the lung [22, 79]. EVs derived from macrophages can promote epithelial regeneration by modulating the regenerative niche (figure 1). For example, human alveolar macrophages (AMs) secrete suppressor of cytokine signalling (SOCS) 1 and SOCS3 proteins in EVs that can be taken up by alveolar epithelial cells to mediate inhibition of signal transducer and activator of transcription (STAT) activation in response to cytokines, such as IL-6 [80]. Janus kinase–STAT signalling is critical in transducing the effects of many cytokines, hormones and growth factors. A family of STAT-induced STAT inhibitors termed SOCS proteins act in a classical negative-feedback loop to inhibit cytokine signal transduction [81]. In addition, secretion of SOCS3 within EVs derived from resident human AMs can inhibit lung tumourigenesis and dampen allergic airway inflammation by inhibiting the production of type 2 immune response-associated cytokines such as IL-4, IL-13 and thymic stromal lymphopoietin from airway epithelial cells [82]. Furthermore, after stimulation by lipopolysaccharide (LPS), macrophage-derived EVs, particularly apoptotic bodies, can promote proliferation of lung epithelial cells *via* shuttling miR-221/222 [83]. Moreover, antifibrotic properties are enriched in EVs from the sputum and plasma of patients with IPF, and these EVs are suggestive of a macrophage origin [84]. These studies highlight that macrophage-derived EVs are crucial in the maintenance of epithelial cells (figure 1).

Lung-resident mesenchymal stem/stromal cell-derived EVs

MSCs are a unique subset of progenitor cells defined by their capacity to differentiate into multiple mesenchymal lineages such as bone, cartilage, muscle and adipose tissue [85]. They are typically found in a variety of human tissue, including bone marrow, adipose tissue, umbilical cords, placenta, lung, and other tissues. Lung-resident MSCs reside in the perivascular niche and can be activated and recruited to the site of injury, which contributes to the maintenance of lung integrity. Emerging evidence indicates that they can orchestrate immunomodulation, lung repair and regeneration [86]. Although the mechanisms of action of these MSCs in lung injuries and diseases have not yet been fully elucidated, the beneficial effects are at least partially dependent on paracrine mechanisms including release of bioactive molecules and EVs [87, 88].

Lung-resident MSC-derived EVs may be involved in repair and regeneration of lung tissue. EVs derived from MSCs from different sources have been shown to be beneficial to experimental viral, bacterial and LPS-induced lung injury models [89–92]. Silva *et al.* [93] showed that MSC-derived EVs modulate alveolar–capillary barrier integrity through mitochondrial transfer. It has been reported that MSC-derived EVs contain mitochondria [94]. The mitochondrial transfer *via* EVs could attenuate mitochondrial dysfunction and restore mitochondrial respiration impaired by LPS stimulation in primary human distal lung epithelial cells. Yi *et al.* [95] found that miR-30b-3p in MSC-derived EVs is transferred to human alveolar epithelial cells, which inhibit expression of serum amyloid A3 (SAA3). SAA3 is a chief member of the SAA family and a major component of the acute phase of inflammation. EVs derived from miR-30b-3p-overexpressing MSCs resulted in an anti-inflammatory and pro-reparative effect in LPS-treated mouse alveolar epithelial cells through inhibition of SAA3. Li *et al.* [96] showed that MSCs could ameliorate ischaemia/reperfusion lung injury *via* EV secretion. MSC-derived EV treatment could reduce lung oedema, pulmonary dysfunction and inflammation that were associated with ischaemia/reperfusion injury in mice. It could also reduce hypoxia/reoxygenation-induced lung epithelial cell apoptosis. Khatri

et al. [89] investigated the effect of MSC-derived EVs in influenza virus-induced acute respiratory distress syndrome in a pig model. In the study, MSC-derived EVs inhibited the replication of influenza virus and the virus-induced apoptosis in human lung epithelial cells. Wei *et al*. [97] demonstrated that miR-21-5p in MSC-derived EVs reduced oxidative stress-induced apoptosis in mouse lung epithelial cells. Although there is limited evidence of lung-resident MSC-derived EVs in the stem cell niche, these results support the notion that they exert protective effects against lung epithelial injury. Further studies are needed to understand their importance in the context of homeostasis and disease of lung, since lung-resident MSC-derived EVs could be instrumental in providing more specific and targeted therapies for lung diseases.

EV-mediated tissue remodelling in pulmonary fibrosis and COPD

Lung injury can induce a dysplastic repair response in the epithelium and mesenchyme. Irreversible lung remodelling by chronic aberrant injury and inflammation is considered a primary driver of lung diseases such as pulmonary fibrosis and COPD. EV cargos have been proved to reflect cell types and their physiological and pathological conditions of donor cells [98]. Although EVs secreted from stem/progenitor cells exhibit beneficial effects on lung repair and regeneration, EVs from other cell types such as senescent cells and activated neutrophils can lead to tissue remodelling in the lung (figure 2).

IPF is the most common form of fibrotic pulmonary disease, representing a progressive, irreversible and fatal lung disease characterised by diffuse alveolar epithelial cell injury and structural remodelling. Although the aetiology has not been clearly delineated, continuing injury to the epithelium, aberrant activation of the epithelium, impaired epithelial regenerative capacity, and fibroproliferative responses in the ageing lung are well-accepted theories for the pathogenesis of IPF [99]. Several recent studies have highlighted the roles of EVs on the epithelial phenotype and fibroproliferative response in IPF pathogenesis. Our recent study showed that LF-derived EVs from IPF patients increase mitochondrial reactive oxygen species (mtROS) and associated mitochondrial damage to lung epithelial cells, leading to mtROS-mediated activation of DNA damage responses and subsequent epithelial cell senescence [69]. Another group showed that Wnt5A secretion is increased in EVs from bronchoalveolar lavage fluid among patients with IPF, promoting the proliferation of LFs mediated by Wnt5A [100]. In addition, fibroblasts undergoing replicative senescence or TGF-β1-induced senescence promote fibroblast invasion, which is caused by direct interaction of fibronectin localised to the EV surface with recipient fibroblasts [101].

COPD is characterised by chronic, progressive and irreversible airway limitations and is caused by the inhalation of cigarette smoke and other noxious particles. The pathologic hallmarks of COPD are characterised by the emphysematous destruction of the alveolar structure and the remodelling and narrowing of small airways. Our study showed that cigarette smoke extract (CSE) triggers the modification of EV components of bronchial epithelial cell-derived EVs, which promoted the differentiation of LFs into myofibroblasts [102]. Mechanistically, an elevated level of miR-210 in CSE-stimulated bronchial epithelial cell can regulate autophagy *via* targeting ATG7 in LFs, leading to myofibroblast differentiation. Furthermore, Genschmer *et al*. [103] revealed the role of neutrophil-derived EVs in the pathogenesis of COPD *via* promoting proteolytic damage. Activated neutrophil-derived EVs could bind and degrade extracellular matrix *via* the integrin Mac-1 and neutrophil elastase, causing the hallmarks of COPD. Soni *et al*. [104] also showed that the bronchoalveolar lavage fluid levels of neutrophil-derived EVs correlated with the disease severity of patients with COPD. Such findings provide evidence that the disruption of proper EV secretion or secretion of pathogenic EVs after injury results in inefficient lung repair and regeneration, and subsequent remodelling of the lung.

EV therapy for lung regeneration

Stem cell-based therapy as a modality of regenerative medicine is considered an attractive treatment option for various lung diseases [105–108]. The therapeutic effects of stem cells can be attributed to three main mechanisms of action: homing, that is, migration to the site of injury; differentiation into various cell types that can repair the damaged tissue; and secretion of bioactive factors [109]. Among these mechanisms, recent studies suggest that stem cell paracrine effects are primarily responsible for the regenerative potential [110, 111] and may be advantageous given the risks associated with stem cell use, such as immune compatibility, tumorigenicity, and transmission of infections [112]. Cell-free secretome-based therapies, including the use of EVs, have thus been proposed as a potentially efficacious alternative to cell injection approaches. EVs secreted from stem cells are naturally loaded with various bioactive molecules derived from stem cells and offer several advantages for clinical application. First, the smaller size of EVs is suitable for delivery and deposition in the small airways and alveolar regions responsible for many respiratory diseases *via* the modality of inhalation therapy. Second, EVs are stable in tissues and body fluids due to their lipid bilayer architecture. Third, EVs exhibit low levels of immunogenicity and toxicity

compared to cell therapies. To date, various types of stem cell-derived EVs have been studied for respiratory diseases in experimental models and in clinical trials, such as MSCs [91, 92, 113], human bronchial epithelial cells [46], lung tissue spheroid cells [114], endothelial progenitor cells and induced pluripotent stem cells [59].

MSC-derived EVs have various effects such as immunosuppression and regeneration. Numerous preclinical and clinical studies have suggested that MSC-derived EVs hold promise as therapeutics for respiratory diseases such as COPD, IPF and acute respiratory distress syndrome [88, 91, 92, 113, 116]. The mechanisms of action and clinical applications of MSC-derived EVs for respiratory diseases have been the topic of several comprehensive reviews, including ours [88, 116].

Lung-resident airway epithelial cell-derived EVs have therapeutic effects for IPF [46]. Human bronchial and small airway epithelial cell-derived EVs can suppress TGF-β-induced myofibroblast differentiation and lung epithelial cell senescence, and the effects are more potent than those observed with MSC-EVs. Mechanistically, the bronchial epithelial cell-derived EV miRNA cargo is primarily responsible for attenuating both myofibroblast differentiation and cellular senescence. This attenuation occurs *via* inhibition of canonical and non-canonical Wnt signalling pathways. Mouse models utilising intratracheal administration of EVs demonstrate efficient attenuation of bleomycin-induced lung fibrosis development accompanied by reduced expression of both β-catenin and markers of cellular senescence [46].

Lung tissue-derived EVs have therapeutic effects on their native tissues. EVs derived from lung spheroid cells, which contain a heterogeneous population of cells expressing lung epithelial and mesenchymal markers, attenuate and resolve bleomycin- and silica-induced fibrosis by re-establishing normal alveolar structure and decreasing both collagen accumulation and myofibroblast proliferation [114]. Although the mechanisms were not fully elucidated in that study, anti-fibrotic miRNAs such as miR-30a and the let-7 and miR-99 family in EVs may be partially involved in the attenuation of fibrosis.

Endothelial progenitor cells (EPCs) are involved in several lung diseases, such as COPD, pulmonary hypertension and bronchopulmonary dysplasia. Systemic administration of EPCs can partially improve alveolar and endothelial damage [117]. These therapeutic effects are caused, at least partially, by paracrine effects. EVs from human EPCs isolated from the cord blood from a healthy pregnant woman reduced lung injury in LPS-induced acute lung injury mice. This effect was partly achieved by delivering the miRNA-126 in EVs into the injured epithelial cells, thus modulating the expression of a number of relevant genes, including cytokines, VEGFα and tight junction components [115].

HiPSCs are derived from adult somatic cells and are reprogrammed into a pluripotent state by induced expression of certain transcription factors, such as Oct4, Sox2, Myc and Klf4 [118, 119]. Induced pluripotent stem cell (iPSC) secretome, including EVs has been reported to improve hyperoxia-induced acute lung injury, bleomycin-induced fibrosis, and postpneumonectomy lung structure and function [59, 120–122]. However, clear evidence for the efficacy of iPSC-derived EVs in improving lung diseases remain elusive, although one study may have shown some beneficial effects [59]. Considering the effects of the iPSC secretome, iPSC-derived EVs may have some therapeutic potential. Future studies are needed to investigate which of the MSCs or many other candidate lung cell populations, such as lung resident progenitors and iPSCs, might have the best relative potential in EV therapy to reconstitute an injured lung.

Conclusions and perspectives

Because of the inherent architectural complexity and region-dependent unique population of cell lineages, highly orchestrated cell-to-cell communications are central to the remarkable regenerative capacity of the lung after injury. Recent work suggests EV-mediated cellular crosstalk as a novel regulator of lung repair and regeneration. In particular, both airway and alveolar epithelial cell-derived EVs can play key roles in lung repair and regeneration. Furthermore, after lung injury, disruption of proper EV secretion or secretion of pathogenic EVs can lead to lung remodelling. Moreover, the containing cargo and function of EVs are dependent on not only cell type but also specific microenvironments including cigarette smoke exposure, cytokines and culturing condition. For example, normal human bronchial epithelial cell-derived EVs showed an anti-fibrotic property through suppressing myofibroblast differentiation and cellular senescence. In contrast, EVs derived from cigarette smoke-exposed human bronchial epithelial cells can induce myofibroblast differentiation associated with airway remodelling. Importantly, in addition to its roles in lung regeneration, stem cell-derived EVs have potential therapeutic functions in respiratory diseases. Thus, a better understanding of the roles of EVs derived from stem cells in the lung will facilitate the discovery and development of novel therapeutic agents for respiratory diseases. However, many questions remain to be addressed in future studies. A major challenge is how to culture lung-resident stem cells while retaining

their phenotype. For example, the distinct pulmonary epithelial phenotype of AT2 cells is lost within few days in two-dimensional cultures. Moreover, current culture media are poorly defined and contain unknown factors derived from fetal bovine serum or bovine pituitary extracts [70]. Such complex conditions represent an obstacle to evaluating cell-type-specific EVs properly, because stem cells can differentiate into other types of cells. In addition, a further understanding of the precise characterisation of EVs remains to be solved for revealing the EV-mediated mechanisms underlying lung repair and regeneration. This limitation is due to the technical difficulty in isolating and characterising pure populations of specific subtypes of EVs. Furthermore, elimination of xenogeneic components is needed, at least during the EV production and harvesting phase, in order to allow use in clinical therapeutics. In recent years, a variety of *in vitro* culture models have been studied, such as three-dimensional organoid culture methods and large-scale mass culture systems [70]. More recently, chemically defined conditions for human AT2 cell expansion and differentiation in alveolosphere cultures have been reported [123, 124]. Future experiments using these stem cell culture methods will be crucial to reveal the EV-mediated crosstalk in lung repair and regeneration, which will facilitate the development of novel EV therapeutics.

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