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# Extracellular Vesicles in Idiopathic Pulmonary Fibrosis: Pathogenesis, Biomarkers and Innovative Therapeutic Strategies

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**Abstract:** Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and irreversible interstitial lung disease caused by aberrant deposition of extracellular matrix in the lungs with significant morbidity and mortality. The therapeutic choices for IPF remain limited. Extracellular vesicles (EVs), as messengers for intercellular communication, are cell-secreted lipid bilayer nanoscale particles found in body fluids, and regulate the epithelial phenotype and profibrotic signaling pathways by transporting bioactive cargo to recipients in the pathogenesis of IPF. Furthermore, an increasing number of studies suggests that EVs derived from stem cells can be employed as a cell-free therapeutic approach for IPF, given their intrinsic tissue-homing capabilities and regeneration characteristics. This review highlights new sights of EVs in the pathogenesis of IPF, their potential as diagnostic and prognostic biomarkers, and prospects as novel drug delivery systems and next-generation therapeutics against IPF. Notably, bringing engineering strategies to EVs holds great promise for enhancing the therapeutic effect of anti-pulmonary fibrosis and promoting clinical transformation.

**Keywords:** extracellular vesicles, idiopathic pulmonary fibrosis, pathogenesis, diagnosis, therapy

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, degenerative, and progressive interstitial lung disease (ILD) characterized by epithelial injury, fibroblast activation, and abnormal extracellular matrix (ECM) deposition, resulting in a progressive decline in lung function and gas exchange that can eventually cause death.[1] The epidemiological survey of IPF indicates that the global incidence ranges from 1 to 13 per 100,000 individuals, whereas prevalence ranges from 3 to 45 per 100,000 individuals, with a yearly increase seen.[2] According to recent observations, IPF is currently incurable, with an average survival rate of 3–5 years following diagnosis, poor patient prognosis, and limited therapy choices. The pathogenesis encompasses a complex interplay of cell types and molecular pathways.[3] Overall, the IPF course remains unpredictable and affected by several variables, including delayed diagnosis, severe exacerbations, and comorbidities.[4] Treatment has focused on reducing progression of fibrosis, and improving quality of life such as oxygen supplementation, anti-fibrotic drugs, management of comorbidities and acute exacerbations, and pulmonary rehabilitation.[5] Nevertheless, these therapies do not stop the progression of IPF or ideally cure the disease. Lung transplantation is the only treatment for IPF that has been shown to increase life expectancy. The primary challenge in this domain is to identify novel therapies that can modify the natural progression of IPF by stabilizing or reversing the fibrotic process. Therefore, there are still significant unmet needs for the exploration of pathophysiological mechanisms and novel therapeutic strategies for IPF.

Extracellular vesicles (EVs) are phospholipid bilayer-enclosed spherical structures secreted from all cell types, and are categorized as exosomes, microvesicles, and apoptotic bodies according to their size, biogenesis, and secretory mechanisms.[6] Microvesicles are produced by direct budding from the plasma membrane, apoptotic bodies are generated by membrane blebbing during the late stages of apoptosis, whereas exosomes are formed through the endosomal system.[7] In the past decade, EVs were

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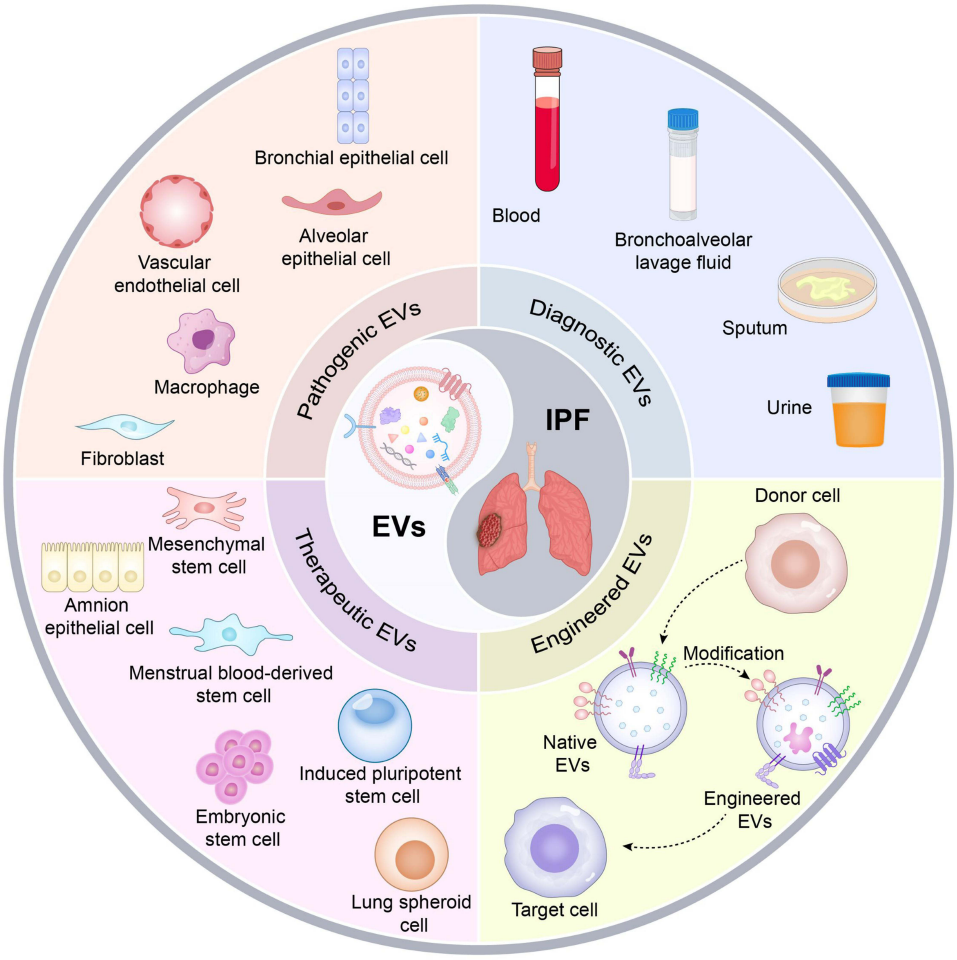
**Graphical Abstract**




reported to mediate intercellular communication by transferring bioactive cargo they carry through paracrine or endocrine effects to activate phenotypic and functional changes in target cells.[8] They are present in various biological fluids such as blood, saliva, bile, urine, cerebrospinal fluid, and bronchoalveolar lavage fluid (BALF), which are being recognized as useful tools facilitate diagnoses, monitoring the lung disease progression, and treatment efficacy.[9] More importantly, EVs can be employed as natural drug delivery systems or cell-free therapeutic modalities based on their intrinsic targeting characteristics and increased biocompatibility for IPF treatment.[10] Zhang et al conducted a single-arm clinical trial to assess the efficacy of intravitreal injection of mesenchymal stem cell (MSC)-derived EVs for treating large and refractory macular holes. Closure of the macular hole was noted in four out of five patients treated with MSC-EVs, which coincided with an enhancement in the best-corrected visual acuity score.[11]

Nevertheless, there are several drawbacks to the use of native EVs for the treatment of IPF, such as their quick removal from the bloodstream and low amounts of effective substances.[12] Recently, engineering to target EVs has gained a lot of interest and is seen to be a potential strategy for EV-based therapy.[13] Van Delen et al performed a systematic review and meta-analysis of clinical trials evaluating the safety and efficacy of human EV-based therapy, and the findings demonstrated that EV-based therapy is both safe and effective.[14] Consequently, in this review, we discuss our current understanding of EVs in the pathogenesis of IPF, and their potential as biomarkers for IPF diagnosis and prognosis. Moreover, we further highlight the prospects of EVs as drug delivery vehicles and next-generation therapeutics in IPF for clinical use.

## IPF Pathogenesis and Therapy

IPF is a chronic, progressive, fibrosing ILD of unknown etiology characterized by dry cough, fatigue, and exertional dyspnea, leading to reduced quality of life and earlier mortality.[15] Pathological features of IPF include alveolar epithelial cells (AECs) dysfunction, fibroblast activation and proliferation, anomalous deposition of ECM, immune dysregulation, interstitial inflammation, and cellular senescence.[16]

Various cells, including MSCs, fibroblasts, immune cells, alveolar capillary endothelial cells, and AECs, work together under normal conditions to maintain the homeostasis of the alveolar environment.[17] Exposure to external stimuli can cause repeated microdamage to AECs, leading to disruption of the basement membrane. Damaged AECs release an array of cytokines such as transforming growth factor β (TGF-β), insulin-like growth factor 1 (IGF-1), connective tissue growth factor (CTGF), fibroblast growth factor 2 (FGF-2), platelet-derived growth factor (PDGF), interleukin 13 (IL-13), and tumor necrosis factor-α (TNF-α) to recruit more immune system cells, induce epithelial-mesenchymal transition (EMT), and facilitate fibroblast proliferation and activation into myofibroblasts, the main collagen-producing cells.[18]

Although the pathogenic mechanisms of IPF remain unknown, it has been shown to occur as a result of repetitive micro-injuries of the AECs influenced by predisposing variables (eg, genetic, epigenetic, environmental, immunologic, and gerontologic),[19,20] which result in metabolic dysfunction, failure of alveolar re-epithelialization and repair. Recurrent alveolar epithelial injury, in combination with dysregulation of the epithelial/mesenchymal crosstalk, promotes fibroblast and myofibroblast activation, leading to accumulation of ECM, pathological tissue remodeling, and irreversible loss of lung function.[21]

As of 2022, the most recent revision to ATS/ERS/JRS/ALAT clinical practice guidelines only recommends two drugs nintedanib and pirfenidone to slow down the progression of IPF.[22] Pirfenidone and nintedanib were approved by the FDA for IPF treatment in 2014 based on their capacity to slow lung function decline and decrease mortality.[23,24] Pirfenidone demonstrates anti-fibrotic effects through the inhibition of TGF-β signaling, leading to a reduction in the transcription of genes associated with collagen synthesis and ECM production. Additionally, it also suppresses the production of pro-inflammatory cytokines, including TNF-α, interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), thereby reducing fibrosis formation.[25] Nintedanib is an oral small-molecule tyrosine kinase inhibitor. It targets receptor tyrosine kinases involved in fibrosis, including PDGF, FGF, VEGF, and TGF-β, as well as non-receptor kinases involved in inflammation and proliferation (Src family kinases). It can also inhibit the proliferation of vascular cells and regulate the activity of fibroblasts.[26]

However, they do not stop disease progression, or reverse established fibrosis, and are associated with adverse events, such as transaminitis, nausea, dyspepsia, rash, and diarrhea.[27] Lung transplantation is the only therapeutic option for selected patients with end-stage IPF with a 5-year survival rate post-transplantation of about 50%. Nevertheless, a protracted waitlist time, suitable donor shortage and medical complications can occasionally be key risk factors.[28] Undoubtedly, there is still a pressing need to develop drugs or therapeutic approaches that can halt the course of the disease, as the majority of IPF clinical studies conducted over the past 20 years have failed to meet the primary endpoint.[29]

## Characteristics of EVs

EVs are lipid bilayer-particles that are classified into exosomes, microvesicles, and apoptotic bodies, depending on their biogenesis, secretion mechanism, and size. Indeed, EVs can be detected in diverse biological fluids, including BALF, blood, sputum, bile, and urine, as well as cell culture-conditioned medium and bacteria.[30] Exosomes are small EVs (30–150 nm) discharged into the extracellular environment following the fusion of late endosomes/multivesicular bodies (MVBs) with the plasma membrane.[31] The formation of MVBs is a critical process in the biogenesis of exosomes. This process begins with early-sorting endosomes which result from the invagination of the cell membrane during endocytosis. Subsequently, intraluminal vesicles (ILVs) are produced by the inward budding of the endosomal membrane, further converting the endosomes into MVBs. Ultimately, ILVs are released into the extracellular space as exosomes when MVBs fuse with the plasma membrane.[32] Microvesicles are medium EVs (100–1000 nm) formed by direct budding at the plasma membrane, resulting in direct release into the extracellular environment. Apoptotic bodies are large EVs (>1000 nm) generated by membrane blebbing during apoptotic cells disassembly[33] (Figure 1).

At present, distinguishing exosomes from microvesicles has always been challenging. Exosomes and microvesicles inevitably contain components from their parental cells, such as DNA, RNA, membrane proteins, cytoplasmic proteins,

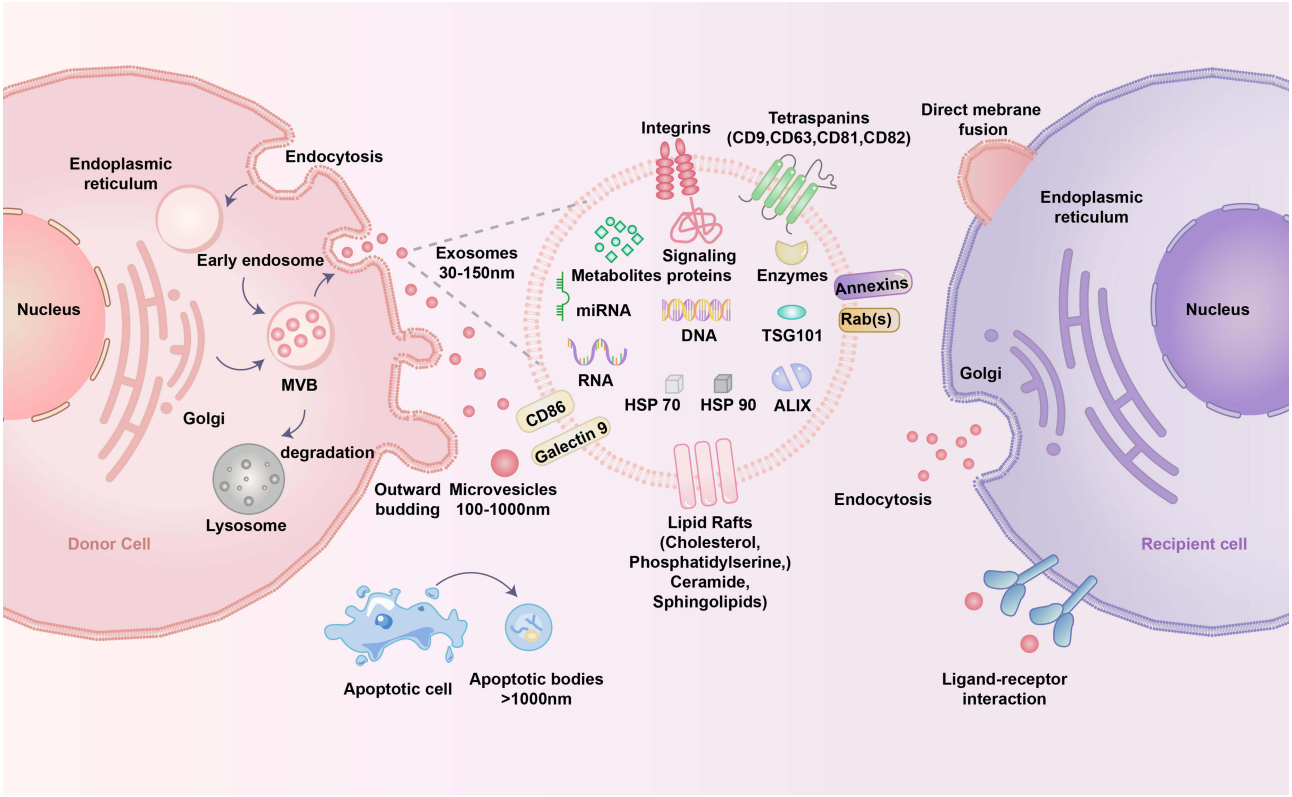



**Figure 1** Biogenesis and uptake of EVs (exosomes, microvesicles, and apoptotic bodies). Exosomes are generated through the endocytic pathway by the fusion of MVBs with the plasma membrane. Microvesicles are formed by directly budding from the plasma membrane. Apoptotic bodies are produced by membrane blebbing during apoptotic cells disassembly. EVs interact with recipient cells and act as messengers for intercellular communication: ligand-receptor interaction, endocytosis into cells, and direct fusion with the plasma membrane.

phospholipids, and metabolites, resulting in some degree of overlap in their molecular composition and particle size. Despite theoretical distinctions, the current technology struggles to entirely differentiate exosomes from microvesicles, only allowing for incremental improvements in purity. The most recent International Society for Extracellular Vesicles (ISEV) guidelines updated in 2023 recommend using "extracellular vesicle" as a generic term to describe vesicles of all sizes,[34] due to difficulty in enriching for EVs produced by different mechanisms, lack of definitive characterization of biogenesis-based subtypes, and no universal molecular markers for each EV subtype. Thus, it is advised that EVs < 200 nm can be described as "small EVs", whereas EVs > 200 nm are called "large EVs", based on the diameter of the separated particles. As such, developing standardized techniques that effectively facilitate the isolation of an EV subpopulation continues to be a major challenge.

Generally, several methods can be used to isolate EVs according to the EV biophysical characteristics of size, density, charge, and surface composition, each with its advantages, limitations, and applications.[35] The most common techniques for their isolation and concentration have been differential ultracentrifugation and ultrafiltration, while alternative approaches include density gradient, size exclusion chromatography, polymer-based precipitation, charge and molecular recognition-based separation, and fluid flow-based separation.[36] Once isolated, EVs can be characterized by dynamic light scattering, nanoparticle tracking analysis, nanoscale flow cytometry, and transmission electron microscopy. Nevertheless, there are still a number of unsolvable problems with the current EVs isolation techniques, such as loss of activity, contamination with non-vesicular substances, and a time-consuming separation process.[37] Indeed, categorizing EVs according to size or immunoaffinity may overlap among different EV subsets and even include some other particles. As a result, most published studies focus on the properties of mixed EV subsets rather than specific groups.

Notably, EVs carry diverse components such as proteins, lipids, mRNAs, microRNAs (miRNAs), DNA, mitochondria, and metabolites, which can vary widely with cellular origin and their microenvironment.[38] Given the diverse functions and characteristics of exosomes and microvesicles, the majority of research on EVs has concentrated on these two types. In contrast to microvesicles, the formation of exosomes entails complex processes including initiation, endocytosis, multivesicular body

formation, and secretion, resulting in a more complex composition, content and structure. Among the three subpopulations of EVs, exosomes have the smallest average particle size, the highest uniformity, the narrowest particle size distribution, relatively intricate composition, and relatively diverse functions. Therefore, it has high theoretical and application value, and has been deeply explored and widely used. Apoptotic bodies have been relatively understudied due to their large size, variability, potential for inducing apoptosis, and ease of clearance. However, they possess unique advantages. Apoptotic bodies can be recruited and phagocytosed by immune cells, especially macrophages, enhancing their applicability in contexts related to immune cell environments or diseases. Exosomes and microvesicles have partially overlapping components, such as tumor susceptibility gene 101 protein (TSG101), ALG-2-interacting protein X (ALIX), HSP70, HSP90, RAB proteins, tetraspanins, integrin, and annexins. Besides their common features, exosomes may express specific cargo, including the endosomal sorting complex required for transport (ESCRT), syntenin, flotillin 1 and 2, peroxidases, and ubiquitin.[39] Additionally, some lipids like sphingolipids, cholesterol, phosphatidylserine, and ceramide are more enriched in exosomes compared to the plasma membrane.[40,41]

As carriers of cell-to-cell information transmission, EVs can release their components and correlate to recipient cells by ligand-receptor interaction or vesicle internalization through phagocytosis, endocytosis, or direct membrane fusion,[42] which can affect a wide range of downstream signaling events. There is compelling evidence that EVs are associated with major (patho)physiological processes, including the immune system, infectious, neurodegenerative, cardiovascular, and chronic lung diseases including IPF, depending on their capabilities to regulate the function and phenotype of recipient cells.[43–46] Briefly, the intrinsic stability and targeting properties of EVs, their dynamic and functional cargo, as well as their capacity to cross biological barriers, make them promising candidates for disease diagnostics and therapy.

## Role of EVs in IPF Pathogenesis

Increasing evidence highlighted the pivotal role of intercellular communication mediated by EVs in the pathogenesis and progression of IPF, including inflammation and immunity, fibroblast proliferation and differentiation, and ECM deposition.[47] To the best of our understanding, pathogenic EVs mainly derived from various cell types in specific microenvironments, including AECs, macrophages, endothelial cells, fibroblasts.[48] Most of those cells secrete EVs that drive the development of pulmonary fibrosis via activation of profibrotic signaling pathways (Figure 2) such as TGF-β pathway, wingless-N-type (Wnt)/β-catenin pathway, and cellular senescence (Table 1).

Mouawad et al[49] suggested that TGF-β-loaded EVs derived from fibrotic lung tissues or activated fibroblasts contained increased levels of fibrotic proteins, and propagated a fibrotic signal driven by TGF-β. Their study highlighted the role of EV communication as a novel platform and mechanism in the propagation of systemic sclerosis related lung fibrosis. The study from Sun et al[50] revealed that angiotensin II type 1 receptor (AT1R)-containing macrophage exosomes could promote collagen synthesis, activate TGF-β/Smad2/3 pathway, and exacerbate pulmonary fibrosis by upregulating angiotensin II (Ang II)/AT1R axis in lung fibroblasts or fibrotic lung tissues. miRNAs are frequently researched cargo in EVs and have been linked to IPF pathogenesis. Another study uncovered that exosomes with low miRNA let-7d secreted by pulmonary vascular endothelial cells (VECs) might trigger lung pericyte fibrosis through TGFBR1/FoxM1/Smad/β-catenin signaling axis in a mouse model with pulmonary fibrosis.[51] Moreover, Zhang et al revealed that exosomes derived from senescent epithelial cells facilitated the activation of pulmonary fibroblasts through targeting the miR-217-5p/SIRT1/β-catenin axis. These findings suggested that overexpression of exosomal miR-217-5p enhanced acetylation and nuclear translocation of β-catenin and Wnt signaling.[52]

In addition, the study by Asghar et al reported that human bronchial epithelial cells-derived small EVs from IPF patients propagated a senescent signal to nearby healthy cells, aggravating the disease state in IPF. Moreover, miR-411, miR-137, miR-195, and miR-7 were upregulated, which might draw attention to potential targets for cutting-edge treatments in IPF.[53] A work from Kadota et al also found that IPF fibroblast-derived small EVs could induce mitochondrial damage and senescence in epithelial cells, and were positively correlated with increased levels of miR-23b-3p and miR-494-3p.[54] Parimon et al showed that syndecan-1-dependent EVs modulated alveolar epithelial preprogramming and plasticity by downregulating the cargo of miRNA profiles (miR-34b-5p, miR-503-5p, miR-144-3p, and miR-142-3p) within EVs that can augment profibrotic pathways, such as TGF-β, Wnt/β-catenin signaling, and cellular senescence.[55]

Furthermore, Chanda et al demonstrated that fibroblast-derived EVs carried fibronectin on the vesicular surface and could mediate fibroblast invasion by interacting with the fibronectin receptor α5β1 integrin, and triggering focal adhesion kinase and steroid receptor coactivator.[56] Chen et al demonstrated that HOTAIRM1 encapsulated in exosomes from hypoxia-induced AECs

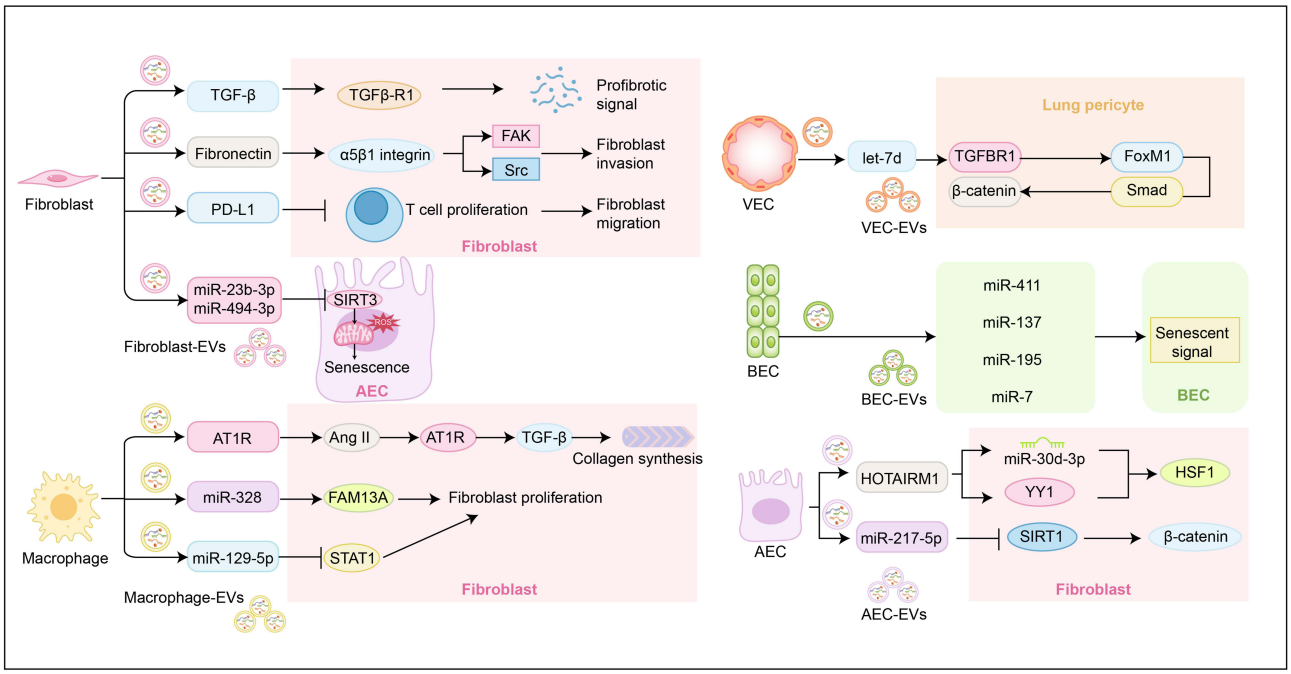



**Figure 2** Diagram of EVs derived from various lung cell types involved in the pathogenesis of IPF. EVs and their cargo modulate key signaling pathways linked to profibrotic processes that promote the progression of IPF.

accelerated IPF progression by regulating the miR-30d-3p/HSF1/YY1 axis.[57] Besides, Kang et al reported that EVs derived from TGF-β stimulated-human lung fibroblasts contained higher levels of PD-L1, potentially reducing T cell proliferation, promoting fibroblast migration, and further contributing to pulmonary fibrosis.[58] The recent study of Hayek et al proved that IPF-derived exosomes contained high miR-143-5p and miR-342-5p levels, which blocked the *de novo* fatty acid synthesis pathway in

**Table 1** Role of EVs in IPF Pathogenesis

| EV Source | EV Cargo | Mechanism to Induce IPF | Ref. |
|---|---|---|---|
| fibroblast | TGF-β | propagate a fibrotic signal throughout the lungs driven by TGF-β | [49] |
| macrophage | AT1R | promote collagen synthesis by upregulating Ang II/AT1R/TGF-β axis | [50] |
| vascular endothelial cell | let-7d | trigger lung pericyte fibrosis through TGFBR1/FoxM1/Smad/β-catenin signaling | [51] |
| senescent epithelial cell | miR-217-5p | activate fibroblasts through modulation of the SIRT1/β-catenin axis | [52] |
| bronchial epithelial cell | miR-411, miR-137, miR-195, miR-7 | propagate a senescent signal to nearby healthy cells, and promote IPF | [53] |
| fibroblast | miR-23b-3p, miR-494-3p | suppress SIRT3, and induce senescence in epithelial cells | [54] |
| MLE-12 cell | miR-34b-5p, miR-503-5p, miR-144-3p, miR-142-3p | promote lung fibrosis by regulating epithelial reprogramming and plasticity | [55] |
| fibroblast | fibronectin | engage integrin receptors on target cells to signal fibroblast invasion | [56] |
| alveolar epithelial cell | HOTAIRM1 | accelerated IPF by regulating the miR-30d-3p/HSF1/YY1 axis | [57] |
| fibroblast | PD-L1 | reduce T cell proliferation, promote fibroblast migration, and stimulate pulmonary fibrosis | [58] |
| IPF | miR-143-5p, miR-342-5p | block the *de novo* fatty acid synthesis pathway in ATII cells, and induce a profibrotic response in lung fibroblasts | [59] |
| fibroblast | TNC, IGFBP7, FBN1, COL1A1, COL1A2, LOXL1 | undefined | [60] |
| M2 macrophage | miR-328 | enhance pulmonary fibroblast proliferation and pulmonary fibrosis by regulating FAM13A | [61] |
| M2 macrophage | miR-129-5p | inhibit STAT1 gene expression, promote fibroblast proliferation and pulmonary fibrosis | [62] |
| macrophage | undefined | activate fibroblast proliferation and migration via endoplasmic reticulum stress | [63] |

alveolar type II (ATII) cells, as well as induced a profibrotic response in lung fibroblasts.[59] Velázquez-Enríquez et al discovered differentially expressed proteins linked with human IPF progression, including tenascin-c (TNC), insulin-like-growth-factor-binding protein 7 (IGFBP7), fibrillin-1 (FBN1), alpha-2 collagen chain (I) (COL1A2), alpha-1 collagen chain (I) (COL1A1), and lysyl oxidase homolog 1 (LOXL1) in EVs isolated from the fibroblast cell lines. This suggests that proteins contained within EVs cargo might have a potential contribution to the pathogenesis of IPF.[60]

Alveolar macrophage-derived exosomes are also involved in accelerating pulmonary fibrosis via mediating intercellular communication. Yao et al discovered that M2 macrophage-derived exosomes overexpressed miR-328 and enhanced pulmonary fibroblast proliferation and the progression of pulmonary fibrosis via regulating FAM13A in a rat model.[61] Qian et al also claimed that M2 macrophage-derived exosomes transported miR-129-5p to pulmonary interstitial fibroblasts and inhibited STAT1 gene expression, leading to fibroblast proliferation and pulmonary fibrosis.[62] In a study by Qin et al discovered that macrophage-derived exosomes contributed to silica-induced pulmonary fibrosis via activating fibroblast proliferation and migration in an endoplasmic reticulum stress-dependent manner.[63]

## Potential of EVs as Diagnostic Biomarkers of IPF

Despite substantial progress in understanding IPF pathogenesis, it remains challenging to accurately forecast the course of a specific patient's condition or response to medication. Consequently, early recognition and precise diagnosis are essential to monitor the disease and improve survival rates.[64] Nonetheless, clinical biomarkers for diagnostic, prognostic, and therapeutic purposes, are not well characterized to date. As another kind of liquid biopsy, EVs have recently attracted attention as novel diagnostic markers since they reflect pathophysiological characteristics and microenvironment of the parental cell, and are stably detectable in body fluids.[65] Additionally, their concentration and profiles are altered depending on the current disease state.[66] Recently, the use of EVs as biomarkers for IPF has been made easier by the remarkable advancement of EV-associated proteins and RNAs (Table 2).

### EVs in Blood

Makiguchi et al found that an upregulation of miR-21-5p in the serum-derived EVs of IPF patients compared with healthy controls. Moreover, during the 30-month follow-up period, a significant correlation was seen between the level of serum EV

**Table 2** Biomarker Potential of EVs in IPF

| EV Source | EV Cargo | Expression | Cohort Selection | Ref. |
|---|---|---|---|---|
| serum | miR-21-5p | upregulated | 41 IPF patients and 21 healthy controls | [67] |
| serum | miR-16, let-7d | downregulated | 61 IPF patients and 15 healthy controls | [68] |
| plasma | hsa_circ_0044226, hsa_circ_0004099, hsa_circ_0008898 | upregulated | 113 IPF patients and 76 healthy controls | [69] |
| plasma | SFTPB, ALDOA, HMGB1, CALML5, TLN1 | upregulated | 20 IPF patients and 19 ILDs patients | [70] |
| serum | CD19, CD69, CD8, CD86, CD209, CD133/1, MCSP, and ROR1<br>CD42a | upregulated<br>downregulated | 90 IPF patients and 19 healthy controls | [71] |
| serum | SFTPB | upregulated | 56 PPF patients and 86 non-PPF cases | [72] |
| BALF | Wnt5a | upregulated | 7 IPF patients and 5 non-IPF cases | [73] |
| BALF | miR-125b-5p, miR-128-3p, miR-21-5p, miR-100-5p, miR-140-3p, miR-374b-5p<br>let-7d-5p, miR-103-3p, miR-27b-3p, miR-30a-5p | upregulated<br>downregulated | 30 IPF patients and 16 healthy controls | [74] |
| BALF | miR-204-5p | upregulated | undefined | [75] |
| BALF | miR-141-3p, miR-200a-3p, miR-200b-3p, miR-375-3p, miR-423-3p<br>miR-22-3p, miR-24-3p, miR-320a-3p, miR-320b | downregulated<br>upregulated | 8 IPF patients and 8 healthy non-smoking controls | [76] |
| BALF | ncRNAs | undefined | undefined | [77] |
| urine | miR-let-7d, miR-29a-5p, miR-181b-3p<br>miR-199a-3p | downregulated<br>upregulated | 16 IPF patients and 10 healthy controls | [78] |
| sputum | miR-142-3p, miR-33a-5p<br>let-7d-5p | upregulated<br>downregulated | 16 IPF patients and 14 healthy controls | [79] |
| sputum | miR-142-3p | upregulated | 19 IPF patients and 23 healthy controls | [80] |

miR-21-5p and mortality in IPF patients; individuals with higher expression of serum EV miR-21-5p had a worse prognosis.[67] Lacedonia et al investigated the expression of five exosomal miRNAs in the serum of patients with IPF versus to healthy controls. Of note, this result showed that miR-16, miR-21, miR-26a, miR-210 and let-7d were downregulated in serum exosomes from IPF patients, with only miR-16 and let-7d showing a statistically significant difference.[68] Notably, Gan et al indicated that IPF patients had higher expression levels of exosomal circular RNAs (hsa_circ_0044226, hsa_circ_0004099, and hsa_circ_0008898) in their plasma compared to non-IPF individuals, which could serve as a new paradigm of biomarkers for the diagnosis of IPF. Moreover, markedly elevated hsa_circ_0044226 not only indicated its potential as an indicator for forecasting IPF progression but also contributed to the advancement of IPF via the TGFβ1 signaling pathway.[69]

However, most research on IPF have focused on miRNA cargo within EVs to uncover biomarkers. Recently, there has been a renewed interest in using EV proteins as promising diagnostic biomarkers. An experiment by Adduri et al identified that a five-protein signature comprising surfactant-associated protein B (SFTPB), aldolase A (ALDOA), high mobility group box protein 1 (HMGB1), calmodulin like 5 (CALML5), and talin-1 (TLN1) from plasma EVs could be used as promising noninvasive biomarkers to differentiate IPF from other ILDs with higher specificity and sensitivity.[70] D'Alessandro et al detected the expression of blood exosome surface epitopes in IPF patients, and showed that many exosomal surface epitopes CD19, CD69, CD8, CD86, CD209, CD133/1, melanoma-associated chondroitin sulphate proteoglycan (MCSP), and receptor tyrosine kinase like orphan receptor 1 (ROR1) were significantly higher in IPF patients, while CD42a was lower than in controls. Furthermore, higher expression of CD25 and CD8 was associated with worse prognosis in IPF survival analysis. These findings supported the use of blood exosome surface epitopes as potential biomarkers for IPF diagnosis and prognosis.[71] Enomoto et al recently revealed that pulmonary SFTPB in serum EVs is a better predictor of ILD progression than serum Krebs von den Lungen-6 (KL-6) and surfactant protein D (SP-D), two recognized biomarkers. It also served as an independent prognostic factor from the ILD-gender-age-physiology index.[72]

## EVs in BALF, Sputum and Urine

EVs in BALF, sputum and urine are appealing biomarkers for IPF based on their accessibility and non-invasiveness. Martin-Medina et al found that BALF-EVs from IPF patients were enriched in functional Wnt5a, which regulated lung fibroblast-to-myofibroblast differentiation and collagen production, and thus contributed to IPF pathogenesis.[73] Besides, Liu et al reported that up-regulation of miR-125b-5p, miR-128-3p, miR-21-5p, miR-100-5p, miR-140-3p, and miR-374b-5p, along with down-regulation of let-7d-5p, miR-103-3p, miR-27b-3p, and miR-30a-5p were observed in BALF-derived exosomes from IPF patients. Of these, decreased expression of miR-30a-5p in BALF exosomes may be a novel predictive biomarker for IPF diagnosis.[74] The results from Zhu et al's study demonstrated that miR-204-5p in BALF-derived exosomes exhibited pro-fibrotic effects, and facilitated the progression of pulmonary fibrosis by inhibiting autophagy and AP1S2 expression.[75] Kaur et al identified five significant downregulated miRNAs (miR-141-3p, miR-200a-3p, miR-200b-3p, miR-375-3p, and miR-423-3p) and four upregulated miRNAs (miR-22-3p, miR-24-3p, miR-320a-3p, and miR-320b) in the BALF-derived exosomes of IPF patients as compared to non-smoking controls.[76] Tang et al found that differentially expressed non-coding RNAs (ncRNAs) in BALF-EVs play an important part in the process of mechanical ventilation-induced pulmonary fibrosis. What's more, 6 downregulated genes (Yes1, Itsn1, Arhgap32, Ehbp1, Norch2 and Hipk2) were confirmed by qRT-PCR in mouse lung fibrosis tissue, which may be potential therapeutic targets for mechanical ventilation-induced pulmonary fibrosis treatment in the future.[77]

The study by Elliot et al showed that urine-derived exosomes from IPF patients carried pro-fibrotic lung phenotype in vivo, suggesting promising prospects for the diagnosis of IPF. Dysregulation of miRNAs (miR-let-7d, miR-29a-5p, miR-181b-3p and miR-199a-3p) packaged in the urine-derived IPF exosomes regulated expression of pro-fibrotic, inflammatory and ECM encoding genes, which involved in the development and progression of fibrosis.[78] Besides, Njock et al found that sputum exosomal miRNA levels differ significantly between IPF patients and healthy individuals. Three miRNAs (miR-142-3p, miR-33a-5p and let-7d-5p) in the sputum-derived exosomes from IPF patients were identified as clinically useful biomarkers for diagnosis and disease severity.[79] When exosomal miR-142-3p and IPF were further investigated, Guiot et al revealed that sputum macrophage-derived exosomes inhibited abnormal ECM deposition and pulmonary fibrosis progression by delivering antifibrotic miR-142-3p to lung epithelial cells and fibroblasts. In addition, miR-142-3p was able to repress the expression of TGFBR1, making it more promising as a sputum biomarker for IPF.[80]

Based on the frequency of IPF biomarkers we investigated, their diagnostic specificity, and the ability to detect them in different clinical samples, we believe that miR-21-5p and let-7d-5p in EVs are appealing and promising biomarkers. Indeed, the diagnostic value of a single biomarker may be limited. Therefore, a comprehensive assessment of multiple biomarkers may provide more accurate diagnostic and prognostic information.

## Therapeutic Roles of EVs in IPF

Stem cell therapy is emerging as a feasible therapeutic approach for IPF based on their capacity of homing to the injury site, and differentiation into specific cell types. Notably, EVs naturally released from stem cells can exert a similar therapeutic effect to their parental cells, and avoid the potential risks of whole-cell transplantation, such as immunogenicity, iatrogenic tumor formation, and thrombosis.[81,82] To date, various types of cell-derived EVs have demonstrated the therapeutic potential in experimental pulmonary fibrosis models, such as MSCs, embryonic stem cells, induced pluripotent stem cells, bronchial epithelial cells, and lung spheroid cells (Table 3).

### MSC-EVs

MSCs are self-renewing multipotent cells isolated from multiple tissues and organs, mainly bone marrow, adipose tissue, menstrual blood, placenta, and umbilical cord tissue.[108] MSCs are the most frequently used stem cells, due to their capacity to modulate the immune system and their simplicity in isolation. Notably, MSCs can also participate in the restoration of injured endothelium through paracrine mechanisms including the release of EVs.[109]

**Table 3** Therapeutic Potential of EVs in Animal Models of IPF

| EV Source | EV Cargo | Model | Delivery | Mechanism to Alleviate IPF | Ref. |
|---|---|---|---|---|---|
| HBMSC | miR-186 | BLM-induced mouse | i.v. | inhibit lung fibroblast activation, and ameliorate IPF by reducing SOX4 and DKK1 expression | [83] |
| HBMSC | undefined | BLM-induced mouse | i.v. | improve collagen deposition, promote immune regulation, and modulate monocyte phenotype | [84] |
| HBMSC | miR-29b-3p | BLM-induced mouse | i.v. | inhibit pulmonary fibroblast proliferation and relieve IPF by downregulating FZD6 expression | [85] |
| RBMSC | undefined | silica-induced rat | i.v. | alleviate pulmonary fibrosis by reversing EMT, and attenuating Wnt/β-catenin signaling | [86] |
| MBMSC | undefined | LPS-induced mouse | i.v. | suppress EMT by inhibiting Wnt/β-catenin signaling, and repair early pulmonary fibrosis | [87] |
| MSC | miR-30b | BLM-induced mouse | i.v. | improve IPF by targeting RUNX1 to reduce the Spred2 transcription level | [88] |
| MADSC | undefined | silica-induced mouse | i.t. | reduce collagen fiber content, granuloma size, and the quantity of macrophages | [89] |
| ADSC | let-7d-5p | PM2.5-induced rat | i.t. | mitigate pulmonary fibrosis by inhibiting ROS levels, inflammation, and TGFBR1 | [90] |
| ADSC | miR-29c, miR-129 | BLM-induced mouse | i.t. | reduce lung fibrosis progression, influence myofibroblast phenotype, and ECM deposition | [91] |
| HUMSC | miR-21, miR-23 | BLM-induced mouse | i.v. | alleviate pulmonary fibrosis, and enhance AECs proliferation by repressing TGF-β pathway | [92] |
| HUMSC | let-7i-5p | silica-induced mouse | i.v. | block fibroblast activation via regulating TGFBR1/Smad3 pathway | [93] |
| HUMSC | miR-218 | BLM-induced mouse | i.v. | inhibit EndMT through MeCP2/BMP2 pathway | [94] |
| HUMSC | undefined | hyperoxia-induced rat | i.t. | decrease α-SMA, reduce collagen deposition and protect against oxidative stress | [95] |
| HUMSC | miR-223-3p | silica-induced mouse | i.v. | decrease inflammation and collagen deposition via the circPWWP2A/miR-223-3p/NLRP3 axis | [96] |
| HUMSC | miR-26a-5p | silica-induced mouse | i.v. | suppress EMT by blocking the Adam17/Notch signaling pathway | [97] |

(*Continued*)

**Table 3** (Continued).

| EV Source | EV Cargo | Model | Delivery | Mechanism to Alleviate IPF | Ref. |
|---|---|---|---|---|---|
| HMenSC | miR-let-7 | BLM-induced mouse | i.v. | inhibit ROS, mtDNA damage, and NLRP3 inflammasome activation | [98] |
| HESC | undefined | BLM-induced mouse | i.v. | inhibit EMT-induced phenotypic changes in epithelial cells, and reduce collagen levels | [99] |
| HESC | miR-17-5p | BLM-induced mouse | i.v. | reduce inflammation, and eliminate collagen deposits by binding to thrombospondin-2 | [100] |
| HAEC | undefined | BLM-induced mouse | i.n. | polarize and increase macrophage phagocytosis, reduce neutrophil myeloperoxidases, and suppress T cell proliferation | [101] |
| HAEC | undefined | BLM-induced mouse | i.n. | reduce airway remodeling and inflammation | [102] |
| LSC | miR-30a, let-7, miR-99 | BLM/silica-induced mouse | i.h. | reduce both collagen deposition and myofibroblast proliferation, and restore normal alveolar structure | [103] |
| iPSC | miR-302a-3p | BLM-induced mouse | i.v. | inhibit the M2-type macrophages via targeting TET1 | [104] |
| DASC | miR-let-7a, miR-let-7b | BLM-induced mouse | i.v. | reduce CCL7 expression of fibroblasts, and inhibit macrophage recruitment | [105] |
| HBEC | miR-16, miR-26a, miR-26b, miR-141, miR-148a, miR-200a | BLM-induced mouse | i.t. | inhibit myofibroblast differentiation and epithelial cellular senescence via suppressing TGF-β-WNT crosstalk | [106] |
| VEC/AEC2s | miR-223, miR-27b-3p | BLM-induced mouse | i.t. | regulate the alveolar macrophages via targeting RGS1 | [107] |

## Bone Marrow MSC-Derived EVs

Bone marrow is one of the most extensively investigated sources of MSCs, Zhou et al described that human bone marrow MSC-derived EVs (HBMSC-EVs) with overexpressed miR-186 suppressed lung fibroblast activation, and ameliorated IPF via reducing SOX4 and DKK1 expression.[83] HBMSC-EVs can also be involved in preventing and reverting bleomycin (BLM)-induced pulmonary fibrosis by systemic modulation of monocyte phenotypes associated with reduced collagen deposition and promoted immune regulation.[84] Indeed, Wan et al found that HBMSC-EVs encapsulated miR-29b-3p could inhibit pulmonary fibroblast proliferation and relieve IPF by downregulating FZD6 expression.[85] Zhang et al demonstrated that rat BMSC-derived exosomes (RBMSC-Exos) inhibited the progression of EMT and reduced the expression of profibrotic factor TGF-β1 potentially via attenuating Wnt/β-catenin signaling to alleviate silica-induced pulmonary fibrosis.[86] Consistently, mouse bone marrow MSC-derived microvesicles (MBMSC-MVs) suppressed LPS-induced EMT by inhibiting Wnt/β-catenin signaling and have a protective effect on early pulmonary fibrosis.[87] Recently, Zhu et al demonstrated that MSC-Exos carrying miR-30b improved IPF by targeting RUNX1 to reduce the Spred2 transcription level. Accordingly, these findings provide reliable evidence and therapeutic targets for IPF patients in clinical practice.[88]

## Adipose MSC-Derived EVs

In comparison to BMSCs, adipose-derived MSCs (ADSCs) and their accompanying EVs provide a more plentiful tissue source in both preclinical and clinical trials for IPF. Bandeira et al investigated therapeutic effects of mouse adipose MSC-derived EVs (MADSC-EVs) in a late-stage model of silicosis. These observations suggested that MADSC-EVs reduced collagen fiber content, granuloma size, and the quantity of macrophages within the granuloma and alveolar septa, hence mitigating lung fibrosis and inflammation.[89] The work from Gao et al indicated that treatment with ADSC-EVs reduced PM2.5-induced apoptosis and necrosis in type 2 alveolar epithelial cells (AEC2s), alleviating lung damage and pulmonary fibrosis in rats. Mechanistically, such EVs could exert antifibrotic activity with reduction of ROS levels and inflammation by transferring let-7d-5p to inhibit TGFBR1.[90] Administration of ADSC-EVs has been shown by Nataliya et al to reduce fibrosis progression and induce the resolution of established pulmonary fibrosis. Mechanistically, miR-29c and miR-129 delivered by ADSC-EVs contributed to targeting myofibroblasts and their progenitors, influencing their phenotype and ECM deposition.[91]

### Umbilical Cord MSC-Derived EVs

Shi et al showed that human umbilical cord MSC-derived EVs (HUMSC-EVs) with highly enriched in miR-21 and miR-23, suppressed pulmonary fibrosis and enhanced the proliferation of AECs in fibrosis mice by repressing the TGF-β signaling pathway.[92] The study of Xu et al further suggested that exosomal let-7i-5p from 3D-cultured HUMSCs blocked fibroblast activation and repaired pulmonary fibrosis via regulating TGFBR1/Smad3 signaling pathway. Endothelial-mesenchymal transition (EndMT) has been implicated in the development and progression of pulmonary fibrosis.[93] Recently, Zhao et al reported that HUMSC-Exos could increase miR-218 expression, inhibit EndMT through MeCP2/BMP2 pathway, and alleviate BLM-induced pulmonary fibrosis in mice.[94] Interestingly, recent research suggested that HUMSC-EVs exhibited antifibrotic actions in the lungs shown by decreased α-SMA, reduced collagen deposition and protection against oxidative stress via interfering with the switch of macrophages toward myofibroblasts.[95] Hou et al found that treatment with HUMSC-EVs reduced silica-induced lung inflammation and collagen deposition, thereby alleviating pulmonary fibrosis via the circPWWP2A/miR-223-3p/NLRP3 regulatory pathway.[96] In a study conducted by Zhao et al observed that miR-26a-5p from HUMSC-EVs suppressed EMT in silica-induced pulmonary fibrosis via blocking the Adam17/Notch signaling pathway.[97]

### Other Stem Cell-EVs

In addition to MSC-derived EVs mentioned above, several other types of stem cell-derived EVs have the potential for the treatment of IPF. Sun et al revealed that exosomal miR-let-7 from human menstrual blood-derived stem cells (HMenSCs) alleviated BLM-induced lung fibrosis and AECs damage through suppressing ROS levels, mtDNA damage, and NLRP3 inflammasome activation.[98] According to Yang et al, EVs of human embryonic stem cell (HESC)-derived immune and matrix regulatory cells showed therapeutic effects on attenuating pulmonary fibrosis, and improving lung function. These effects of EVs on pulmonary fibrosis were probably achieved by inhibiting EMT-induced phenotypic changes in epithelial cells, and reducing collagen levels.[99] Liu et al reported that HESC-Exos significantly reduced inflammation, eliminated collagen deposits, and restored alveolar architecture in the pulmonary fibrosis mice model by the miR-17-5p/thrombospondin-2 axis.[100] Human amnion epithelial cells (HAECs), one of the perinatal stem cells isolated from healthy term placenta, possess therapeutic potential for fibrotic diseases with pluripotent differentiation capability and immunomodulatory properties. Tan et al reported that HAEC-Exos reduced lung inflammation, improved tissue-to-airspace ratio and relieved fibrosis via increasing macrophage phagocytosis, reducing neutrophil myeloperoxidases, and suppressing T cell proliferation.[101] Furthermore, co-administration of serelaxin with HAEC-Exos demonstrated their effectiveness in treating pulmonary fibrosis, which may represent a promising treatment option for fibrosis-related diseases.[102]

Lung spheroid cells (LSCs) are therapeutic adult lung cells and express both lung epithelial and mesenchymal markers. Dinh et al found that inhaling LSC-EVs reduced both collagen deposition and myofibroblast proliferation, and restored normal alveolar structure in an IPF model, outperforming MSC-EV treatments in some cases. Importantly, specific EV miRNAs, such as miR-30a, the let-7, and miR-99 families, may contribute to relieving lung fibrosis and regeneration.[103] Induced pluripotent stem cells (iPSCs), reprogrammed from somatic cells with defined factors, possess self-renew and differentiation properties similar to embryonic stem cells. Zhou et al reported that BLM-induced pulmonary fibrosis was relieved, with less collagen deposition after treatment with iPSC-Exos. Consistently, iPSC-Exos transferred miR-302a-3p to inhibit the M2-type macrophages via targeting TET1, further mitigating pulmonary fibrosis.[104] Deer antler stem cells (DASCs) have developed as a novel type of adult MSCs. Notably, miR-let-7a and miR-let-7b from DASC-Exos reduced CCL7 expression of fibroblasts, leading to inhibiting recruitment of monocyte-derived macrophages, thus alleviating symptoms of pulmonary fibrosis.[105]

## Mature Somatic Cell-EVs

The antifibrotic and regenerative characteristics of EVs released from healthy adult lung resident cells, including bronchial epithelial cells, pulmonary VECs, and some immune cells, have attracted extensive attention in the treatment of pulmonary fibrosis. Kadota et al found that human bronchial epithelial cell-derived EVs (HBEC-EVs) attenuated BLM-induced lung fibrosis in mouse models, and have a stronger impact than MSC-EVs. They further clarified that HBEC-EVs loaded miRNAs such as miR-16, miR-26a, miR-26b, miR-141, miR-148a, and miR-200a, thereby inhibiting myofibroblast differentiation and epithelial cellular senescence via negative regulation of TGF-β-Wnt crosstalk.[106] More importantly, Feng et al discovered that

EVs from VECs and AEC2s conveyed miR-223 and miR-27b-3p, and influenced the immune balance of alveolar macrophages via targeting regulator of G protein signaling-1 (RGS1), thus ameliorating pulmonary fibrosis.[107]

## Potential of Engineered EVs as IPF Drug Delivery Systems

Given that EVs are cell-derived particles coated with robust phospholipid membrane, EVs are good carriers for various biomolecules, such as proteins, lipids, DNA, RNA, and drugs, demonstrating therapeutic potential as natural drug delivery vehicles.[110] In addition, EVs contain transmembrane and membrane-anchored proteins that can enhance endocytosis, thereby promoting drug delivery. Thus, EVs possess lower toxicity, higher stability, better capacity to cross biological barriers, and inherent targeting characteristics, compared to synthetic drug delivery systems, such as liposomes and nanoparticles.[111] Cai et al utilized ADSC-Exos as carriers for nintedanib, leading to an attractive enhancement in therapeutic efficacy. Further studies demonstrated that ADSC-Exos-Nintedanib alleviated BLM-induced pulmonary fibrosis in mice by inhibiting EndMT through regulation of TGF-β/Smad pathway and reduction of oxidative stress.[112]

In fact, the technology of EVs application for drug delivery systems is still in its infancy and needs to face many challenges.[113] More research requires to be invested in tackling these problems such as how to completely and efficiently enrich EVs, boost their capability to load drugs, modify their membrane surface to enhance specificity and stability, and what kinds of drugs can be delivered.[114] Compared to natural EVs, engineered EVs are modified in a more precise manner through biotechnological engineering, either on the EVs themselves or on the donor cells. This modification results in a longer circulation half-life and stronger targeting capabilities, reducing the systemic distribution of drugs and adverse reactions, further enhancing therapeutic effects.[115] Recently, cargo loading and surface modification are the most common engineering strategies for EVs in the therapy of IPF (Figure 3). Cargo loading can be further divided into pre-loading before EV isolation

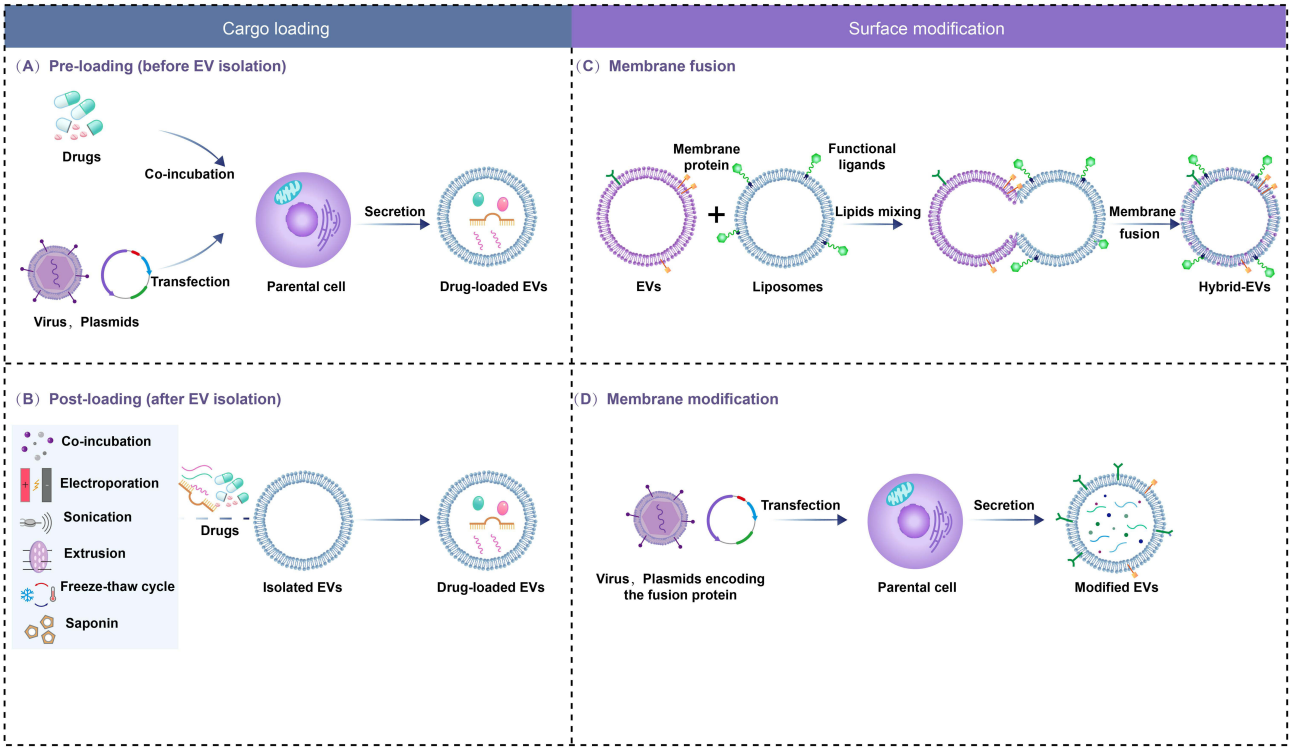



**Figure 3** Engineering strategies for EVs in the therapy of IPF. (**A**) Parental cells are incubated with drugs, encapsulating the drugs within the cells, and secreted EVs will then contain the desired drugs; viral vectors or plasmids can be used as endogenous loading methods to express genes of interest in the parental cells, thereby promoting the expression of proteins, nucleic acids, or other molecules. (**B**) Therapeutic drugs are transferred into EVs through active binding methods, such as co-incubation, electroporation, sonication, extrusion, freeze-thaw cycle, or saponin-assisted methods. (**C**) Liposome mediate membrane fusion endows EVs with specific contents and ligands. (**D**) By transducing cells with viral vectors or plasmids that fuses the gene sequence encoding the target protein or peptide with the gene sequence of EVs membrane protein, genetic engineering of the EV surface is achieved.

and post-loading after EV isolation methods. Surface modification can be achieved either by modifying the parental cells or directly modifying the isolated EVs, thus being categorized into membrane modification and membrane fusion (Table 4).

Low drug loading efficiency limits application of EVs as drug delivery vehicles. Several techniques have been conducted to enhance the loading capacity of EVs, including liposome-mediated EVs membrane fusion, which can change vesicle contents and add specific ligands. Sun et al developed a fibroblast-derived exosome and clodronate-loaded liposome (EL-CLD) hybrid system using non-specific phagocytosis inhibition and fibroblast homing properties for enhanced drug delivery to pulmonary fibrosis (Figure 4). Nintedanib encapsulated in the EL-CLD hybrid system has the potential to reduce macrophage-induced inflammatory response, and improve anti-fibrotic effects by increased pulmonary fibrotic tissue accumulation and delivery. This system has the ability to diminish hepatic uptake and increase penetration inside pulmonary fibrotic tissue, providing efficient orientation for fibroblast specific therapy.[116] Wang et al prepared liposome-exosome hybrid bionic nanovesicles encapsulated cryptotanshinone-loaded microparticles (LE/CTS) by membrane fusion method, aiming to achieve enhanced drug loading capacity and specific targeting efficiency to lung myofibroblasts through the homing effect of exosomes on the parent cells. Moreover, LE/CTS inhalation every two days

**Table 4** Anti-Fibrosis Effect of Engineered EVs in Animal Models of IPF

| Engineering Strategy | EV Source | Method | Model | Mechanism to Alleviate IPF | Ref. |
|---|---|---|---|---|---|
| cargo loading | ADSC | load nintedanib to ADSC-Exos | BLM-induced mouse | inhibit EndMT through regulation of TGF-β/Smad pathway and reduction of oxidative stress | [112] |
| cargo loading, membrane fusion | fibroblast | fuse the membrane of clodronate loaded liposome with fibroblast-Exos and load it with nintedanib | BLM-induced mouse | deplete Kupffer cells, target fibroblast, accumulate in fibrotic lung, and reduce lung fibrosis | [116] |
| cargo loading, membrane fusion | fibroblast | liposome-exosome hybrid nanovesicles encapsulated cryptotanshinone | BLM-induced rat | enhance drug loading capacity, and target lung myofibroblasts through homing ability | [117] |
| cargo loading, membrane modification | fibroblast | overexpress the fusion protein CBD-Lamp2b and miR-29 | BLM-induced mouse | repair pulmonary fibrosis by inhibiting TGF-β1/Smad3 pathway | [118] |
| membrane modification | HUMSC | transfect MSCs with a lentivirus loaded with a CD38 antigen receptor−CD8 transmembrane fragment fusion plasmid | aged mouse | elevate $NAD^+$ levels, attenuate mitochondrial dysfunction, and inhibit EMT | [119] |
| cargo loading, membrane modification | HUMSC | adenovirus infection to overexpress SARS-COV-2-S-RBD and miR-486-5p | radiation-induced mouse | inhibit Smad2 and ferroptosis, and activate Akt phosphorylation | [120] |
| cargo loading, membrane fusion | MLg2908 cell | engineered exosome membranes by cationic lipid to load siRNAs against Smad4 | BLM-induced mouse | inhibit the Smad4 expression, suppress the muscle fiber proliferation and collagen deposition | [121] |
| cargo loading, membrane modification | HEK293T cell | construct MMP19-PTGFRN-Flag-modified exosomes and siRNA-loaded exosomes | BLM-induced mouse | inhibit mitochondrial fission, restore mitochondrial function, and alleviate IPF | [122] |
| cargo loading | milk | design an inhalable milk-derived EVs encapsulating glycyrrhetinic acid system | BLM-induced mouse | relieve fibrosis and enhance lung function by inhibiting TGF-β1/Smad3 | [123] |

**Abbreviations**: EVs, extracellular vesicles; IPF, idiopathic pulmonary fibrosis; TGF-β, transforming growth factor β; AT1R, angiotensin II type 1 receptor; HOTAIRM1, HOXA transcript antisense RNA myeloid specific 1; TNC, tenascin-c; IGFBP7, insulin-like-growth-factor-binding protein 7; FBN1, fibrillin-1; COL1A2, alpha-2 collagen chain (I); COL1A1, alpha-1 collagen chain (I); LOXL1, lysyl oxidase homolog 1; Ang II, angiotensin II; TGFBR1, transforming growth factor beta receptor 1; FoxM1, forkhead box M1; Smad, small mothers against decapentaplegic; SIRT1, sirtuin 1; SIRT3, sirtuin 3; HSF1, heat shock transcription factor 1; YY1, Yin-Yang 1; ATII cells, alveolar type II cells; FAM13A, family with sequence similarity 13 member A1; STAT1, signal transducer and activator of transcription 1; BALF, bronchoalveolar lavage fluid; SFTPB, surfactant-associated protein B; ALDOA, aldolase A; HMGB1, high mobility group box protein 1; CALML5, calmodulin like 5; TLN1, talin-1; MCSP, melanoma-associated chondroitin sulphate proteoglycan; ROR1, receptor tyrosine kinase like orphan receptor 1; Wnt5a, wingless type MMTV integration site family member 5A; ILDs, interstitial lung diseases; PPF, progressive pulmonary fibrosis; HBMSC, human bone marrow mesenchymal stem cell; RBMSC, rat bone marrow mesenchymal stem cell; MBMSC, mouse bone marrow mesenchymal stem cell; MSC, mesenchymal stem cell; MADSC, mouse adipose mesenchymal stem cell; ADSC, adipose mesenchymal stem cell; HUMSC, human umbilical cord mesenchymal stem cell; HMenSC, human menstrual blood-derived stem cells; HESC, human embryonic stem cell; HAEC, human amnion epithelial cell; LSC, lung spheroid cell; iPSC, induced pluripotent stem cell; DASC, deer antler stem cell; HBEC, human bronchial epithelial cell; VEC, vascular endothelial cell; AEC2s, type 2 alveolar epithelial cell; BLM, bleomycin; i.v., intravenous; i.t., intratracheal; i.n., intranasal; i.h., inhalation; SOX4, SRY-box transcription factor 4; DKK1, Dickkopf-1; FZD6, frizzled 6; EMT, epithelial-mesenchymal transition; RUNX1, Runt-related transcription factor 1; Spred2, sprouty related EVH1 domain containing 2; ECM, extracellular matrix; AECs, alveolar epithelial cells; EndMT, endothelial-mesenchymal transition; MeCP2, methyl-CpG-binding protein 2; BMP2, bone morphogenetic protein 2; Adam17, disintegrin and metalloproteinase domain-containing protein 17; TET1, tet methylcytosine dioxygenase 1; CCL7, C-C motif chemokine 7; RGS1, regulator of G protein signaling-1; ADSC-Exos, adipose mesenchymal stem cell derived exosomes; fibroblast-Exos, fibroblast derived exosomes; CBD-Lamp2b, collagen-binding domain (CBD)-Lamp2b; SARS-COV-2-S-RBD, severe acute respiratory syndrome coronavirus-2 spike glycoprotein receptor binding domain; MMP19, matrix metalloproteinase-19; PTGFRN, prostaglandin F2 receptor negative regulator.

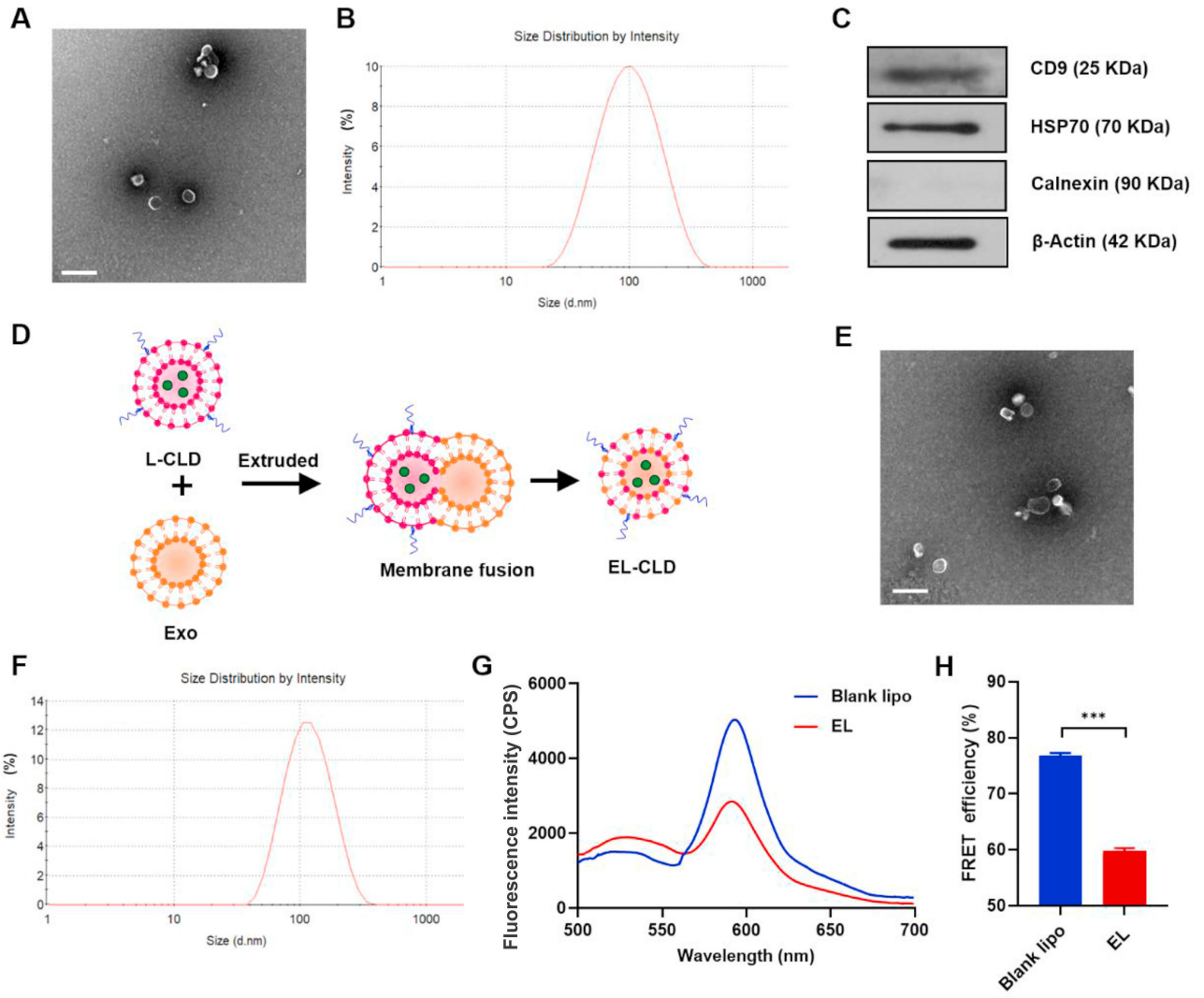



**Figure 4** Characterization of EL-CLD hybrid system. (**A**) TEM image of the exosome. Scale bar: 200 nm. (**B**) The size and distribution of L-929 cell-derived exosomes were determined by DLS. (**C**) Western blot analysis of the protein levels of CD9, HSP70, calnexin, and β-actin in the exosomes released by L-929 cells. (**D**) Diagrammatic representation of the process utilized to produce the EL-CLD hybrid, which involves membrane extrusion to hybridize exosomes with L-CLD. (**E**) TEM image of the EL-CLD hybrid. Scale bar: 200 nm. (**F**) The size and distribution of EL-CLD determined by DLS. (**G**) The FRET study conducted using fluorescent donor NBD (λem = 525 nm) and fluorescent acceptor RhB (λem = 595 nm) at an excitation wavelength of 470 nm. (**H**) Quantitative analysis of FRET efficiency of the blank liposome and EL, ***$p < 0.001$. Reprinted from *Biomaterials*, 271, Sun L, Fan M, Huang D et al. Clodronate-loaded liposomal and fibroblast-derived exosomal hybrid system for enhanced drug delivery to pulmonary fibrosis, 120761, Copyright (2021), with permission from Elsevier.[116]

exhibited similar anti-fibrotic effects, and excellent sustained drug release ability compared with daily administration of conventional microspheres and positive control drug pirfenidone, providing new strategies for the treatment of pulmonary fibrosis.[117]

To optimize EVs isolation and enrichment, Yu et al designed a kind of functional ECM biomaterial enriched with miR-29-loaded exosomes for pulmonary fibrosis treatment employing the repeated freeze-thaw technique without breaking the exosome membrane. They overexpressed miR-29 and the fusion protein collagen-binding domain (CBD)-Lamp2b (CBD-Lamp2b) in human foreskin fibroblast cells to enrich miR-29 into exosomes and entrap exosomes into ECM. This resulted in the production of a functional ECM biomaterial enriched with miR-29-loaded exosomes, which relied on the specific binding between collagen I in the ECM and CBD on the exosomal membrane. In addition, this miR-29-loaded exosome-enriched ECM suppressed TGF-β1/Smad3 signaling pathway in vitro, and efficiently repaired pulmonary fibrosis in vivo.[118]

Surface modification plays a vital role in EVs engineering, contributing to a more specific interaction with target cells or tissues, and minimizing off-target effects. Long et al employed CAR technology to construct CD38 antigen receptor membrane-modified umbilical cord MSC-EVs (CD38-ARM-MSC-EVs) by transfecting MSCs with a lentivirus loaded with a CD38 antigen receptor-CD8 transmembrane fragment fusion plasmid to target senescent AEC2s, and alleviate aging-related pulmonary fibrosis (Figure 5). Strikingly, more details indicated that CD38-ARM-MSC-EVs effectively elevated $NAD^+$ levels, attenuated mitochondrial dysfunction, and inhibited EMT, thereby mitigating multiple age-associated phenotypes and ameliorating pulmonary fibrosis in aged mice.[119] Based on the interaction between severe acute respiratory syndrome coronavirus-2 spike glycoprotein receptor binding domain (SARS-COV-2-S-RBD) and angiotensin converting enzyme 2 (ACE2), Zhang et al constructed miR-486-5p and SARS-COV-2-S-RBD-engineered MSC exosomes (miR-486-RBD-MSC-Exos) for the targeted delivery of miR-486-5p to $ACE2^+$ cells to alleviate radiation-induced pulmonary fibrosis. Adenovirus infection was employed to genetically alter exosomes to overexpress SARS-COV-2-S-RBD and miR-486-5p in MSCs. Mechanistically, miR-486-RBD-MSC-Exos exerted anti-fibrotic effects via targeted regulation of miR-486-5p-Smad2-Akt phosphorylation, and suppression of ferroptosis.[120]

Since the discovery of RNA interference, a post-transcriptional gene silencing mechanism, small interfering RNAs (siRNAs) have been widely exploited for treating diseases that are characterized by excessive gene expressions (eg, IPF).[124,125] Considering the short half-life of unmodified siRNA in systemic circulation, nanocarriers are well suited for siRNA-targeted drug delivery.[126] Compared to lipid- and polymer-based nanocarriers, bio-mimetic delivery vector EVs, have overcome barriers such as potential immunogenicity and non-specific targetability, and have recently attracted significant research interest. Lu et al designed novel EVs membrane (EM)/cationic lipid (ie, DOTAP) hybridized systems to load siRNAs against Smad4 (DOTAP/siRNA@EM), and investigated their specific delivery to pulmonary fibroblasts for treating IPF in a mouse model via pulmonary administration. The outcomes showed that, in comparison to the two

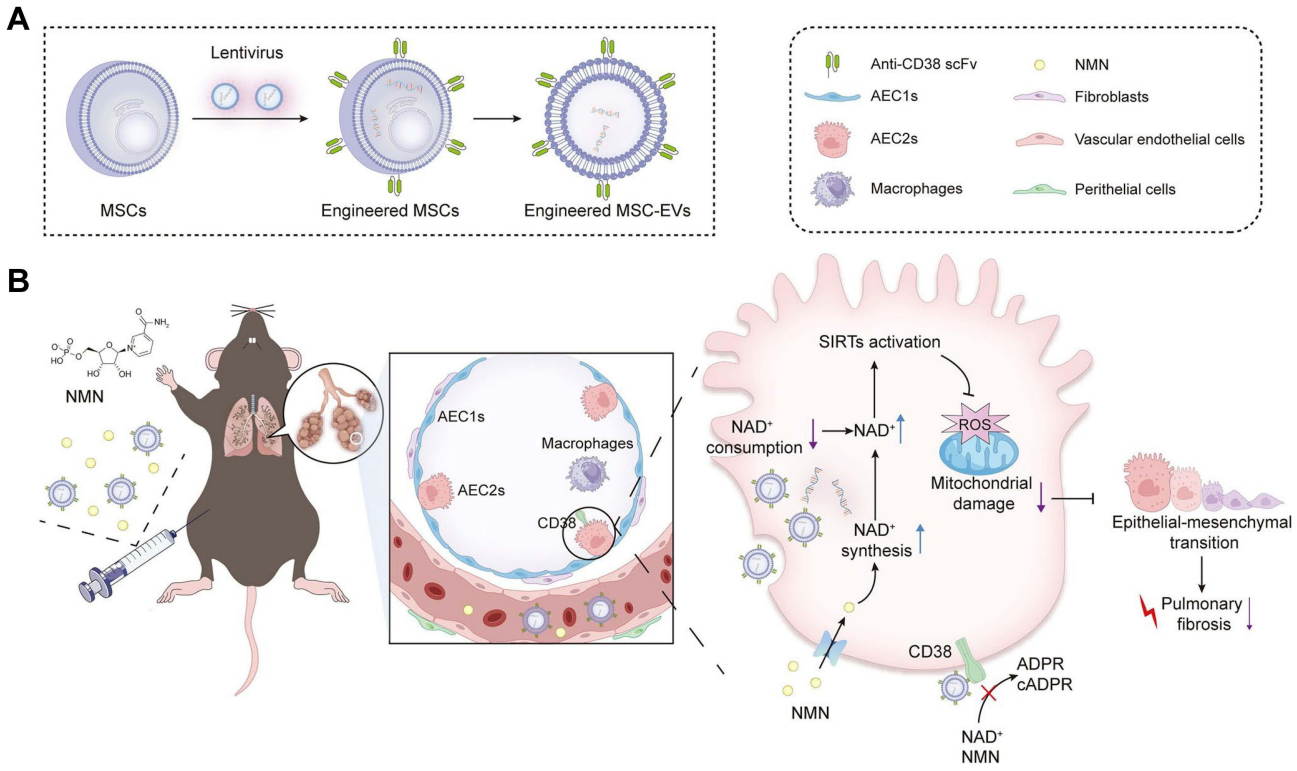



**Figure 5** CD38 antigen receptor membrane-modified MSC-EVs for pulmonary fibrosis. (**A**) Construction of CD38-ARM-MSC-EVs using lentivirus-enclosed anti-CD38 scFv plasmid transfection. (**B**) After intraperitoneal injection, CD38-ARM-MSC-EVs demonstrated a strong tissue tropism for CD38 expression. Subsequently, CD38-ARM-MSC-EVs bound to CD38 on the AEC2 surface and blocked CD38-hydrolyzed $NAD^+$ and NMN. As a result, reducing $NAD^+$ consumption and increasing $NAD^+$ synthesis helped to reverse epithelial-mesenchymal transition and alleviate lung fibrosis by activating the SIRT family and restoring mitochondrial function. Reprinted with permission from Long Y, Yang B, Lei Q et al. Targeting Senescent Alveolar Epithelial Cells Using Engineered Mesenchymal Stem Cell-Derived Extracellular Vesicles To Treat Pulmonary Fibrosis. *ACS Nano 2024*, 18:7046–7063. Copyright (2024) American Chemical Society.[119]

reference nanoscaffolds, DOTAP/siRNA@EM demonstrated a higher cellular uptake and gene silencing efficacies in mouse pulmonary fibroblasts, and could significantly inhibit the Smad4 expression with increased anti-fibrosis efficiency.[121] Subsequently, the Zhang et al group developed an engineered exosome-based formula composed of pathfinder and therapeutics (Figure 6). Exo$^{MMP19}$, a pathfinder exosome, was engineered to carry matrix metalloproteinase-19 (MMP19) on its surface, facilitating the local degradation of excessive ECM in fibrotic lung tissue. A therapeutic exosome (Exo$^{Tx}$) was designed to encapsulate siRNA for dynamin-related protein 1 (siDrp1) and display D-mannose on the outside. The formulated exosome therapy targeted Drp1-mediated mitochondrial fission, restored mitochondrial functions, and alleviated pulmonary fibrosis significantly, allowing Exo$^{MMP19}$ to break down collagen barriers and thus pave the way for Exo$^{Tx}$ delivery into profibrogenic macrophage.[122]

Efforts to engineered EVs for facilitated therapeutic efficacy against IPF have gained momentum. However, yields from EVs production are currently poor. Dietary dairy products could be a breakthrough in extending EVs manufacturing. Ran et al designed an inhalable milk-derived EVs (mEVs) encapsulating glycyrrhetinic acid (mEVs@GA) system for treating IPF. In BLM-induced IPF mice, repeated noninvasive inhalation delivery of mEVs@GA relieved fibrosis and enhanced lung function at a lower dosage than pirfenidone oral administration through significantly inhibiting TGF-β1/

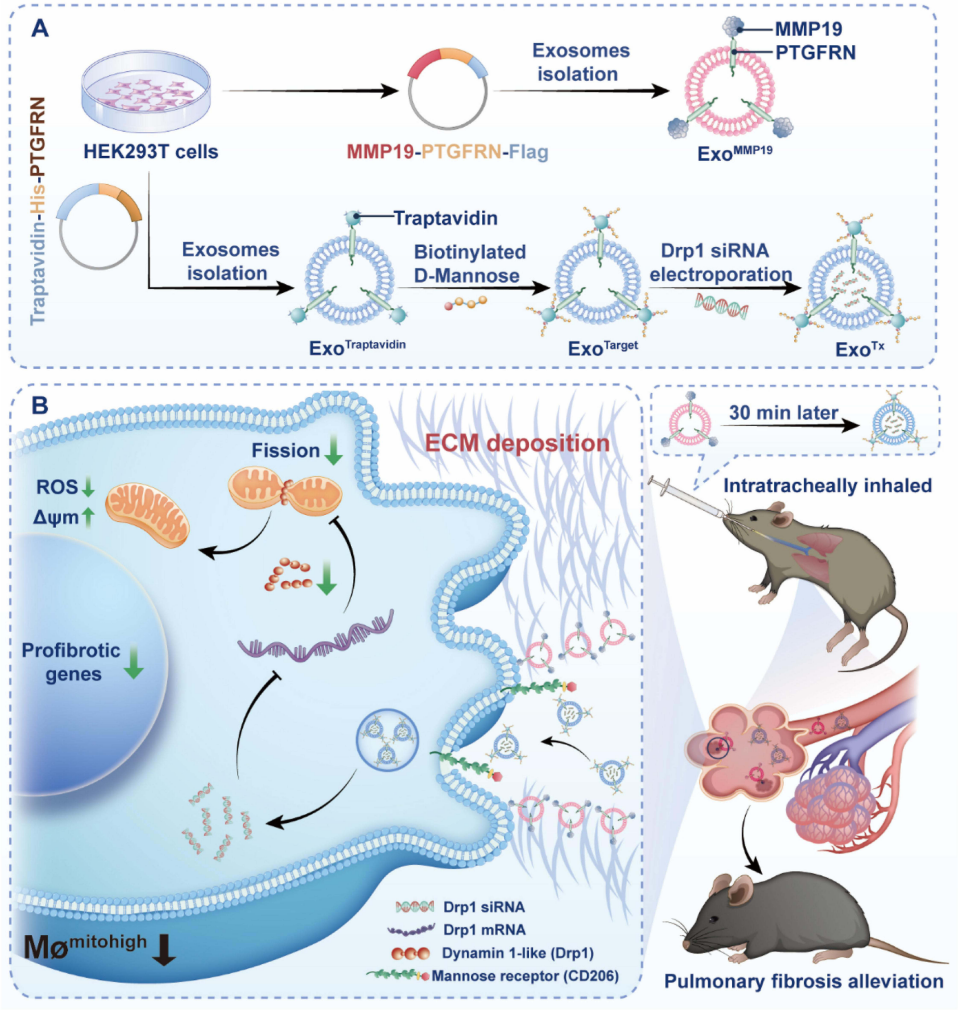



**Figure 6** Schematic illustration of the study. (**A**) Exo$^{MMP19}$, the pathfinder exosome, and Exo$^{Tx}$, the therapeutic exosome, were engineered. (**B**) As prior delivery of Exo$^{MMP19}$ degraded excessive ECM deposition, the route was paved for Exo$^{Tx}$ delivery into Mø$^{mitohigh}$, the novel profibrogenic macrophage subtype was identified. The formulated exosome treatment reduced mitochondrial fission and alleviated pulmonary fibrosis efficiently. Reprinted from Zhang W, Wan Z, Qu D, et al. Profibrogenic macrophage-targeted delivery of mitochondrial protector via exosome formula for alleviating pulmonary fibrosis. *Bioactive Materials*. 2024;32:488–501. Under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).[122]

Smad3, and the levels of IL-6, IL-1β, and TNF-α. Overall, the non-invasive pulmonary inhalation administration with mEVs could provide novel insights into the treatment of chronic pulmonary fibrosis.[123]

## Conclusions and Future Prospects

EVs have garnered significant attention in the fields of IPF therapy as well as diagnostic and prognostic biomarker development due to their inherent nature such as carrying tissue-specific information substances, low immunogenicity, and easy penetration of biological barriers. Researchers have also made encouraging breakthroughs in these areas. Recent studies have shown the essential role of EVs and their miRNAs cargo in the pathogenesis of IPF, contributing to a better understanding of the disease, as well as the identification of candidate biomarkers for the early diagnosis and prognostication of IPF. In the process where EVs participate in the development of IPF, some of the most extensively studied pathways are TGF-β, Wnt/β-catenin signaling, and cellular senescence, which are also involved in the regulation of EMT, fibroblast proliferation and differentiation, ECM deposition, and immunity in the lungs, as well as influencing cytokines such as TGF-β, TNF-α, and PDGF. Interestingly, EV cargo can be influenced by the physiological or pathological state of parent cells. EVs derived from particular cell types such as injured epithelial cells, activated fibroblasts, senescent cells, and immune cells in specific microenvironments can promote pulmonary fibrosis, while EVs derived from various MSCs and other stem cells have shown promising therapeutic potential in pulmonary fibrosis. Regarded as natural drug delivery systems, EVs show good biocompatibility and low toxicity compared to conventional synthetic nanoscale carriers. To enhance the therapeutic efficacy of EVs-based interventions, extensive exploration of engineered EVs makes them a promising next-generation nanomedicine treatment platform. In particular, EV engineering to improve scalability, cargo loading efficiency, targeted delivery capability, and stability in circulation are the major objectives for medicinal therapies of EVs in the near future.

However, there are still some important issues that need to be resolved in this field, which restrict EVs as biomarkers and therapeutics in IPF from the laboratory to clinical application. Firstly, scaling up the production of EVs to a level that can produce clinical-grade quality and quantity still poses challenges. Secondly, optimizing storage condition that can maintain the stability of EVs is also crucial, though, as EVs from different cell sources and/or EV subsets may have varied optimal storage requirements. Additionally, a more in-depth study of the relationship between the biological properties, distribution, and transport mechanisms of EVs is required to better predict and control their behavior. Significant difficulties remain in the labeling and tracking technology for EVs, particularly the methods for accurately quantifying and monitoring EVs in vivo. More importantly, the application of EVs in various scenarios such as early disease screening, diagnosis, treatment, and prognosis monitoring requires extensive clinical sample validation. Proteomics and genomics, comprehensive multi-omics research for analyzing proteins and nucleic acids, are laying the groundwork for future research and expanding the current clinical use of EVs as reliable biomarkers. Despite the possible advantages of EV-based therapy, the present obstacles hindering its advancement encompass the absence of standardized cell-based platforms for EV production, the need for standardization in isolation and characterization techniques, the identification of optimal dosing and treatment protocols, and the elucidation of long-term safety and effectiveness. Accordingly, because of the heterogeneity and complexity of EVs, the guidelines and protocols for cell culture, purification, characterization, quality control, as well as safety assessment and toxicity evaluation of EVs require further standardization and unification. It is necessary to reach a consensus in the fields of basic research, clinical application and industrial transformation and lead the standardized implementation of EVs industry. Regulators can enhance repeatability and reliability by mandating that clinical trials report parameters in accordance with internationally established guidelines. Additionally, international societies and relevant publishers should be required to upload data following publicly accessible standardized reporting protocols. Researchers must also contribute by providing adequate experimental details regarding both upstream and downstream production processes. Upon resolution and implementation of the quality control issue in this field, the progression of clinical trials will be significantly enhanced. As the clinical application of EVs in IPF gradually matures, more IPF patients will benefit.

## Abbreviations

IPF, idiopathic pulmonary fibrosis; EVs, extracellular vesicles; ILD, interstitial lung disease; ECM, extracellular matrix; BALF, bronchoalveolar lavage fluid; AECs, alveolar epithelial cells; MSCs, mesenchymal stem cells; TGF-β, transforming growth factor β; IGF-1, insulin-like growth factor 1; CTGF, connective tissue growth factor; FGF-2, fibroblast growth factor 2; PDGF,

platelet-derived growth factor; IL-13, interleukin 13; TNF-α, tumor necrosis factor-α; EMT, epithelial-mesenchymal transition; IL-1β, interleukin-1 beta; IL-6, interleukin-6; MVBs, multivesicular bodies; ILVs, intraluminal vesicles; ISEV, international society for extracellular vesicles; miRNAs, microRNAs; ESCRT, endosomal sorting complex required for transport; TSG101, tumor susceptibility gene 101 protein; ALIX, ALG-2-interacting protein X; Wnt, wingless-N-type; AT1R, angiotensin II type 1 receptor; Ang II, angiotensin II; VECs, vascular endothelial cells; ATII, alveolar type II; TNC, tenascin-c; IGFBP7, insulin-like-growth-factor-binding protein 7; FBN1, fibrillin-1; COL1A2, alpha-2 collagen chain (I); COL1A1, alpha-1 collagen chain (I); LOXL1, lysyl oxidase homolog 1; SFTPB, surfactant-associated protein B; ALDOA, aldolase A; HMGB1, high mobility group box protein 1; CALML5, calmodulin like 5; TLN1, Talin-1; MCSP, melanoma-associated chondroitin sulphate proteoglycan; ROR1, receptor tyrosine kinase like orphan receptor 1; KL-6, Krebs von den lungen-6; SP-D, surfactant protein D; ncRNAs, non-coding RNAs; HBMSC-EVs, human bone marrow MSC-derived EVs; BLM, bleomycin; RBMSC-Exos, rat BMSC-derived exosomes; MBMSC-MVs, mouse bone marrow MSC-derived microvesicles; ADSCs, adipose-derived MSCs; MADSC-EVs, mouse adipose MSC-derived EVs; AEC2s, type 2 alveolar epithelial cells; HUMSC-EVs, human umbilical cord MSC-derived EVs; EndMT, endothelial-mesenchymal transition; HMenSCs, human menstrual blood-derived stem cells; HESC, human embryonic stem cell; HAECs, human amnion epithelial cells; LSCs, lung spheroid cells; iPSCs, induced pluripotent stem cells; DASCs, deer antler stem cells; HBEC-EVs, human bronchial epithelial cell-derived EVs; RGS1, regulator of G protein signaling-1; EL-CLD, fibroblast-derived exosome and clodronate-loaded liposome; LE/CTS, liposome-exosome hybrid bionic nanovesicles encapsulated cryptotanshinone-loaded microparticles; CBD, collagen-binding domain; CD38-ARM-MSC-EVs, CD38 antigen receptor membrane-modified umbilical cord MSC-EVs; SARS-COV-2-S-RBD, severe acute respiratory syndrome coronavirus-2 spike glycoprotein receptor binding domain; ACE2, angiotensin converting enzyme 2; miR-486-RBD-MSC-Exos, miR-486-5p and SARS-COV-2-S-RBD-engineered MSC exosomes; siRNAs, small interfering RNAs; EM, EVs membrane; MMP19, matrix metalloproteinase-19; siDrp1, siRNA for dynamin-related protein 1; mEVs@GA, milk-derived EVs encapsulating glycyrrhetinic acid.

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## Disclosure

The authors declare that they have no competing interests in this work.

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