Advances in common in vitro cellular models of pulmonary fibrosis

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Abstract

The establishment of in vitro models plays a vital role in understanding and investigating pulmonary fibrosis (PF) at the cellular and molecular levels. In this paper, we conduct a literature review and provide an analysis of various cellular models used in scientific experiments, along with their applications in understanding the pathogenesis of PF. Our studies indicate that a comprehensive understanding of PF should not be based on a single cell type or organ, but on a multi-organ, multi-level, and multi-perspective approach. Primary cells demonstrate superior cell growth characteristics and gene expression profiles. However, challenges such as limited availability, difficulties in maintenance, inability for continuous propagation, and susceptibility to phenotype loss over time significantly restrict their utility in scientific research. On the other hand, replacement cell lines can be easily obtained, cultured, and continuously propagated, but their phenotypic characteristics are somewhat different compared to primary cells. In vitro co-culture models offer a more practical and precise means to elucidate the intricate interactions between cells, tissues, and organs. Therefore, when constructing pathology models of PF, researchers should carefully consider the advantages, limitations, and relevant mechanisms associated with different cell models for selection according to the research objectives.

Introduction

Pulmonary fibrosis (PF) is a chronic fibrotic interstitial lung disease characterized by progressive dyspnea and declining lung function. While pirfenidone and nintedanib have been approved for PF treatment, demonstrating efficacy in improving lung function and slowing disease progression during clinical trials, they are associated with adverse effects and tolerability issues. Furthermore, the underlying pathomechanisms of PF remain insufficiently understood(1-3).

The incomplete understanding of PF and the lack of safe and effective therapies have emerged as significant challenges in recent years. As a result, extensive research has been conducted in this field using various in vitro and in vivo models to investigate PF(4). In vitro models enable intricate investigations within cellular and tissue contexts, playing a crucial role in comprehending the pathogenic mechanisms of PF at the cellular and molecular levels(5). Processes such as cellular communication, metabolic activities, and signal transduction mechanisms are central to the pathogenesis of PF(6). At present, researchers commonly utilize PF patients, experimental murine models, genetically modified mouse strains, and in vitro-cultured cells to study PF. However, a definitive representative in vitro cell model remains elusive, hindering a comprehensive understanding of PF pathogenesis.

Therefore, the main objective of this review is to present and compare different in vitro cellular models used in the study of PF, as well as to review their application in various mechanistic studies.

A Comprehensive Review of Current In Vitro Models for PF

1. Alveolar Epithelial Cells (AECs)

AECs are classified into two main types: type I AECs (AEC1s) and type II AECs (AEC2s). AEC2s play a crucial role as the initial responders to lung injury and perform essential functions such as maintaining the alveolar environment, transitioning into AEC1s during lung injury, releasing various pro-fibrotic and chemotactic factors, and are closely associated with the occurrence and progression of PF. In contrast, AEC1s are large, flat cells with limited proliferative capacity. They are challenging to harvest and maintain in primary cultures, with AEC2s replenishing them after injury(7). Presently, there are two main types of AEC2 cells used to construct in vitro models of PF mainly include primary and replacement cell lines. Primary cells refer to human or mouse-derived primary AECs, while alternative cell lines include: (1) Human lung cancer AEC line (A549 cell line); (2) SV40 large T transgenic mouse-derived AEC line (MLE-12 cell line); and (3) Rat-derived type II AEC line (RLE-6TN cell line).

1.1 Primary AECs

The isolation and cultivation of primary AECs are crucial for current scientific experiments, providing essential insights into the cellular and molecular understanding of PF pathogenesis. Mechanisms such as cellular senescence, cell death, and endoplasmic reticulum stress play pivotal roles in AEC2 depletion and impaired self-renewal, hindering the regenerative capacity of damaged lung tissue.

Numerous pathogenesis studies of PF have utilized primary AECs to construct pathology models. For example, Lv X et al. investigated the regenerative capacity of lung alveoli by examining AEC2 senescence induced by a single dose of bleomycin (BLM) and multiple doses of BLM in a PF model(8). Liu Y et al. studied silica-treated THP-1-induced macrophages and AEC2s, discovering that ferroptosis specifically occurs in AEC2s rather than macrophages or fibroblasts during PF(9). To confirm that IL-17 drives pulmonary endoplasmic reticulum stress and apoptosis, ultimately influencing fibrosis, Cipolla E et al. used RNA isolated from normal human AECs exposed to IL-17A for 24 hours(10).

However, the application of primary cells is limited due to unreliable supply, challenges in isolation and in vitro culture, and the loss of unique phenotypes over time, which hinders in vitro study.

1.2 AEC Lines

To overcome the limitations of primary cells, cell lines are often used as substitutes for primary cells. These cell lines are typically derived from cancer tissues or obtained by inducing immortalization through retroviral transduction or transfection of primary cells. Compared to primary cells, cell lines are easier to culture with the advantages of higher proliferation rates and longer lifespans while maintaining their original phenotypes during cultivation. Currently, cell lines are commonly used in research to study mechanisms such as epithelial-mesenchymal transition (EMT), cell senescence, cell communication, and post-translational protein modifications.

The A549 cell line is a genetically mutated human non-small cell lung cancer cell line. Though A549 cells exhibit some characteristics of AEC2s, their structural and barrier properties, as well as cell phenotypes, significantly differ from those of AEC2s, and they do not express pulmonary surfactant proteins. A549 cells have been extensively utilized as an in vitro model for lung epithelial cell injury and EMT progression. For example, Lin C et al. conducted in vitro experiments by inducing A549 cells to undergo EMT through TGF-β1 stimulation, demonstrating that Valproic acid (VPA) inhibits EMT in AECs in a time- and dose-dependent manner(11).

The MLE-12 cell line is derived from SV40 large T transgenic mice and exhibits typical characteristics of cancer cells. MLE-12 cells can express surfactant proteins B and C (SP-B, SP-C), similar to AEC2s. However, like A549 cells, MLE-12 cells significantly differ from AEC2s in terms of structure, barrier properties, and cell phenotypes. Various studies have utilized MLE-12 cells in different mechanistic studies. Phosphorylation, the most widespread form of post-translational protein modification, was investigated by Pedroza M et al., who demonstrated that STAT-3 phosphorylation is involved in TGF-β and IL-6-induced injury and fibrosis in AECs and lung fibroblasts using immortalized AEC2s (MLE-12 cells)(12).

The RLE-6TN cell line is isolated from 56-day-old male F344 rats through airway perfusion using a protease

solution to separate type II AECs. These cells do not express SV40-T antigen, indicating their spontaneous immortalization. RLE-6TN cells exhibit characteristics similar to AEC2s and express several chemokines comparable to primary cultures of type II alveolar cells. Furthermore, they do not form tumors in nude mice. Zhou T et al. found that melatonin prevents cellular focal death through nrf2-triggered ROS downregulation by exposing RLE-6TN cells to different doses of Lipopolysaccharides (LPS) and Adenosine triphosphate (ATP) while incubating the injury-inducing constructs in a PF pathology model(13).

However, cell lines used as substitute models for primary cells may express phenotypes inconsistent with their main counterparts. Therefore, when using cell lines in medical research to simulate primary AT2 cells, researchers should carefully consider the limitations associated with these cell lines.

2. Bronchial Epithelial Cells

Bronchial epithelial cells serve as both physical barriers against external damage and active participants in maintaining airway structure and promoting lung tissue repair. Growing evidence suggests that abnormal responses of airway epithelial cells, including AEC2 cells, are involved in PF(14). In PF, there are also cells expressing markers of proximal airway epithelial cells found in the distal lung, such as goblet cells, basal cells, and ciliated cells(15). Through the release of cytokines and growth factors, bronchial epithelial cells induce cell differentiation, alter matrix deposition, chemotaxis, and activation, thereby enhancing lung host defense mechanisms(16). Currently, bronchial epithelial cell models, including primary cells and alternative cell lines, are commonly used to construct pathological models of PF. Primary cells consist of human primary bronchial epithelial cells (HBECs) and mouse primary bronchial epithelial cells (MBECs). Alternative cell lines include the human bronchial epithelial-like cell line (16HBE) and the human bronchial epithelial cell line (BEAS-2B).

2.1 Primary Bronchial Epithelial Cells

Primary bronchial epithelial cells exhibit the most natural characteristics of epithelial cells. Isolating and culturing these cells from lung tissue better preserves their morphology, functional features, and expression of lung epithelial lineage characteristics. Additionally, primary bronchial epithelial cells retain their potential to proliferate, migrate, and differentiate into various cell types, making them valuable models for studying the mechanisms of lung fibrosis and drug development(17). These cells are often utilized to investigate mechanisms such as EMT, autophagy, cellular senescence, and apoptosis in the pathogenesis of PF.

Interestingly, Asghar S et al. demonstrated that small extracellular vesicles (EVs) from idiopathic PF (IPF)-injured distal human bronchial epithelial cells (DHBEs) induce senescence in normal HBECs, potentially creating a feed-forward loop leading to epithelial cell senescence(18). Studies have reported that after apoptosis of AECs, bronchial epithelial cells undergo abnormal re-epithelialization and exhibit resistance to pro-apoptotic stimuli, contributing to the pathological activation of epithelial-stromal interactions. Minagawa S et al. showed that effective degradation of p21 protein through the proteasome pathway inhibits TGF-β-induced cellular senescence in HBECs, thereby alleviating PF(19).

However, isolating and culturing primary cells is relatively costly, and they share common disadvantages associated with primary cells, such as strict culture environment requirements and experimental operations.

2.2 Bronchial Epithelial Cell Lines

Due to the limited feasibility of culturing primary bronchial epithelial cells, which lose vitality after passaging, researchers often employ alternative cell lines such as the human bronchial epithelial cell lines 16HBE and BEAS-2B. Compared to primary cell models, cell lines offer advantages such as being of human origin, possessing physiological barrier characteristics, and ease of culture. Human bronchial epithelial cell lines are widely used to study the pathogenesis of PF, including EMT, post-translational protein modification, and mitochondrial autophagy.

The 16HBE cell line is a transformed human airway epithelial cell line that exhibits many characteristics of differentiated bronchial epithelial cells, including differentiated morphology and normal function. These cells

can uptake silica particles without structural changes and express proteins typically produced by epithelial cells, such as laminin and type IV collagen. Bodo M et al. found that when silica particles enter the epithelium, they alter the phenotype of bronchial cells. Using radiolabeled precursors, they studied protein synthesis, collagen, and fibronectin in 16HBE cells maintained in vitro, providing evidence that bronchial epithelial cells directly participate in the pathogenesis of lung fibrosis(20).

The BEAS-2B cell line is a continuously growing cell line derived from pathological sections of normal human bronchial epithelium, which was infected with adenovirus 12-SV40 hybrid virus to establish an immortalized cell line resembling respiratory lung epithelial cells. BEAS-2B cells retain the capacity to differentiate into squamous cells in response to serum, exhibiting traits of epithelial cells and terminal differentiation. For example, Li X et al. investigated the ability of mitochondrial autophagy in lung epithelial cells to reduce mitochondrial reactive oxygen species (ROS) accumulation and protect bronchial epithelial cells from bleomycin-induced cell death using BEAS-2B cells(21).

Cell lines are continuously growing and differentiating cell populations, and due to their genetic modifications, they possess the characteristic of immortality, resulting in their potential for unlimited growth. However, with prolonged passaging, cell lines may undergo changes in genotype and phenotype, potentially losing the characteristics of primary cells.

3. Macrophages

AMs are crucial innate and adaptive immune cells that originate from progenitor cells in the bone marrow and can either circulate in peripheral blood or migrate to different tissues. There are three major macrophage populations: tissue-resident alveolar macrophages (TR-AMs), monocyte-derived alveolar macrophages (Mo-AMs), and interstitial macrophages (IMs)(22). Understanding their role in PF is of great interest, and researchers commonly utilize primary macrophages and alternative cell lines to study macrophage behavior and function(23). Primary macrophages are often derived from AMs and mouse bone marrow-derived macrophages (BMDMs), while alternative cell lines include: (1) Human peripheral blood monocyte cells (THP-1 cell line); (2) Mouse alveolar macrophages (MH-S cell line); and (3) Mouse monocyte macrophage leukemia cells (RAW264.7 cell line).

3.1 Primary Macrophages

Recent research has highlighted the importance of primary macrophages in studying PF. These cells closely resemble macrophages found in the body and have been widely recognized and accepted by researchers. Using primary macrophages avoids variations that may occur with genetically modified cell lines, providing more stable and reliable experimental results.

Notably, there are two distinct subsets of AMs in IPF: Mo-AMs and TR-AMs. Mo-AMs produce pulmonary surfactant protein E (ApoE), which is one of the major characteristic markers distinguishing Mo-AMs from TR-AMs. Cui H et al. found by using Mo-AMs and TR-AMs as in vitro models that ApoE directly binds to type I collagen and mediates its phagocytosis leading to the alleviation of PF(24). BMDMs are terminally differentiated cells that do not proliferate and cannot be passaged; they can only be used for primary culture, and their survival time is relatively short. However, these advantages also become drawbacks when using BMDMs. The extraction process of primary cells is relatively cumbersome and operationally challenging. Freshly isolated cells are required for each experiment, which can be quite troublesome, and BMDMs need to be induced for seven days before they can be used. Boutanquoi PM et al. isolated BMDMs from trim33-floating mice and induced an in vitro model with TGF-β1 or BLM. Liang Q et al. studied how macrophages converted mechanical signals into IL-1β production by conducting experiments on primary AMs and differentiated BMDMs(25, 26).

Although monocyte cell lines have significant advantages in terms of easy accessibility compared to primary macrophages, their differentiated state means that the conclusions drawn from these experiments regarding the behavior of differentiated tissue macrophages may not always be accurate. Therefore, the cytokine response induced by monocyte cells often varies depending on the stimulation and the type of differentiated

macrophage used(27). Primary macrophages need to be collected from specific tissues through blood donation or invasive procedures such as bronchoscopy or tissue biopsy, which means that the number of primary macrophages is limited and not easily expanded in vitro.

3.2 Macrophage Cell Lines

Macrophage cell lines are frequently utilized as alternative models in scientific research to simulate macrophage functions through various degrees of monocyte cell line differentiation. These cell lines, such as monocyte-derived M1 and M2 macrophages, as well as foam cells, are commonly employed in inflammation studies. Additionally, researchers often utilize macrophage cell lines to investigate mechanisms related to inflammation, cellular autophagy, and apoptosis in PF.

The THP-1 cell line, derived from the peripheral blood of an 83-year-old male acute monocytic leukemia patient, exhibits characteristics similar to primary human monocytes regarding morphology, function, and differentiation markers. Unlike peripheral blood monocytes (PBMCs), THP-1 has a more consistent genetic background and is easier to culture, minimizing issues arising from PBMC variability and enhancing experimental repeatability. Consequently, THP-1 is widely used in various laboratories for studying immunity and inflammation. Two classic inflammation models are commonly employed for induction and differentiation experiments: (1) M1-type macrophage induction achieved by lipopolysaccharide (LPS) and IFN-γ, and (2) M2-type macrophage induction simulated by IL-4, IL-13, and macrophage colony-stimulating factor (M-CSF) to mimic late-stage tissue repair after inflammation(28). She Y et al. have used phorbol 12-myristate 13-acetate (PMA) to differentiate THP-1 cells into macrophages, examining TGF-β expression levels. Furthermore, PMA has been used to induce differentiation of THP-1 cells into M0 macrophages, which were later polarized into M1 or M2 phenotypes(29).

The MH-S cell line, derived from mouse alveolar macrophages transformed by SV40, retains numerous characteristics of alveolar macrophages, including their typical morphology and adhesive, phagocytic, esterase-positive, and peroxidase-negative properties. Macrophage autophagy, leading to mitochondrial autophagy, can reduce excessive ROS release, subsequently decreasing the secretion of inflammatory factors and ultimately alleviating CS particle-induced PF. For instance, Du S et al. utilized cigarette smoke (CS) to induce cellular autophagy in MH-S cells, constructing a model for PF(30). Similarly, Qian Q et al. exposed MH-S cells to CS to establish an in vitro silicosis cell model, aiming to determine the significance of macrophage autophagy in silicosis development(31).

The RAW264.7 cell line was established by W.C. Raschke at the Salk Institute in California using ascites from male BAB/14 mice induced with A-MuLV (Abelson murine leukemia virus) through intraperitoneal injection. This cell line serves as one of the most commonly used inflammation cell models. RAW264.7 cells exhibit a strong phagocytic capacity and, upon antigen phagocytosis, release chemotactic factors that promote differentiation, extend pseudopods, and enhance crawling ability. However, during cell culture, excessive antigen phagocytosis, poor culture conditions, or sparse cell distribution after passage may cause cells to exhibit spindle or elongated shapes, making digestion more challenging. Wang Y et al. studied various in vitro cell models, including the LPS/IL-4-induced macrophage inflammation model and the TGF-1-induced fibroblast activation model(32). Moreover, Zhang Y et al. induced RAW264.7 polarization into the M2 phenotype by overexpressing TREK-1(33).

It is important to note that due to the unique characteristics of alternative macrophage cell lines, they cannot fully replace primary macrophages in scientific research. Thus, it is essential for researchers to carefully select suitable cell models when utilizing alternative macrophage cell lines in their scientific investigations.

4. Fibroblasts

Fibroblasts were first observed by Virchow and Duvall in the mid-19th century. They are cells present in the fibrous or loose connective tissues of most mammalian organs(34). Abnormal activation of fibroblasts, mainly originating from the transformation of normal resident fibroblasts, as well as epithelial or endothelial cells in lung tissue, is responsible for many fibrotic diseases, including PF(35). Myofibroblast foci form due to

migration, proliferation, and activation of mesenchymal cells by activated AECs. Excessive secretion of extracellular matrix (ECM) proteins by myofibroblasts disrupts pulmonary homeostasis and structure, causing pulmonary interstitial matrix sclerosis and pathological matrix deposition(36, 37). In studies investigating the pathogenesis of PF, fibroblasts are classified into primary fibroblasts and alternative cell lines. Primary fibroblasts include human primary fibroblasts and mouse primary fibroblasts. Alternative cell lines consist of: (1) human embryonic lung fibroblast cell line (MRC-5 cell line), (2) lung fibroblast cell line (HLF1 cell line), and (3) mouse embryo fibroblast cell line (NIH3T3 cell line).

4.1 Primary Fibroblasts

Fibroblasts are typically undifferentiated but possess a robust proliferative capacity. In experimental research, primary fibroblasts are primarily utilized to examine the relationship between fibroblast activation, ECM deposition, and PF. Under in vitro culture conditions, primary fibroblasts maintain good division and proliferation capabilities.

The excessive activation and proliferation of lung fibroblasts contribute to extensive ECM deposition; however, the specific mechanisms driving this process remain unclear. Hence, researchers often employ fibroblasts to construct pathological models for their investigations. Li JM et al. observed that fibroblasts deficient in argininosuccinate synthase 1 (ASS1), isolated from patients with IPF, display an invasive phenotype characterized by increased migration, proliferation, and matrix deposition capacities(38). Similarly, Nguyen XX et al. found that overexpression of hIGFBP5 alters the expression of several structural and functional macromolecules of ECM in primary fibroblasts(39).

Furthermore, primary fibroblasts have a limited number of passages in culture, and as time progresses, they gradually lose their functionality and stability.

4.2 Fibroblast Cell Lines

Due to the limitations of primary cells, fibroblast cell lines have become the preferred choice for cellular-level research. They offer easy cultivation, diverse types, and substantial yield, making them valuable in studying mechanisms such as EMT, activation of signaling pathways, and fibroblast activation in PF.

MRC-5 cell line was derived from human lung tissue obtained from a 14-week-old male fetus. It exhibits a fibroblast-like morphology and maintains a normal diploid karyotype during long-term in vitro expansion. The MRC-5 cell line displays mesenchymal characteristics, including elongated and spreading morphology, highly dynamic cellular protrusions, and enhanced migration and invasion potential(40). However, due to its properties, the MRC-5 cell line is not suitable for studying a single mechanism alone. For instance, Kim HS et al. demonstrated that inhibition of the Smad2/3 and ERK pathways reduced fibroblast EMT activation using a TGF-β1-induced MRC-5 cell model(41). Nonetheless, being a diploid cell line, MRC-5 cells have limited passaging capacity, with extended passages leading to cell senescence, which presents challenges for large-scale production.

HLF cell line comprises normal fetal lung fibroblasts with a normal karyotype but has a limited lifespan. Recent evidence suggests that bioactive lipid mediators participate in the pathological processes of IPF and experimental pulmonary fibrosis. Huang LS et al. treated the HLF cell line with PF543 and found that the lung fibroblast SPHK1/S1P signaling axis regulates the expression of mtROS, FN, and α-SMA induced by BLM or TGF-β through the YAP1 pathway(42). Notably, urocanic acid, an anti-fibrotic factor in mouse lungs, impairs HLF cell line activity. Ogger PP et al. reversed IPF metabolic reprogramming by adding exogenous urocanic acid to cultured HLF cells, reducing proliferation and wound healing ability(43).

The mouse embryo fibroblast cell line (NIH3T3 cell line) was derived from NIH Swiss Mouse Embryonic Fibroblasts and is a highly contact-suppressed continuously passaged cell line. To establish a sub-strain suitable for transformation analysis, the NIH3T3 cell line underwent more than 5 rounds of sub-cloning. The NIH3T3 cell line has been utilized in mechanistic studies of PF. For example, Liu H et al. established an in vitro model by stimulating NIH3T3 cells with TGF-β1 to demonstrate that inhibition of the MEK/ERK

signaling pathway alleviates the process of PF(44). In another study, Sun W et al. investigated the relationship between miR-320a-3p/FOXM1 axis activation and lung fibrosis using a silica-induced NIH3T3 cell model(45).

It is important to note that fibroblast cell lines undergo continuous mutations during continuous cultivation, and prolonged multiple passages can lead to changes in the genotype and phenotype of the cell lines, potentially affecting experimental results.

5. Co-culture System

In contrast to single-cell culture, cell co-culture involves cultivating two or more types of cells (from the same or separate tissues) in the same culture system. Co-culture systems offer several advantages over single-cell culture as they better simulate the in vivo environment and allow for comprehensive observation of cell-cell and cell-environment interactions. In recent scientific studies, two-dimensional (2D) and three-dimensional (3D) co-culture systems have been utilized to create in vitro pathology models of PF(46).

5.1 Two-dimensional Co-culture System

In scientific research on PF, researchers mostly use 2D co-culture systems to investigate underlying mechanisms. Two main constructs are used: the direct contact co-culture model and the indirect contact co-culture model.

In the direct contact co-culture system, two or more cells are mixed in specific ratios and plated on the same interface under predetermined conditions. This system allows for the demonstration of cellular interactions. For example, Brookes et al. established a representative AEC culture model using an air-liquid interface culture with cell lines exhibiting type I (hAELVis) and type II (NCI-H441) AEC characteristics. This co-culture system, representing type I and type II lung cells, can be maintained for over 21 days, providing a promising alternative model for studying toxic compounds and treatment effects(47).

Indirect contact co-culture involves cultivating different cell types in such a way that they interact through chemokines within the culture medium. In this case, chemical signals generated in the culture environment play a significant role in regulating or influencing cell behavior, rather than direct physical contact between cells. A co-culture technique that prevents direct contact between cells is necessary for studying signaling pathways involved in macrophage M2 polarization. For instance, C. Gan et al. treated fibroblasts with a conditioned medium from M2 macrophages to determine the effects of substances released by M2 macrophages on fibroblasts(48).

Compared to cells under in vivo physiological settings, cells grown in 2D culture exhibit distinct growth patterns, shapes, and functions. In 2D culture, cells display flattened growth conditions, undergo aberrant division, and may lose their differentiated phenotypes, thereby affecting various cellular functions.

5.2 Three-dimensional Co-culture System

The 3D co-culture system provides a means to study complex cell-cell interactions within an environment that simulates living conditions. Although attempts have been made to replicate the in vivo microenvironment under 2D conditions, cells exist in a three-dimensional (3D) environment with specific spatial structures. The 3D co-culture model enables the observation of unique gene expression patterns resulting from the complex interactions between cells and the microenvironment in PF, which cannot be fully captured by other reductionist systems(49).

By utilizing cell culture systems based on biomaterials, researchers can study changes in cell crosstalk dynamics and mechanical properties of the microenvironment during the initiation of fibrosis in the distal lung. Caracena T et al. described a 3D model based on a polyethylene glycol (PEG) hydrogel in which alveolar type II (ATII) cells started differentiating into alveolar type I (ATI) cells when embedded with fibroblasts in a stiff gel, resulting in the highest fibroblast activation rate among the co-culture conditions(50).

Various cancer organoids have been developed for applications such as drug screening, radiotherapy screening, genome editing, transplantation, and oncogene identification(51). Similarly, the use of organoid models represents a novel and critical step in PF research. Tan Q et al. generated airway organoids by combining human primary bronchial epithelial cells, lung fibroblasts, and lung microvascular endothelial cells under supportive 3D culture conditions(52). Suezawa T et al. established a new in vitro PF model using alveolar organoids composed of AECs derived from human pluripotent stem cells and primary human lung fibroblasts(53).

Undoubtedly, the new three-dimensional cell culture models offer an attractive approach to overcome the limitations of traditional monolayer culture. In particular, the 3D co-culture system holds great potential for simulating physiological and pathological conditions in the human body. Moreover, the organoid model, as the latest technology for experimental research on human tissues, is still in the research stage compared to the traditional model. Its stability, reproducibility, scalability, and ability to precisely control microenvironmental conditions are all issues that need to be addressed during its development(54).

Discussion

In recent years, significant progress has been made in the study of PF and its related fibrotic mechanisms. PF is generally caused by multiple pathogenic factors, triggering a wound-healing process that leads to an inflammatory response in macrophages. Subsequently, dysregulated epithelial cells interact with various cells, including mesenchymal cells, immune cells, and endothelial cells, through multiple signaling mechanisms. This process ultimately recruits fibroblasts and activates myofibroblasts, resulting in excessive deposition of collagen-rich ECM and the formation of fibrosis(55). In this review, we focus on the application and research of in vitro models in PF, specifically exploring strategies for uncovering disease pathways, repurposing drugs, and investigating cell-cell interactions.

Throughout our literature review, we found that different cellular models have been used to study the various pathogenic aspects of PF, although there is a lack of strict uniform criteria for selecting cell types in mechanistic studies. Macrophages persist during inflammation and injury, while monocyte-derived macrophages are recruited to aid in repair. Researchers commonly employ macrophages to construct inflammatory models of PF to investigate its pathogenesis in terms of inflammation and immunity. The repair process driven by airway epithelial cells leads to epithelial hyperplasia and the emergence of abnormal basal-like cells around the lungs. Bronchial epithelial cells are commonly used to construct pathological models of lung fibrosis, focusing on the pathogenesis of tracheal, bronchial, and fine bronchial-associated lung fibrosis. Chronic damage to AECs impairs the effective repair of damaged epithelium by AT2 cells. AECs are often employed to construct pathological models of PF, primarily studying the pathogenesis of epithelial-mesenchymal transition (EMT), cellular senescence, endoplasmic reticulum stress, mitochondrial dysfunction, and telomere shortening. Abnormally activated fibroblasts and myofibroblasts contribute to excessive ECM production in the alveolar interstitial space, ultimately leading to PF. Developing in vitro models of fibroblasts is crucial for studying excessive ECM deposition and tissue remodeling in lung fibrosis. Current studies often utilize multiple cell lines for comparison, enhancing the scientific validity and accuracy of experiments compared to using a single cell line.

However, the complex and diverse pathogenesis of PF necessitates new disease models for more intricate mechanism studies. The advent of co-culture systems enables the study of intercellular relationships and cell-environment interactions in vitro. Traditional culture systems consist of single cell types isolated from naturally or complexly growing in vivo environments, resulting in simplified characteristics. However, cells require information exchange and substance metabolism within their survival microenvironment. Intercellular signal transduction plays a vital role in cellular behaviors. Understanding the crosstalk mechanisms between different cell types is significant for investigating the pathogenesis of certain diseases. Thus, comprehending PF should be based on a multi-organ, multi-level, and multi-perspective understanding, rather than solely relying on a single cell type or organ. While the research paradigm traditionally involves studying in vivo models and extrapolating findings to humans, in vitro co-culture models offer a simpler and reproducible system for researching PF, providing a more comprehensive cellular characterization compared to single-cell

models.

In conclusion, this article reviews the construction and application of in vitro models for PF and their relevance to mechanistic studies. In the future, optimizing co-culture systems in disease research, particularly in the early stages, will enable better understanding of disease mechanisms and support the development of new therapeutic strategies for PF.

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Conflict of interest disclosure

All authors report no conflict of interest.

Contributors

Weibin Qian and Xinrui Cai supervised the project, design, interpretation, manuscript revision, and final approval of the version to be submitted. Xinyue Zhang and Ziqiong Song were responsible for literature search. Shan Zhao and Yuan Huang were responsible for the documentation work. Die Li was responsible for literature collection, article writing and charting.

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Table 1: Commonly Used Cell Models for Alveolar Epithelial Cells

Cell	Representative Species	Advantages
Alveolar Epithelial Cell	Primary Alveolar Epithelial Cells (Human/Mouse)	Resemble cells' characterist
	Human Lung Cancer Alveolar Epithelial Cell Line (A549 Cell Line)	Easy to cultivate; exhibits c
	Mouse Alveolar Epithelial Cell Line (MLE-12 Cell Line)	Retains many characteristic
	Rat Alveolar Epithelial Cell Line (RLE-6TN Cell Line)	Exhibits characteristics of a

Table 2: Commonly Used Cell Models for Bronchial Epithelial Cells

Cell	**Representative Species**	**Advantages**
Bronchial Epithelial Cell	Primary Bronchial Epithelial Cells (Human/Mouse)	Most closely resemble natural e
	Human Bronchial Epithelial Cell Line (BEAS-2B Cell Line)	Human origin, physiological bar
	Human Bronchial Epithelial-Like Cell Line (16HBE Cell Line)	Human origin, physiological bar

Table 3: Commonly Used Cell Models for Macrophages

Cell	**Representative Species**	**Advantages**
Macrophage	Primary Macrophages (Human/Mouse)	Resemble cells' physiological state; preserv

Cell	Representative Species	Advantages
	Human Peripheral Blood Monocyte Cell Line (THP-1 Cell Line)	Similar morphology and functional feature
	Mouse Alveolar Macrophage Cell Line (MH-S Cell Line)	Retains many characteristics of alveolar m
	Mouse Macrophage Cell Line (RAW264.7 Cell Line)	Circular or elliptical, darker in color, firm

Table 4: Commonly Used Cell Models for Fibroblasts

Cell	Representative Species	Advantages
Fibroblast Cell Line	Primary Fibroblasts (Human/Mouse)	Minimal differentiation ability, bu
	Human Embryonic Lung Fibroblast Cell Line (MRC-5 Cell Line)	Fibroblast-like morphology; main
	Lung Fibroblast Cell Line (HFL1 Cell Line)	Normal karyotype, easy to cultiva
	Mouse Embryonic Fibroblast Cell Line (NIH3T3 Cell Line)	Continuous passaging, easy cultiv