The role of autophagy in idiopathic pulmonary fibrosis: from mechanisms to therapies

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Abstract: Idiopathic pulmonary fibrosis (IPF) is an interstitial pulmonary disease with an extremely poor prognosis. Autophagy is a fundamental intracellular process involved in maintaining cellular homeostasis and regulating cell survival. Autophagy deficiency has been shown to play an important role in the progression of pulmonary fibrosis. This review focused on the six steps of autophagy, as well as the interplay between autophagy and other seven pulmonary fibrosis related mechanisms, which include extracellular matrix deposition, myofibroblast differentiation, epithelial–mesenchymal transition, pulmonary epithelial cell dysfunction, apoptosis, TGF-β1 pathway, and the renin-angiotensin system. In addition, this review also summarized autophagy-related signaling pathways such as mTOR, MAPK, JAK2/STAT3 signaling, p65, and Keap1/Nrf2 signaling during the development of IPF. Furthermore, this review also illustrated the commonly used autophagy detection methods, the currently approved antifibrotic drugs pirfenidone and nintedanib, and several prospective compounds targeting autophagy for the treatment of IPF.

Keywords: autophagy, autophagy detection, idiopathic pulmonary fibrosis, therapies

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease with progressive onset, while its etiology is still unknown. IPF affects 0.9 to 13 out of every 100,000 individuals worldwide, and its incidence is rising year after year according to an IPF epidemiological survey. Among the numerous countries studied, South Korea, Canada, and also the United States took the top three places in terms of the incidence rate. The prevalence of IPF increases with age, with a high rate in men and individuals over 50. In the United States, the median age is 62 in patients newly diagnosed, of which 54% of them are male.

Histopathological features of IPF include spatially and temporal heterogeneous fibrosis, fibroblast and myofibroblast clusters, massive aggregation of extracellular matrix (ECM), and disorganized collagen. These pathological changes eventually lead to the disruption of normal lung tissue structure and an irreversible decline in pulmonary function. The current findings suggested that the complicated interactions between the environment and host factors lead to abnormal lung tissue repair and the development of IPF. However, the specific factors that trigger this process remain unknown. IPF presents with progressive cough, dyspnea, and some signs of reduced quality of life. The characteristic manifestation of IPF on chest high-resolution computed tomography (HRCT) is usual interstitial pneumonia (UIP), mainly distributed in both lower lungs and outer bands. At present, the pharmacological treatment for IPF recommended by evidence-based guidelines includes pirfenidone and nintedanib, both of which have pleiotropic antifibrosis effects. However, these two drugs have limited efficacy in improving quality of life and preventing disease progression, and have been associated with tolerance issues. The limitation of IPF treatment is mainly caused by its uncertain pathogenesis; therefore, clarifying its specific pathogenesis is in extremely urgent need.

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A range of molecular mechanisms, including ECM deposition, epithelial cells damage, oxidative stress, fibroblasts differentiation to myofibroblasts, immunity, and inflammation, are involved in pulmonary fibrosis. However, recent research has identified that there is insufficient autophagy during lung fibrosis, indicating that autophagy may be a significant driving factor for IPF development. Autophagy is a collection of normal physiological processes that respond to various stress, such as nutritional deficiencies, radiation exposure, and infection. Activated autophagy maintains pulmonary homeostasis in a cellular protective manner, it can selectively degrade potentially detrimental cytoplasmic substances, uneliminated proteins, and some unfavorable microorganisms, such as damaged organelles, viruses, protists, and bacteria. Interestingly, under conditions of energy deficiency and other pressures, autophagy also secretes cytoplasmic components and provides nutrients for important cellular functions. In recent years, more and more research has been committed to investigating the regulatory network of autophagy in IPF. Therefore, in this review, we mainly generalized the patterns and molecular pathways of autophagy in regulating IPF, the commonly used methods for detecting autophagy, as well as promising therapeutic strategies.

The overview of autophagy

Autophagy is a significant degradation pathway that relies on lysosomes and is evolutionarily conserved. Autophagy is classified into macro-autophagy, micro-autophagy, and chaperone-mediated autophagy according to the way substrates enter lysosomes, all of which ultimately transport cargoes to lysosomes for degradation. Macro-autophagy utilizes autophagosomes to transport cytoplasmic contents to lysosomes. Autophagosomes are vesicles formed by intracellular degenerated or damaged organelles surrounded by a bilayer membrane, which subsequently fuses with lysosomes to form autolysosomes and degrades the contained substances. In micro-autophagy, the lysosomal or vesicular endosomes directly invaginate and then wrap and degrade the intracellular material. On the contrary, chaperone-mediated autophagy is highly selective, the substrate proteins recognized by molecular chaperone bind to lysosomal-associated membrane protein 2A (LAMP-2A) for translocation across lysosomal membrane, leading to its unfolding and degradation. Even if these three kinds of autophagy have distinct pathways of occurrence, they all function critically in removing damaged substances and responding to external stimuli. Compared with micro-autophagy and chaperone-mediated autophagy, macro-autophagy has been studied in great detail. Therefore, herein we refer to macro-autophagy simply as ‘autophagy’.

The process of autophagy includes initiation, nucleation, elongation, maturation, fusion, and degradation, which is strictly controlled by the coordinated activity of diverse regulatory components (Figure 1). In mammalian cells, the initiation step is the induction of autophagosome formation in response to autophagy activation signals. The endoplasmic reticulum (ER)-associated structures called omegasome may be the starting site for mammals. The process is regulated by the Unc-51-like kinase 1 (ULK1) or ULK2 complex, which is made up of ULK1/2, focal adhesion kinase-family-interacting protein of 200 kDa (FIP200), autophagy-related 13 (ATG13), and ATG101. Several signals can act on various sites of the ULK complex to regulate its autophagic activity. In the case of adequate nutrition, the ULK1 complex associates with the mammalian target of rapamycin complex 1 (mTORC1) and then phosphorylates the Ser758 or Ser757 sites of ULK1, causing the inhibition of autophagic activity via suppressing ULK1 catalytic activity. Equally, mTORC1 can phosphorylate ATG13 and inhibit the process of the ULK1 complex migrating to the autophagy initiation sites. On the contrary, 5-AMP-activated protein kinase (AMPK) can stimulate ULK complex to deliver stress signals for autophagosome generation during energy scarcity. However, except for AMPK and mTORC1, many other signals can also regulate the ULK complex activity. The nucleation formation is the immediate next step. During this process, the vacuolar protein sorting 34 (VPS34) complex would be stimulated by the activation of ULK1 complex, which is engaged in vesicle nucleation and consists of VPS34, VPS15, ATG14, AMBRA, and Beclin-1. The nucleation formation is the immediate next step. During this process, the vacuolar protein sorting 34 (VPS34) complex would be stimulated by the activation of ULK1 complex, which is engaged in vesicle nucleation and consists of VPS34, VPS15, ATG14, AMBRA, and Beclin-1.

In the subsequent phase of elongation, the phagophore keeps expanding and engulfing the cytoplasm and its content. The extension of phagophores mainly depends on two ubiquitination complex systems. The first ubiquitination system is correlated with the formation of ATG12-ATG5-ATG16 complex. ATG12 binds to ATG5 in a covalent manner to form a complex
through a ubiquitin-like (UBL) response that demands ATG7 and ATG10. Then ATG16 L binds to ATG12-ATG5 conjugate noncovalently and dimerizes to compose ATG12-ATG5-ATG16 L complex, which binds to the phagophore membrane then dissociates after autophagosome completion.49–53 Another UBL system involving phagophore expansion is the ATG8/LC3 system. Light Chain 3 (LC3) in mammals is encoded by homologues of the ATG8 gene, which serves as a pivotal indicator of the level of cellular autophagy.49,52 During the maturation and fusion steps, the phagophore is completely enclosed, which contributes to the formation of round or elliptical autophagosomes encapsulated by bilayer membranes. This process is promoted by autophagy receptor or adaptive proteins such as p62, acrylonitrile-butadiene rubber 127 (NBR127), autophagy-linked FYVE protein 28 (Alfy28), and optineurin. Following this, the mature autophagosome transports cargoes to the lysosome through microtubules, which subsequently enter the lysosome to form an autophagic lysosome.54–57 In the final step of degradation, the endosomal portion of autophagosomes would be degraded along with its encapsulated contents and then the components of which are released into the cytoplasm for cycling by lysosomal membrane permeases.58

**The role of autophagy in IPF**

A growing number of evidence have verified that both autophagic stream and autophagic function are inhibited in the lung tissues of IPF, as manifested by a decline in autophagosomes,
the inability of fusion of autophagosomes with lysosomes, elevated expression of intracellular p62, and ubiquitinated proteins.59 This phenomenon reveals that deficient autophagy is closely associated with the pathogenesis of IPF. The next step is to figure out how the abnormality of autophagic process results in the occurrence of pulmonary fibrosis (Figure 2).

**Regulation of ECM deposition**

Loss of balance between ECM production and degradation can lead to massive aggregation of ECM, which in turn promotes tissue fibrosis progression, the most significant pathological change during pulmonary fibrosis.60,61 Collagen I and III are essential elements of human connective tissues, which are secreted by the activated myofibroblasts. However, Type I collagen is mainly associated with the pathogenesis of severe cases of IPF, while type III collagen is primarily accumulated in mildly fibrotic lung tissues. During pulmonary fibrosis, the critical functions of autophagy for degrading pathogens, misfolded proteins, and ECMs are impaired.62 The research found that Beclin1-deficient mice showed an increase in type I collagen and ECM deposition.63,64 The study found that autophagy induced by etoposide-induced protein 2.4 (Ei24) can inhibit the deposition of ECM in a mouse model of pulmonary fibrosis. While the suppression of autophagic activity by 3-methyladenine (3-MA) promotes the production of ECM protein, which is manifested by increased levels of collagen I and fibronectin.24 The above results indicate that autophagy is a significant channel for intracellular collagen degradation. Inhibiting autophagic activity can contribute to the failure of collagen clearance and ECM deposition, thus promoting the development of IPF.

**Regulation of myofibroblast differentiation**

Studies have found that fibroblast infiltration and myofibroblast activation can lead to pathological changes in fibrosis, which is a critical step in the generation of pulmonary fibrosis.65 Knockdown of Beclin1 or LC3B promoted transforming growth factor-β (TGF-β)-mediated myofibroblasts differentiation, and increased the expression of fibronectin and alpha-smooth muscle actin (α-SMA) in myofibroblasts. Another study collected primary
lung fibroblasts from patients with IPF to detect changes in autophagy activity, and they found that under starvation conditions, p62 expression was reduced in fibroblasts, while LC3B-II levels were markedly increased, and the cells exhibited distinct features of myofibroblast activation. However, the knockdown of ATG7, or autophagy inhibitors, can inhibit starvation-induced myofibroblast activation. In addition, a cellular assay demonstrated that activation of mTORC1 in MRC-5 cells by rapamycin can downregulate the levels of myofibroblast markers α-SMA and fibronectin. As described previously, defective autophagy can give rise to fibroblasts differentiation into myofibroblasts during the generation of pulmonary fibrosis. The lung architecture will then be remodeled by fibroblasts and myofibroblasts through the production of matrix and media, as well as metalloproteinase secretion.

In addition to fibroblasts, there are several other cell types that can transdifferentiate into myofibroblasts and participate in the pulmonary fibrosis process. The research found that markers of myofibroblasts, vimentin, and α-SMA, were detected in alveolar macrophages in response to TGF-β stimulation. Furthermore, the researchers demonstrated that myofibroblasts were mainly differentiated from M2-type macrophages. In vivo rat experiments have also confirmed the occurrence of M2-type macrophage-myofibroblast, leading to pulmonary fibrosis. Several experiments have shown that alveolar epithelial and endothelial cell lines can express the myofibroblast phenotype through mesenchymal transformation. Recognizing the origin of myofibroblasts is essential for finding effective targets to suppress fibrosis.

**Regulation of epithelial–mesenchymal transition (EMT)**

Another significant pathogenesis of IPF is the development of EMT in alveolar epithelial cells, which is manifested by a decline in cell adhesion molecules, the formation of vimentin-dominated cytoskeleton, and morphological characteristics of mesenchymal cells. Alveolar epithelial cells that undergo EMT lose cell polarity and connectivity with basement membranes, and in the presence of tissue damage, they can be converted into fibroblasts to repair the damage. Nevertheless, if the inflammatory reaction continues to persist, the EMT process may continue and ultimately promote the process of pulmonary fibrosis. Inhibition of autophagy treated with Bafilomycin-A1 leads to EMT, as demonstrated by a decline in E-cadherin, a rise in vimentin, and upregulation of EMT transcription factor Snail2. Furthermore, a broad range of studies has revealed that in the presence of blocked autophagic flux and impaired autophagic function, epithelial cells cannot eliminate a great number of misfolded proteins, and can cause the accumulation of some nuclear factors, which can induce the occurrence of EMT and chronic inflammation. Leptin inhibits autophagic activity in A549 cells through PI3K/AKT/mTOR signaling, thereby accelerating the EMT process. Inhibition of autophagy activity has been verified to participate in EMT during the development of pulmonary fibrosis. At present, most studies related to EMT focus on tumor cells, and the relationship between EMT and pulmonary fibrosis needs to be further explored.

**Regulation of lung epithelial cell dysfunction**

Epithelial cells are known to secrete not only anti-fibrotic and anti-inflammatory mediators but also profibrotic molecules that participate in the generation of pulmonary fibrosis. It has been hypothesized that epithelial cell stress promotes fibrosis by causing chronic or persistent lung injury. Therefore, the relationship between the autophagy formation in epithelial cells and fibrosis becomes a research hotspot. The research demonstrated that several essential genes in the autophagic pathway, including ATG14 and Beclin1, were downregulated in the lung epithelial cells treated with profibrotic factor IL-17, that is mostly released by neutrophils. It has been speculated that IL-17-induced attenuation of autophagy contributes to the promotion of fibrogenesis and may inhibit the collagen degradation pathway. Except for IL-17, several bioactive lipids like lysophosphatidic acid also have a role in inhibiting autophagy and promoting fibrogenesis. In addition, it has been demonstrated that mice with knockout of ATG4b exhibited more pronounced apoptosis of bronchial and alveolar epithelial cells at day 7 post bleomycin treatment, along with a stronger inflammatory response, resulting in more severe fibrosis and collagen accumulation. Likewise, in the case of conditional knockout of tuberous sclerosis-1 (TSC1) gene in mouse epithelial cells, the mice appear to have a higher risk of fibrosis caused by bleomycin, while rapamycin or chloroquine, that induce autophagy,
could reverse this phenomenon. Therefore, insufficient autophagy leads to epithelial cell dysfunction and subsequent pulmonary fibrosis, while activating autphagic flux can enhance the repairability of epithelial cells and attenuate pulmonary fibrosis. The exact molecular mechanism on how autophagy formation is regulated and its effect on epithelial cell function remains unclear and needs to be explored in future research.

Regulation of apoptosis
Fibroblasts and myofibroblasts have been discovered to have high anti-apoptotic properties in IPF. Beclin1 is downregulated in pulmonary fibroblasts from IPF patients, whereas the anti-apoptotic protein Bcl-2 is upregulated. Apoptosis of alveolar and bronchial epithelial cells in mice deficient in the autophagy-related gene ATG4b was increased after bleomycin treatment for 7 days. At the same time, it was also observed in pulmonary fibroblasts of IPF patients that when the mTOR pathway was continuously activated, its anti-apoptotic ability was significantly enhanced, and when the expressions of forkhead box-class O3a (FOXO3a) and LC3B were reduced, the type I collagen matrix-mediated apoptosis reduced. However, fibroblasts can recover the sensitivity to collagen matrix-induced apoptosis upon restoration of FOXO3a or LC3B expression. Some studies found that aberrantly activated human chromosome 10 deleted phosphatase and tensin homolog (PTEN)/AKT/mTOR signaling can induce autophagy in myofibroblasts by regulation of collagen synthesis, which leads to cell survival. Therefore, autophagy may reduce fibroblast apoptosis through the mTOR pathway, thereby increasing its anti-apoptotic ability. Currently, the mechanism regarding how autophagy is involved in apoptosis remains obscure and further research is needed to improve the understanding of the detailed relationship.

Regulation of TGF-β1 signaling pathways
TGF-β1 is capable of activating the TGF-β1/Smad and non-Smad signaling pathways, causing the activation of myofibroblast and excessive aggregation of ECM, which contributes to pulmonary fibrosis in the progression of IPF patients. TGF-β1 is a major contributor to fibrosis. Multiple autophagy genes including PARK, PINK1, p62, aminobutyric acid receptor-related protein, ATG4c, ATG5, ATG7, ATG16l1, ATG16l2, and ubiquitin-like kinase 2, are associated with the regulation of TGF-β1. Overexpression of TGF-β1 could induce the generation of pulmonary fibrosis in animal models. In addition, TGF-β can induce MRC-5 cell differentiation and increase the expression of fibronectin, collagen I, p62, and α-SMA, and also activate mTOR pathway, reducing the level of LC3B instead. Silencing of LC3 and ATG5 genes increased type I collagen and α-SMA expression, while TGF-β treatment could further increase their expression levels. In addition, TGF-β treatment also promoted differentiation of alveolar macrophages in mice, and autophagic activity could enhance this effect. TGF-β1 has been shown to reduce the number of autophagic vesicles and inhibit autophagic gene expression in normal human lung fibroblasts (NHLFs).

Collectively, autophagy participates in conjunction with TGF-β1 signaling pathways in lung fibrosis.

Regulation of angiotensin
The renin-angiotensin (Ang) system is composed of two major axons, which act as a critical part in lung fibrosis. Abnormality in AngII is related to several respiratory diseases. In addition, Ang-(1~7) performs antagonistic effects on AngII by acting on its specific receptor Max. AngII is a key factor in inducing collagen synthesis, autophagy occurrence, and collagen degradation in pulmonary fibrosis. The evidence shows that autophagy activity not only increases reactive oxygen species caused by AngII but also activates nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome via redox. Ang-(1~7) attenuates the impairment of autophagy caused by an increase in reactive oxygen species and ameliorates pulmonary fibrosis associated with cigarette smoking. Furthermore, overexpression of angiotensin-converting enzyme 2 (ACE2) enhances autophagy and attenuates collagen deposition in the lung tissue. At present, the reports regarding the interactions between autophagy and Ang in the occurrence and progression of IPF remain limited. More and more studies are needed.

Underlying signaling pathways of autophagy involved in IPF
Substantial studies have rectified that several signaling pathways could cause pathological alterations in IPF through regulation of cell differentiation and migration. Therefore, in order to discover
pivotal signaling targets in IPF pathologies and improve the integrated signaling pathway spectrum of IPF, it is necessary to figure out how these signaling pathways are involved in these processes (Figure 3).

**mTOR signaling pathway**

A growing body of research indicates that mTOR-mediated pathways may play indispensable roles in facilitating IPF. mTOR is a serine-threonine kinase that has two distinct forms of complex: mTORC1 and mTORC2. mTORC1 mainly modulates cell growth and autophagy in an unfavorable environment, thereby altering the proliferation and viability of fibroblasts. In contrast, mTORC2 acts mostly on cytoskeletal protein construction and cell survival. In addition, mTOR can recognize various intracellular signaling molecules and inhibit autophagy via suppression of the phosphorylation state of ATG1/ULK1 protease complex.

**PI3K/akt/mTOR signaling.** The PI3K/akt/mTOR pathway is a widely studied signal axis that is correlated with autophagic regulation. The studies showed that the suppression of PI3K/akt/mTOR signaling alleviated pulmonary fibrosis in bleomycin-induced pulmonary fibrosis animal models. Phosphatidylinositol 3-kinase (PI3K) is a network of lipid kinases that is participated in numerous processes, which can activate the downstream protein kinase Akt. There are three isoforms of Akt: Akt1, Akt2, and Akt3. Research on pulmonary fibrosis has focused on isoforms of Akt1 and Akt2, as Akt3 is predominantly expressed in brain tissue. PI3K activates Akt by phosphorylating threonine (Thr308) and serine (serine 473), and then Akt signals to several downstream effectors including mTOR. Phosphorylated-mTOR (p-mTOR) can phosphorylate downstream effector molecules such as ribosomal protein S6 kinase 1, thus promoting the production of protein and inhibiting autophagy.

The activity of mTOR is enhanced in IPF fibroblasts incubated on collagen I through Akt activation, resulting in a proliferative and anti-apoptotic fibroblast phenotype by changing autophagic activity. Long-term treatment with paraquat (PQ) can stimulate the PI3K/ AKT signaling pathway, thereby increasing mTOR activation, reducing the expression of Beclin1 and LC3-II expression. Ultimately, the autophagic activity was inhibited and the process of pulmonary fibrosis induced by PQ was aggregated as well.

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**Figure 3.** Autophagy-related molecular pathways in IPF. Six autophagy pathways are summarized in this figure: PI3K/Akt/mTOR pathway, AMPK/mTOR/ULK1 signaling, p38MAPK/eEF2 K/eEF2 signaling, JNK signaling, JAK2/STAT3 signaling, p65, and Keap1/Nrf2 signaling. These six autophagic pathways are involved in the autophagic process through cascade reactions.
autophagy mediated by PI3K/AKT signaling was significantly reversed after LY294002 treatment, as evidenced by decreased levels of phosphorylated S6K protein, a downstream target of mTOR signaling. In addition, the PI3K/AKT/mTOR signal axis can be induced and activated by lipopolysaccharide (LPS), which is manifested by the elevated expression of p-mTOR and p-Akt. The activation of this pathway would further upregulate p62 expression and reduce the number of autophagic vesicles, which would inhibit autophagy in lung fibroblast. Collectively, the numerous findings above show that studying the specific regulatory network of PI3K/AKT/mTOR signaling could provide an effective target for IPF management.

AMPK/mTOR/ULK1 signaling. AMPK is able to diminish ATP consumption by inhibiting anabolism and stimulate metabolism to promote ATP production, which functions as a significant regulator in the metabolic process. It was found that in case of hunger and energy deprivation, activated AMPK directly phosphorylates the Thr1227 and Ser1345 sites of the mTOR upstream regulator TSC2, and the Ser722 and Ser792 sites of the mTORC1 subunit PAPTOR. These two phosphorylation processes inhibit the action of mTOR, thus attenuating the inhibitory phosphorylation effect on ULK1 and activating autophagy. The silica-induced pulmonary fibrosis could be inhibited by metformin by activation of autophagy through AMPK/mTOR pathway, as demonstrated by the increased levels of phosphorylated AMPK, LC3B, and Beclin1, and decreased p-mTOR and p62 expression.

Except for phosphorylating ULK1, AMPK can also phosphorylate other key elements in autophagic pathways, such as Ser91 and Ser94 on Beclin1, Ser761 on ATG9, and so forth. However, some AMPK phosphorylation sites mentioned above have not been clarified. Therefore, the regulatory role of AMPK and ULK1 on autophagy and its relationship with lung fibrosis remains to be explored in future studies.

Mitogen-activated protein kinase (MAPK) signaling pathway

MAPK is a key transmitter of signal transduction, which is activated by various external stimulants. MAPKs consist of p38MAPK, c-Jun N-terminal kinase (JNK)1/2/3, extracellular signal-regulated kinase (ERK)1/2, ERK7/8, ERK3/4, and ERK5/BMK1, which exerts a signal transduction role in the occurrence of autophagy and other metabolic activities.

P38MAPK/eEF2K/eEF2 signaling. p38MAPK has been shown to have four isoforms in mammals, which have different tissue-specific distributions and can be coupled to different upstream kinases. The p38MAPK pathway is a critical regulator of autophagic process. Eukaryotic elongation factor-2 (eEF2) kinase (eEF2K) is a well-conserved protein kinase that is encoded by genes in the calcium- or calmodulin-mediated pathway. Protein eEF2K participates in regulating the process of protein synthesis through the control of peptide chain elongation. Once activated by the upstream p38MAPK, eEF2K would phosphorylate the Thr56 site of its sole substrate eEF2, making it unable to bind to the ribosome and thus inhibiting the function of eEF2. Recent studies have shown that inhibiting eEF2K promotes myofibroblast proliferation and differentiation through the p38MAPK signaling pathway. Furthermore, the inhibition of myofibroblast autophagy could be aggravated by inactivating eEF2K, which facilitates the formation of myofibroblasts and subsequently accelerates the procedure of lung fibrosis. Overall, more extensive studies of p38MAPK/eEF2K/eEF2 signaling are needed to find more effective treatments for IPF.

JNK signaling. The JNK family is a key molecule in signaling transduction initiated by diverse stimuli during cellular stress reactions. Previous research has shown that the JNK signaling acts as a key player in the process of autophagy induced by multiple stimulations, such as hypoxia, infection, and DNA damage. JNK activation regulates autophagy mainly by promoting the phosphorylation of Bcl-2/Bcl-XL. Beclin-1 is a protein containing the pro-autophagy BH3 domain, and its binding to Bcl-2 can be disturbed by phosphorylation of Bcl-2 mediated by JNK. Research data has suggested that JNK may upregulate the expression of autophagy gene Beclin-1 by regulating the phosphorylated state of Bcl-2, thereby enhancing autophagy. In addition, JNK also leads to the upregulation of diastasis of the rectus abdominis (DRAM). DRAM regulates autophagosome-lysosome fusion, which can promote the production and accumulation of autophagic phagosomes. One study found that
JNK activation promoted the initiation of autophagy and alleviated the progression of liver fibrosis in a mouse model. Nevertheless, the current research concerning JNK and autophagy in IPF is still limited. Broadening the understanding of their interactions would provide new perspectives on the treatment of IPF patients.

**JAK2/STAT3 signaling**

Janus kinases (JAK) is a family of intracellular non-receptor type protein tyrosine kinase, and four members have been found, namely, JAK1, JAK2, JAK3, and TYK2. It is a key part of signal transduction initiated by multiple receptor molecules. Signal transducer and activator of transcription (STAT) is a unique group of proteins that can bind to DNA, including seven structurally and functionally related proteins, which can respond to a variety of extracellular cytokine and growth factor signals. Cytokine and growth factor can phosphorylate and activate JAK after receptor binding, which can phosphorylate tyrosine residues of downstream target proteins, recruit and phosphorylate the transcription factor STAT. Once activated, STAT dimerizes and enters the nucleus to combine with target genes, regulating the transcription of downstream genes and the process of cell proliferation, differentiation, and apoptosis. Among these JAK/STAT isoforms, it was found that JAK2/STAT3 pathway was mainly involved in the pulmonary fibrosis process. The studies have indicated that JAK2 and STAT3 play independent roles in the process of autophagy and aging. Compared with the inhibition of either protein alone, the dual inhibition of JAK2 and STAT3 can lead to higher levels of autophagy. Nevertheless, the independent mechanisms of JAK2 and STAT3 causing pulmonary fibrosis remain unclear, and the potential mechanisms of the synergistic effect of double inhibition have yet to be studied in the future.

**p65 and Keap1/Nrf2 signaling**

p65 is a subunit of nuclear factor (NF)-κB, and its expression level was elevated in lung fibrosis caused by lipopolysaccharide and TGF-β1. Upregulating p65 expression could reverse PQ-induced suppression of autophagic flux and progression of fibrosis in lung tissues. In addition, p65 can bind to Kelch-like ECH-associated protein 1 (Keap1) to induce the production of nuclear factor erythroid-derived 2-like 2 (Nrf2). Nrf2 knockout can reverse the protective effects of p65 in pulmonary fibrosis, and reduce autophagy gene expression. Furthermore, the study has found that p62 could also competitively binds to Keap1, resulting in the release of Nrf2. As a key marker for autophagy, the accumulation of p62 indicates that autophagy is impaired. Similarly, Nrf2 promotes autophagy by activating the positive feedback loop of the downstream target gene p62. In conclusion, both P65 and P62 can regulate autophagic activity in pulmonary fibrosis through the Keap1/Nrf2 signaling pathway. However, studies on the Keap1/Nrf2 signaling axis and its relationship with pulmonary fibrosis are still limited, and further studies in this area are needed in the future.

**Routine methods monitoring autophagy after IPF**

It has been widely established that inadequate autophagy causes the generation of pulmonary fibrosis in IPF. Hence, observing the status and activity of autophagy is quite necessary to investigating autophagic function, which facilitates autophagy-targeted IPF therapy. A large number of methods for detecting autophagy have their own merits. However, one single autophagy detection method is unable to precisely identify this process. In practical studies, to precisely illustrate the process of autophagy, it might be necessary for the researchers to use complementary approaches.

**LC3 detection**

Western analysis. It is well known that LC3 is a specific marker for autophagy. Under normal physiological conditions, LC3 level in mammalian cells is usually remained within a certain range. The mammalian LC3 gene has a high genetic identity of 94%, reflecting the evolutionary conservation of autophagy. When autophagy is formed, cytoplasmic LC3-I cleaves off a small segment of polypeptides and subsequently binds to phosphatidylethanolamine (PE) to transform into membrane-bound LC3-II. Therefore, the total quantitative changes of LC3 merely represent the transformation between LC3-I and LC3-II, and detecting LC3-II or LC3-I alone is unable to reflect the status of autophagic activity. In order to ascertain the authentic activity of autophagy, it is of great necessity to detect the dynamic change of these two isoforms. Usually, converting
LC3-I to LC3-II or elevated LC3-II expression represents activated autophagy, whereas the declined LC3-II content indicates that autophagy is inhibited. Intriguingly, blocking of autophagic activity can contribute to a failure in converting LC3-I to LC3-II, resulting in a decline in LC3-II levels. Besides, the overactivation of autophagic flux can also reduce LC3-II content according to the removal of LC3B-II by autophagic lysosomes. Both of the abovementioned situations can lead to similar results in Western blot, but they represent unequal biological ending points. Hence, clarifying the relationship between LC3 protein content and different autophagy states is crucial to ensure a proper assessment of autophagy activation. To distinguish between these two possible conditions, it is advisable to utilize lysosome-dependent degradation inhibitors, such as Bafilomycin A1 and pepstatin A, which can prevent autophagosome-to-autolysosome fusion and block the proteolytic activity of autophagosome-degrading enzymes.

Fluorescence microscopy detection. Except for the Western blot assay, the contents of LC3 can also be detected by fluorescence microscopy. The antibodies against endogenous LC3 or LC3-expressing plasmid tagged with green fluorescent protein (GFP) can be used to detect puncta via immunofluorescence microscopy. Intracellular aggregation of GFP-LC3B under fluorescence microscopy is considered as an indicator of activated autophagic flux. Unlike the soluble cytosolic LC3-I, the membrane-bound LC3-II protein can bind to the outer membrane of autophagosome. Thus, when autophagy is formed, the GFP-LC3 fusion proteins appear as multiple bright green fluorescent spots under fluorescence microscopy, while LC3-I shows only diffuse fluorescence. Each spot represents one autophagosome, so counting the amounts of GFP-LC3 spots per cell can help assess the autophagic activity. In addition, the utilization of tandem fluorescent-labeled LC3 expression vectors is also a proper method to detect autophagy flux by LC3 puncta. For instance, the utilization of mRFP/mCherry-GFP-LC3 tandem fluorescent proteins in cellular experiments could accurately determine the autophagic activity through simultaneously observing changes in fluorescence intensity.

Flow cytometry. The flow cytometry can determine the autophagy activity as well. In the state of activated autophagy, the intracellular LC3B-I convert to membrane-bound LC3B-II. As the activation of autophagy continues, LC3B-II localized on the surface of autophagosomes or autophagic lysosomal membranes will be gradually degraded, resulting in a decrease in LC3B fluorescence intensity on the flow cytometer. Nevertheless, due to the generation of LC3B-I also improved after the initiation of cellular autophagy, the decline of LC3B fluorescence intensity is implicit. Therefore, the use of Saponin is essential for observing obvious changes in fluorescence intensity on flow cytometers, which can damage the membranes of cells via producing micropores on the cell surface. Collectively, both the immunofluorescence and flow cytometry detection of autophagy demands the use of fluorescent dyes or proteins for labeling. While immunofluorescence can observe punctate aggregation of LC3, flow cytometry can offer high-throughput analysis for autophagic flux.

Transmission electron microscopy (TEM) In cellular experiments, TEM is widely utilized to directly observe the morphological alterations in autophagy during different periods. Monitoring autophagic flux via TEM enables qualitatively observing the autophagy ultrastructures inside cells. Autophagy inhibitors can be used to observe the morphology and number of autophagic ultrastructure during various periods, and they can realize the dynamic detection of autophagic flux. For instance, the significantly higher proportion of autophagosomes than autolysosomes may be due to overactivation upstream of the autophagic flux, or hindered formation and maturation of autolysosomes. Besides, if substantial late autophagic structures accumulate in cells, it may mean that the process of autolysosome degradation is blocked. At present, TEM can observe various ultrastructures of autophagy, such as phagocytic vesicles and autolysosomes, which is the most direct method to monitor autophagy activity.

The SQSTM1/p62 and LC3B binding protein turnover assay Sequestosome1 (SQSTM1)/p62 is involved in autophagosome composition as a regulator with substrate specificity and serves as a bridge connecting LC3B-II to the ubiquitinated substrates to be degraded. p62 binds to the ubiquitinated proteins to enter the autophagosomes, and then
eventually cleared within autolysosomes. In the absence of certain ATG genes or blocked autophagosome-lysosome fusion, p62/SQSTM1 accumulates significantly in cells with impaired autophagic flux, and the expression of overall p62 levels in cells is negatively correlated with autophagic activity. In addition, p62 is also associated with the proteasomal degradation process, and when the proteasomal degradation pathway is blocked, the p62 expression levels increase as well.\(^{168,169}\) Hence, to ensure that only the autophagic degradation of p62 is monitored in experiments, it is necessary to appropriately use proteasome degradation system inhibitors for observing the correct p62 protein degradation rate. In addition, it is worth noting that with the fluctuation of autophagic flux, there is a certain lag in the changes of soluble and insoluble p62. The level of LC3 protein changes rapidly, whereas p62 has a longer adaptation time as an autophagic substrate, which makes it difficult to detect.\(^{170}\) In the process of detection, different time points need to be set to dynamically analyze the changes of p62 during the autophagic flux.

**Potential therapeutic agents targeting autophagy**

IPF is a rare respiratory disease with an extremely terrible clinical outcome, which can dramatically affect the physical and psychological health of patients. Nevertheless, at present, treatment options for IPF are severely limited, and there is no medicine could reverse or completely prevent the development of IPF. At present, lung transplantation is the only curative therapy for IPF, while the average survival time is only about 5 years and there still exist extremely limitations in clinical treatment, such as the lack of donors. Along with the study on mechanisms of autophagy in IPF, several drugs have been marketed to ameliorate IPF progress, in addition, a variety of compounds that have therapeutic effects on IPF through modulating autophagy are gradually being discovered.

**Current effective drugs**

In recent years, evidence-based treatment guidelines have recommended two antifibrotic drugs for clinical treatment of IPF patients. Clinical trials have verified that pirfenidone and nintedanib can delay pulmonary fibrosis progression and reduce mortality. Studies have found that pirfenidone exerts its antifibrotic effects mainly by inhibiting TGF-\(\beta\)1 and the downstream molecules of its pathway. In the bleomycin-induced lung fibrosis model in mice, pirfenidone inhibited TGF-\(\beta\)1-mediated phosphorylation of SMAD3 and \(\alpha\)-SMA expression, thereby reducing fibroblast proliferation and myofibroblast trans-differentiation. In addition, pirfenidone has been shown to interfere with collagen generation and the fibrillogenic process by decreasing the production of some profibrotic factors and growth factors to reduce ECM deposition.\(^{172-174}\) A recent study confirmed the ability of pirfenidone to activate the formation of autophagic vesicles in lung fibroblasts by detecting an increase in EGFP-LC3 sites and the conversion of LC3-I to LC3-II.\(^{175}\) However, the exact mechanism of how pirfenidone exerts its antifibrotic effects through autophagy remains to be investigated. Furthermore, pirfenidone can detoxify mitochondrial peroxidase to improve mitochondrial respiration, and maintain normal mitochondrial function.\(^{172}\) In addition, pirfenidone can also...
Therapeutic role in IPF through its antioxidant and anti-inflammatory effects. Despite the obvious clinical efficacy of pirfenidone in the treatment of IPF, it still has some pharmacokinetic deficiencies. Several clinical studies have verified that feeding remarkably reduces the absorption and utilization of pirfenidone, affecting its bioavailability. Another study proved that inhaled pirfenidone significantly improved this condition and had the same therapeutic effect, which holds great potential for the treatment of IPF.

Another therapeutic drug with antifibrotic properties is nintedanib. In clinical trials, nintedanib has been validated to reduce the rate of lung function deterioration and attenuate the fibrosis process. Nintedanib can decrease the deposition of collagen induced by TGF-β and inhibit fibroblast proliferation, fibroblast motility and contraction stimulated by growth factor, and fibroblast to myofibroblast transformation, thereby inhibiting the underlying process of progressive pulmonary fibrosis. In addition, some research has confirmed that nintedanib inhibits the proliferation of some pulmonary vascular cells, such as endothelial cells and pulmonary artery vascular smooth muscle cells. However, it is still uncertain whether these effects are related to its antifibrotic properties. In addition, nintedanib may also slow disease progression through an anti-inflammatory response. Importantly, the study found that nintedanib was able to enhance autophagy via detecting the ratio of LC3-I/II. Another study yielded the same results, but found that nintedanib induced a form of autophagy dependent on Beclin-1 and independent of ATG7. Currently, several therapeutic strategies for IPF that target autophagy have been discovered as a result of the intensive study of autophagy regulation.

**Potential compounds**

Amounting research focus on the development of new molecular targets and treatment options. Berberine is a plant quaternary alkaloid segregated from natural sources, which is characterized by a wide spectrum of pharmacological properties that have gained significant interest in clinical applications. The treatment of berberine exerts a beneficial effect on inducing autophagy to resist pulmonary fibrosis. Berberine can promote Beclin-1 and LC3-II production with p-mTOR reduced, and stimulate autophagosome formation as well, leading to autophagy initiation. Meanwhile, in bleomycin-induced animal models, berberine can also reduce the levels of α-SMA, fibronectin, and collagens I and III, restore the normal alveolar structure, and reverse the ultrastructural changes in the lung. In addition, berberine ameliorates the fibrotic progression induced by bleomycin via purposefully inhibiting PI3K/AKT/mTOR signaling axis.

Spermidine is a natural polycation that serves as a physiological autophagy inducer, which can reduce bleomycin-induced production of profibrogenic mediators and structural disorders in mouse lung tissue. The research confirmed that spermidine can increase the levels of critical autophagic marker molecules, such as ATG7 and Beclin-1 in bleomycin-induced fibrotic lung tissues and IPF fibroblasts, thus enhancing the formation of autophagosomes. Moreover, it can reverse autophagy impairment in IPF fibroblasts by suppressing mTOR. This mentioned evidence shows that spermidine may be a promising direction for IPF therapy.

Programmed cell death ligand 1 (PD-L1) has been confirmed to be highly expressed in lung tissues of patients with pulmonary fibrosis. The anti-PD-L1 monoclonal antibody (anti-PD-L1 mAb) was found to greatly reduce the expression of fibrotic marker proteins and relieve pulmonary fibrosis in mouse models. The study has shown that anti-PD-L1 mAb can increase the immunofluorescence intensity of LC3B, promote the transition from LC3I to LC3II, and autophagosomes formation, resulting in promoting autophagy in lung fibrosis. The findings indicate that anti-PD-L1 therapy is capable of relieving pulmonary fibrosis and provides a new strategy for IPF therapy.

Bergenin is a compound isolated from various herbal plants, such as Saxifragaceae. A recent study confirmed that bergenin improved lung function in mice with pulmonary fibrosis, attenuated lung tissue structural disorders caused by bleomycin, and reduced the degree of pulmonary fibrosis. The research found that bergenin reduced phosphorylation levels of mTOR, ULK1, and S6, and inhibited fibroblast activation and collagen deposition, thereby promoting autophagic activity and alleviating pulmonary fibrosis. Furthermore, bergenin can regulate energy metabolism through recovering normal
ATP levels in activated fibroblasts and promote the apoptotic process of fibroblasts. In general, further research and animal model tests are in great need to develop and validate more IPF therapeutic drugs that target autophagy.

Conclusion and prospect

In recent years, accumulating evidence demonstrates that autophagy exerts some unprecedented functions during IPF pathogenesis, which offers novel targets for the therapy of IPF. Targeted autophagy for lung fibrosis is a hot field of research, and there are a variety of elusive mechanisms that need to be further investigated. This review summarizes the interplay of autophagy with other mechanistic processes leading to pulmonary fibrosis. Among them, the exhaustive regulatory mechanisms linking autophagy to epithelial cell dysfunction, apoptosis, and the renin-angiotensin system remain poorly defined. In addition, besides epithelial cells, future research ideas can also focus on other types of cells such as endothelial cells, vascular smooth muscle cells in the lung. Studies on the intricate regulatory network within the renin-angiotensin system and its role with autophagy in the fibrotic process will provide a broad perspective for clinical diagnosis and therapy. Currently, the spectrum of autophagy regulatory pathways in IPF remains to be refined. Six related pathways are summarized in this review; however, studies on JNK signaling axis, JAK2/STAT3 axis, and p65 and Keap1/Nrf2 signaling are still insufficient, and more studies are needed in the future to elucidate their specific regulatory networks in pulmonary fibrosis and to develop molecular drugs targeting these pathways to activate autophagic activity in fibrosis and treat IPF. It must be beneficial to apply these experimental findings to clinical diagnosis and treatment.

Currently, in vivo experiments on pulmonary fibrosis are mainly based on bleomycin-induced mouse models, and most of the available findings are derived from these pulmonary fibrosis models. However, the distribution of bleomycin-induced pulmonary fibrosis lesions and their stability are defective and differ from those of human IPF. Therefore, it is necessary to continuously explore and establish lung fibrosis models that are more consistent with the pathological process of IPF, so that the experimental results can be more accurate.

Although there are numerous pharmaceutical drugs approved for treatments of pulmonary fibrosis, the exact mechanism remains incompletely understood. In recent years, it has been identified that pirfenidone and nintedanib can activate autophagic activity in the fibrotic process, but it remains poorly understood about its specific action process and targets. These two drugs have certain drawbacks in clinical treatment and need to be continuously improved later to fully exploit their antifibrotic effects. Many cellular animal experiments confirmed that some compounds were able to alleviate pulmonary fibrosis progression by mediating autophagy, but the research only stayed at the experimental stage, and further development of these compounds for clinical trials is needed to clarify their therapeutic effects. Meanwhile, researchers can focus on mining other autophagy targets and exploring broader molecular regulatory networks to provide a theoretical basis for drug development.

Therefore, it is extremely urgent to elucidate new IPF pathogenesis and search for more effective drug targets to block the development of pulmonary fibrosis. Furthermore, studying the signaling transduction pathways and detailed molecular mechanisms of autophagy will potentially minimize the impairment to normal tissues and cells. Collectively, developing efficient and safe therapeutic agents through animal disease models and preclinical trials and applying them to the clinic will greatly benefit the treatment and prognosis of IPF patients.

Declarations

Ethics approval and consent to participate
Not applicable.

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Author contributions
Yue-Liang Yue: Writing – original draft; Writing – review and editing.
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