Molecular Diagnosis & Therapy (2020) 24:665–681
https://doi.org/10.1007/s40291-020-00490-7

REVIEW ARTICLE

More than a Genetic Code: Epigenetics of Lung Fibrosis

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Published online: 14 September 2020

Abstract
At the end of the last century, genetic studies reported that genetic information is not transmitted solely by DNA, but is also transmitted by other mechanisms, named as epigenetics. The well-described epigenetic mechanisms include DNA methylation, biochemical modifications of histones, and microRNAs. The role of altered epigenetics in the biology of various fibrotic diseases is well-established, and recent advances demonstrate its importance in the pathogenesis of pulmonary fibrosis—predominantly referring to idiopathic pulmonary fibrosis, the most lethal of the interstitial lung diseases. The deficiency in effective medications suggests an urgent need to better understand the underlying pathobiology. This review summarizes the current knowledge concerning epigenetic changes in pulmonary fibrosis and associations of these changes with several cellular pathways of known significance in its pathogenesis. It also designates the most promising substances for further research that may bring us closer to new therapeutic options.

Key Points

Epigenetic information is crucial for appropriate proliferation, maturation, and functioning of pulmonary fibroblasts and epithelial cells.

Lung tissue affected by fibrosis presents numerous alterations in epigenetic code, many of them associated with regions that code proteins of known contribution to the pathogenesis of fibrosis.

Specific agents that can modify the epigenome arise as potential medications for pulmonary fibrosis.

1 Introduction

Interstitial lung diseases (ILDs) are a group of entities that predominantly affect lung interstitium. These diseases correspond to at least 15% of all known respiratory diseases. These are classified into subgroups, including idiopathic interstitial pneumonias (e.g., idiopathic pulmonary fibrosis [IPF]) and ILD with granuloma formation (e.g., sarcoidosis). Importantly, some ILDs can be induced by drugs or occupational factors (asbestos, beryllium, etc.), while others are associated with connective tissue disease (CTD)—mainly rheumatoid arthritis, scleroderma, polymyositis, or dermatomyositis.

The diagnosis and management of these diseases rely on clinical, radiological, and histopathological findings. The patient's smoking status, exposure to toxins, coexistent diseases, and drug history should be noted carefully during an examination. The vast majority of symptoms of ILDs are limited to the respiratory tract (these would include dyspnea, cough, and less frequently wheezing and hemoptysis), but extrapulmonary symptoms may be present, and in many cases they are associated with underlying CTD (muscle weakness, arthritis, gastroesophageal reflux, skin lesions, digital clubbing, etc.). High-resolution CT (HRCT) of the chest plays a significant role in the diagnosis and follow-up of patients with ILDs [1]. Lung function tests may be helpful to estimate the severity of the ongoing disease, and blood tests can indicate the presence of an underlying autoimmunization. In some cases, HRCT combined with lung function test may be used to predict disease prognosis and to assess current staging [2].

When the diagnosis is uncertain, bronchoscopy with bronchoalveolar lavage (BAL) may be beneficial to support initial diagnosis or indicate other conditions. Differential

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cell count is recommended to be performed with the BAL fluid [3], as well as microbiological assessment and cytopathology in specific situations. During bronchoscopy, a transbronchial biopsy may be needed to evaluate granulomatous conditions (e.g., sarcoidosis). Finally, surgical lung biopsy is the essential last step when other results are equivocal and the data are not highlighting a specific disease. Finally, a multidisciplinary team meeting is a reasonable step in demanding cases, as this will augment confidence in the final diagnosis.

What escalates the complexity is that the late stages of many ILDs present with pulmonary fibrosis and the prognosis and treatment differ between specific diagnoses [4, 5].

With our increasing knowledge, researchers have discovered more and more alterations in gene expression among the ILD-lung or animal models; therefore, treating ILDs as a genetic disease like cancer is not an exaggeration. Targeted therapies already exist in pneumology, e.g., sirolimus in lymphangioleiomyomatosis (LAM), which is known to be caused by a fault in the mTOR signaling pathway—though it is not a fibrotic ILD.

2 Basics of Epigenetics

Every somatic cell in the human body has the same unique DNA sequence, and major genomic alterations that occur during ILD are described in the review by Furukawa et al. [6].

Epigenetics, on the other hand, is defined as a DNA-independent carrier of genetic information that can be modified by stress, nutrition, and other environmental factors. Moreover, epigenetics is the language of the cell; it can activate or silence gene expression via various mechanisms, and it plays a fundamental role both in the human physiology and pathology [7]. Pathogenesis of other pulmonary diseases like cancer [8], asthma [9], and chronic obstructive pulmonary disease (COPD) [10] have been already linked to epigenetics. Below we briefly describe particular epigenetic mechanisms.

2.1 DNA Methylation

Human DNA contains many CpG sites where cytosine occurs next to guanine, and these regions are often concentrated in gene promoters [11]. The methylation process occurs when a methyl group is added to the cytosine in CpG dinucleotides. Methylation of the CpG island via DNA methyltransferases (DNMTs) blocks RNA polymerase complex from binding to the promoter region and therefore suppresses gene expression. The majority of human CpG sites are methylated [12, 13]. Hypomethylated gene promoters should result in increased gene expression, while hypermethylated conducts decreased gene expression—a general rule discussed later in this review.

2.2 Histone Modifications

The activity of cells depends on nucleosomes, which consist of DNA strands and histone core proteins (H2A, H2B, H3, H4) [14]. The H1 family is the most alkaline histone and wraps the whole nucleosome together [15]. The discovery of histone post-translational modifications and rapid progress in our knowledge of the role of histones has provided an opportunity to create histone code theory—another crucial hypothesis in epigenetic inheritance [16]. These modifications have considerable influence on chromatin condensation level and consequently on gene expression rate.

To the present day, a couple of histone modifications have been described. The best elucidated modifications include acetylation by histone acetyltransferases (HATs) and deacetylation by histone deacetylase (HDAC), methylation by histone methyltransferase (HMT), demethylation, phosphorylation, ubiquitination, sumoylation, and polyADP-ribosylation. According to current knowledge, acetylation activates cell transcription by decompression of the chromatin. The function of other modifications is more complex, e.g., methylation may cause either activation or inhibition of gene expression because functional impact depends on the modified region [13, 17].

2.3 MicroRNA (miRNA)

MicroRNAs (miRNAs) belong to the family of non-coding RNA and contain 17–25 nucleotides each. They interact with mRNA's 3′ untranslated region (3′-UTR) and recruit an miRNA-induced silencing complex (miRISC) [18], which leads to consequent mRNA inhibition or degradation—thereby controlling various biological processes. Approximately 2650 human miRNAs have been described [19]. Each miRNA molecule can affect a variety of genes, and each single gene can be controlled by multiple different miRNAs [20].

2.4 Long Non-coding RNA (lnc-RNA)

Long non-coding RNAs (lnc-RNAs) are poorly understood in terms of their regulatory function [21], in opposition to extensively studied short miRNAs. The first promising research has already shown up [22–24], but their role in pulmonary fibrosis still needs to be established. Therefore, we do not describe them in detail in this review.

3 Epigenetics and Interstitial Lung Diseases

3.1 DNA Methylation

3.1.1 Whole-Genome Studies

In search of differences in DNA methylation and RNA expression between lung samples from patients suffering IPF and normal tissue, Sanders et al. used microarrays [25]. The researchers merged the methylation data from almost 15,000 genes and data from gene expression microarrays, and managed to identify 16 genes showing inverse DNA methylation and gene expression levels—assuming that methylation of CpG dinucleotides selected by this method plays a crucial role in their regulation. Eight of these candidate genes had been previously reported as associated with lung fibrosis, e.g., matrix metalloproteinase 7 (*MMP7*) and collagen 3α1 (*COL3A1*) associated with extra-cellular matrix (ECM). A detailed validation of four genes (*ZNF465*, *CLDN5*, *TP53INPI*, and *DDAH1*) not previously associated with IPF has also been performed, suggesting their possible role in its pathogenesis [25].

Rabinovich et al. sought a global methylation pattern in IPF lung tissue and analyzed the methylation of 25,406 human CpG sites, finding 625 of these to be differentially methylated compared to control lung tissue [26]. Hypomethylation of the promoter region associated with increased transcription was proven by the examples of the *STK17B* and *HIST1H2AH* genes; however, only 8.8% of differentially methylated CpG islands were located in promoter regions. It has been shown that 65% of the CpG islands with an altered methylation pattern in IPF lung samples are also modified in lung cancer samples, which advocates for the similarity between IPF and cancer [26]. Unfortunately, small study groups and methodological differences may be the cause of the limited overlap between the differently methylated regions (DMRs) in the two studies mentioned above [27].

Huang et al. also analyzed the methylation profiles of lung fibroblasts from IPF patients and compared them to fibroblasts from histologically normal lung regions from patients undergoing resection for lung nodules and commercial fibroblast lines from the lungs of healthy individuals. Comparison revealed several hundred differently methylated loci (787 in IPF vs normal lung group and 333 in IPF vs human fibroblasts) and an overlap of 125 genes between these groups [28]. Most of the differentially methylated loci were located outside promoter regions in this study. Unfortunately, IPF fibroblasts from different cell lines showed significant heterogeneity in their individual methylation profiles.

Yang et al. identified 2130 DMRs in the IPF lung tissue genome (71% of these within gene bodies, 10% in promoter regions), the majority located in CpG island shores (60%; only 5% in CpG islands themselves). Among the multiple DMRs identified, some had been previously associated with IPF, namely CXCR4, thrombin, Wnt/β-catenin, vascular endothelial growth factor (VEGF), and epithelial adherens junction signaling [29]. Methylation changes were consistent with IPF genome-wide association studies (GWASs) [30, 31] in five out of the ten IPF GWAS loci. Five DMRs were influenced by combined use of corticosteroids and immunosuppressants. A total of 172 out of 1315 differently methylated genes showed a significant relationship between methylation and expression. Further analysis revealed the ten most affected networks [29]. Intriguingly, while mice deficient in amyloid protein precursor (APP) (presenilin 2) spontaneously develop lung fibrosis [32], the most significant network centers were located around *APP* [29].

Investigation of global methylation patterns in rat fibroblasts co-cultured in vitro with silica-exposed alveolar macrophages has shown 2842 DMRs in genes representing many of the pathways leading to myofibroblast differentiation [33]. Noteworthy clusters are associated with 'regulation of actin skeleton' characteristic for these cells, 'focal adhesion' that has its role in cell migration, 'extracellular matrix', and the 'MAPK (mitogen-associated protein kinase) signaling pathway' that is activated via transforming growth factor beta (TGFβ) [34]. The increased expression of MAPK9, one of the MAPK terminal kinases associated with negative regulation of a cell cycle, was confirmed with increased mRNA and translation product levels [33]. Intriguingly, elevated protein levels were not observed in reference to DMRs in the TGFβ1 pathway despite the increased mRNA levels. These findings most likely result from the overlap of different gene expression regulatory mechanisms.

The studies described above show that DMRs are only partially located in promoter regions, while many are found, for example, in introns. Further studies are required to determine the significance of these alterations. Below, we describe the actual data according to the specific pathways known to play significant roles in pulmonary fibrosis. The order of events involving DNA methylation that leads to developed pulmonary fibrosis is presented in Fig. 1.

3.1.2 Prostaglandin E_2 (PGE_2) Pathway

Prostaglandin E_2 (PGE_2) participates in maintaining fibroblasts' suppression and their proper apoptosis. The decreased levels and tissue effects of PGE_2 are simultaneous mechanisms in IPF pathogenesis [35]. Huang et al. sought to determine whether decreased EP2 (PGE_2 receptor subtype) expression in fibroblasts is conditioned by DNA hypermethylation of its promoter, *PTGER2* [36], both in the murine model and humans with pulmonary fibrosis. After proving

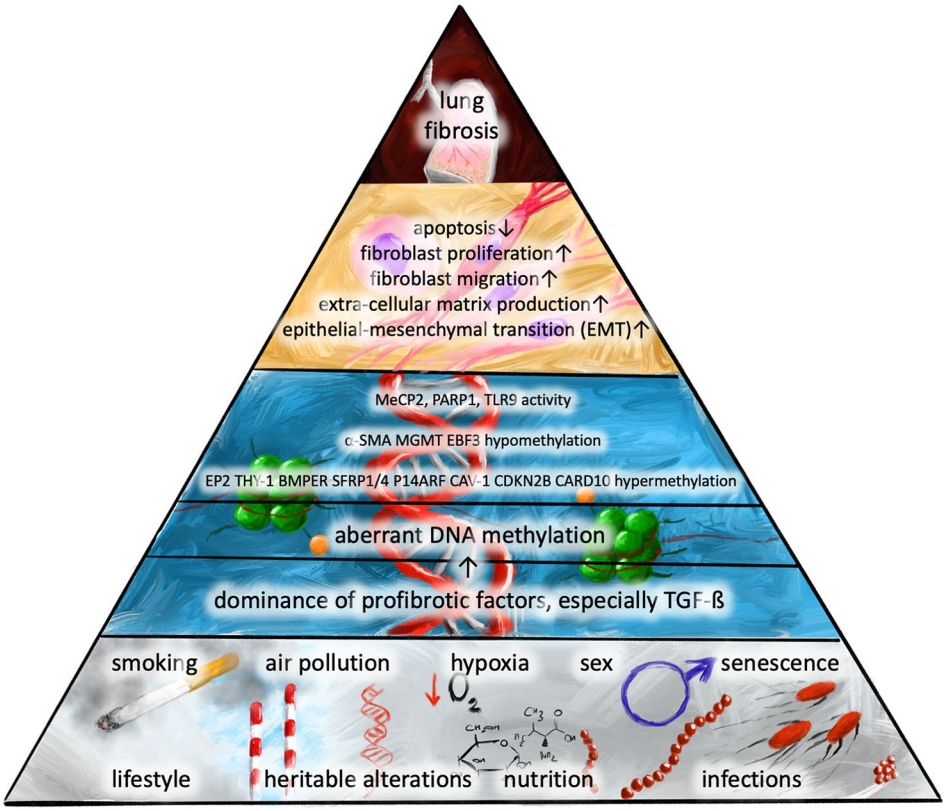

Fig. 1 The sequence of events including impaired DNA methylation that leads to pulmonary fibrosis. *α-SMA* α-smooth muscle actin, *BMP* bone morphogenetic protein, *BMPER* BMP endothelial cell precursor-derived regulator, *CARD10* caspase recruitment domain-containing protein 10, *Cav-1* caveolin 1, *CDKN2B* a cyclin-dependent kinase 4 inhibitor B, *EBF3* early B-cell factor 3, *EP2* prostaglandin E_2 receptor subtype EP2, *MeCP2* methyl CpG binding protein, *MGMT* O^6-alkylguanine DNA alkyltransferase, *PARP1* poly(ADP-ribose)polymerase 1, *SFRP* secreted fizzled protein, *TGF-β* transforming growth factor β, *Thy-1* thymocyte differentiation antigen 1, *TLR9* toll-like receptor 9

this fact, the attempt to restore EP2 gene expression with the use of DNA methylation inhibitors (DNMTi) was made, resulting in an increase in EP2 expression in fibrotic fibroblasts and no influence on non-fibrotic fibroblasts. A rise in EP2 expression resulted in the restored ability of PGE_2 to inhibit collagen synthesis and cell proliferation in these cells [36]. DNMTi in this study were 5-aza-2′-deoxycytidine and zebularine, the first of which is already approved for use in myelodysplasia and acute myeloid leukemia [37, 38]. To avoid confusion, the agent 5-azacytidine and its derivate 5-aza-2′-deoxycytidine are called 5-aza later in this review.

With regard to the underlying cause of reduced PGE_2 levels, other researchers have found that not only are cyclooxygenase-2 (COX-2) mRNA basal levels reduced in IPF and systemic sclerosis (SSc) lung fibroblasts, but TGFβ1 induction is also restricted [39]. A significant reversion of this state has been shown after the treatment of IPF- and SSc-derived cells with 5-aza. The intervention partially corrected aberrant PGE_2 and collagen 1α expression and restored sensitivity to FasL-induced apoptosis [39]. As a whole, these results advocate a positive change in cell phenotype assigned to 5-aza treatment. Interestingly, CpG promoter methylation did not show significant differences. Looking for a potential mechanism, investigators have found five DMRs in the *c8orf4* promoter, while *c8orf4* itself was further proven to interact with COX-2 promoter and regulate its expression [39]—forming a probable direct connection between DNA methylation and impaired COX-2 expression.

On the other hand, human fetal and adult lung fibroblasts, when incubated with PGE_2, tend to increase transcription factors for DNMT3a [40] along with increased DNMT expression and elevated overall DNA methylation level. This effect might be unique for fibroblasts and pathogenesis of lung fibrosis, as it stands opposite to lowering DNMT1 and DNMT3a levels in macrophages.

3.1.3 Thymocyte Differentiation Antigen 1 (*Thy-1*)

Several studies refer to thymocyte differentiation antigen 1 (Thy-1), a cell outer membrane glycoprotein also known as CD90—a thymocyte differentiation antigen. It has long been known that *Thy-1* knockout mice demonstrate enhanced lung fibrosis when treated with intratracheal bleomycin [41]. Additionally, cells have rarely shown Thy-1 expression in fibrotic areas, as opposed to the diffuse presence of phosphorylated Smad2/3, active TGFβ, and α-smooth muscle actin (α-SMA).

The absence of Thy-1 [42] along with increased α-SMA has been confirmed in human lung specimens from patients with fibrotic lung disease. Human lung fibroblasts that have lost Thy-1 expression via incubation with tumor necrosis factor (TNF) and interleukin-1 (IL-1) also

demonstrated elevated levels of α-SMA, pSMAD2/3, and overall SMAD—consistent with the fibrogenic phenotype identified in murine models [41]. These experiments suggest that suppression of Thy-1 expression promotes myofibroblast differentiation.

How is DNA methylation and altered *Thy-1* gene expression associated? The *Thy-1* promoter hypermethylation was proven in Thy-1 non-expressing cells and IPF-derived fibroblasts, in opposition to Thy-1 expressing cells [43]. Another interesting observation has been made by Robinson et al. who showed that hypoxia results in escalated methylation and reduced Thy-1 transcription in normal human pulmonary fibroblasts [44]. The study also suggests that hypoxia promotes myofibroblast differentiation, as it increases α-SMA, collagen 1 and 3 mRNA levels, and α-SMA protein level—specific for this cell phenotype. As hypoxia is a result of smoking and oxidative stress, strongly associated with lung fibrosis, these findings might be an important part of the disease's pathogenesis.

Coal and silica exposure induces a type of lung fibrosis commonly known as pneumoconiosis. In RLE-6TN cells (rat alveolar epithelial cells [AECs]) and rat lungs, coal and silica exposure resulted in accumulation of collagen 1 and 3, indicating fibrotic changes. At the same time, DNMT1 levels were raised, which corresponds to *Thy-1* promoter hypermethylation and a decrease in Thy-1 protein expression. 5-Aza administration 24 h after coal and silica exposure significantly inhibited pneumoconiosis [45].

An increase in Thy-1 expression after demethylation with 5-aza was proved in primary mouse lung fibroblasts [46] and rat and human lung fibroblasts [42]. Similar results were obtained after transfecting mouse fibroblasts with DNMT1 silencing RNA [46]. These interventions reversed the effect of TGFβ1, consistently with lowered DNMT1 activity and collagen type 1, alpha 1 (Col1α1) expression.

Thy-1 was also upregulated in Thy-1 age-deficient cells after incubation with 5-aza. Unfortunately, a study by Sueblinvong et al. despite showing a decrease in Thy-1 mRNA expression of fibroblasts from old mice lungs compared to young ones, did not show an increase in mRNA levels after treatment with this DNMTi [47].

Nevertheless, the findings strongly suggest an attenuation of the profibrotic cell phenotype with the use of a demethylation agent, which provides an opportunity for clinical studies and, potentially, for the development of a new treatment approach.

Sanders et al. stated that treatment with DNMTi and, alternatively, histone deacetylase inhibitors (HDACi) restored expression of *Thy-1* gene and inhibited myosin expression [43]. Regulation of Thy-1 expression in the context of histone acetylation is reviewed in the following chapter.

3.1.4 Myofibroblast Differentiation

Fibroblast to myofibroblast differentiation has usually been measured by the level of α-SMA. *α-SMA* gene methylation has shown DMRs in at least four CpG sites in rat fibroblasts and AECs. When compared to epithelial type II cells that do not express α-SMA, fibroblasts exhibit significantly lower *α-SMA* gene methylation and DNMT levels. During inhibition of methylation activity via 5-aza or short interfering RNA (siRNA) specific for DNMT1, DNMT3a, and DNMT3b, α-SMA expression increased, while transfection with plasmids containing DNMT genes did the opposite [48], underlying the importance of these mechanisms in epithelial–mesenchymal transition (EMT) regulation. Other methyltransferase inhibitors such as arginine N-methyltransferase inhibitor-1 (AMI1) and sinefungin also present the potential to disrupt the ECM architecture generated by IPF fibroblasts, as recently discovered by Sala et al. [49].

While TGFβ is the most recognizable fibrosis-leading factor, the balance between TGFβ and bone morphogenetic proteins (BMPs), especially BMP4, is crucial for lung repair mechanisms, fibroblast proliferation, and EMT [50–52]. BMP endothelial cell precursor-derived regulator (BMPER), which binds BMPs, is overexpressed along with α-SMA in the fibrotic foci of IPF lung tissue. In a series of experiments, BMPER revealed its influence on migration and invasion of fibroblasts and matrix production measured by hyaluronan levels [53]. It also affects TGFβ/BMP signaling in favor of the profibrotic TGFβ pathway. Elevated levels of BMPER were attenuated via administration of 5-aza, which indicates DNA methylation is the main regulatory mechanism. However, in this study, even more 5-aza effects have been shown, including reduced matrix production and fibroblast migration in IPF fibroblasts, as well as lowering the degree of lung fibrosis in bleomycin-treated mice. The phenotypic changes in primary and IPF lung fibroblasts after diminishing *BMPER* via siRNA and via 5-aza are convergent [53].

Toll-like receptor 9 (TLR9), which is excessively expressed in histological specimens of UIP and NSIP patterns characteristic for ILDs, appears to be a significant factor that drives myofibroblast differentiation dependent on type 2 T helper cell (T_H2) lymphocyte/IL-4 expression [54]. Hypomethylation of fibroblastic DNA through the TLR9-associated pathway leads to defective miRNA processing (via the loss of Argonaute proteins—AGO1 and AGO2), causing the progressive phenotype of IPF [55].

Hu and colleagues studied the role of poly(ADP-ribosyl)ation in IPF. The reaction of poly(ADP-ribosyl)ation is performed by the poly(ADP-ribose)polymerase (PARP) enzyme family, mainly by PARP1. They revealed that PARP1 promotes myofibroblast differentiation via the upregulation of α-SMA expression, while the lack of PARP1 causes hypermethylation of CpG islands of the *α-SMA* gene. The authors

also reported higher expression of PARP1 in the IPF fibroblasts and reduced fibrotic response in bleomycin-induced lung fibrosis (BILF) mice with PARP1 deficiency [56].

Methyl CpG binding protein 2 (MeCP2) binds to the *α-SMA* gene, increases its expression [57], and drives fibroblast differentiation into myofibroblasts, which is in opposition to its known function as a gene repressor. Mice deficient in MeCP2 surprisingly presented with much less fibrosis, as measured by hydroxyproline and collagen type 1 [58]. Upregulation of α-SMA via MeCP2 association to the *α-SMA* gene can be significantly reversed with 5-aza in human lung fibroblasts (IMR-90 line).

The Wnt signaling pathway that plays a crucial role in fibroblast activation has a significant role in pulmonary fibrosis [59]. Secreted fizzled proteins (SFRPs) bind to the Wnt ligand, forming non-functioning complexes, and their downregulation is associated with excessive fibrosis in various organs. Zhou et al. showed that SFRP1 and SFRP4 were downregulated in the mouse model of pulmonary fibrosis, that their promoter regions were progressively hypermethylated, along with the duration of the disease, and that this effect was attenuated via incubation of fibroblasts with 5-aza [60]. Intraperitoneal injection of 5-aza has also reduced the levels of COL1α1 and α-SMA, corresponding to the clinical effect of decreasing pulmonary density and pulmonary fibrosis scores in this study.

CDKN2B (a cyclin-dependent kinase 4 inhibitor B considered to be a tumor suppressor), CARD (caspase recruitment domain-containing protein 10, involved in apoptosis), and MGMT (O^6-alkylguanine DNA alkyltransferase, a crucial DNA repair enzyme) were the focus of interest in a study by Huang et al. *CDKN2B* and *CARD10* genes were proven to be hypermethylated, with an accompanying reduced expression, and *MGMT* was hypomethylated with overexpression in IPF fibroblasts [28]. Treatment of these cells with 5-aza resulted in a significant decrease in *CARD10* methylation and a modest decrease in *CDKN2B* methylation, with increased protein expression of both gene products.

The effect of silencing CDKN2B expression via siRNA in normal fibroblasts is not yet clear; it resulted in increased cell proliferation among unstimulated cells [28], but this appeared to decrease with growth factor stimuli [61], which is opposite to the reaction of epithelial cells. In IPF, a similar paradox is described in the context of higher sensitivity of epithelial cells and lower sensitivity of fibroblasts to FasL-mediated apoptosis [62]. The authors suggest that the loss of CDKN2B in fibroblasts promotes EMT rather than proliferation, as both the mRNA and protein levels of collagen 1 and α-SMA increase, along with the transcription factors associated with myofibroblast differentiation such as serum response factor (SRF) and myocardin-related transcription factor A (MRTF-A).

Finally, a positive correlation between pulmonary fibrosis among pigeon breeder's lung patients and *EBF3* (early B-cell factor 3, a B-cell maturation factor) hypomethylation has been shown. T_H2 dominance and excessive B-cell activation are thought to be important factors in fibrosis that complicate chronic stages of hypersensitivity pneumonitis [63]. The methylation rate presented statistically significant differences between patients who were breeding pigeons with and without consequent ILD [64]. The EBF3 mRNA and IL-10 levels were increased in the study group, negatively correlating with *EGF3* gene methylation levels.

3.1.5 Apoptosis

Regarding the reduced apoptotic activity of fibroblasts in the fibroblastic foci of IPF lungs, Cisneros et al. evaluated promoter methylation of the proapoptotic (tumor suppressor) gene *p14ARF* in fibroblasts obtained from IPF and normal lungs. Half (four) of the fibroblast cell lines from IPF patients presented hypermethylation of *p14ARF* promoter, while the other four IPF fibroblast and four normal cell lines remained unmethylated. The hypermethylation resulted in the underexpression of p14ARF, which consequently reduced the apoptosis rate and p53 levels in fibroblasts [65]. The researchers demonstrated these effects were reversible via incubating the cell lines in, once more, 5-aza.

3.2 Histone Modifications

3.2.1 Epithelial–Mesenchymal Transition

In search of epigenetic changes associated with epithelial-to-mesenchymal transition, Noguchi et al. performed RNA sequencing of A549 cells (alveolar epithelial type II cells) stimulated with the profibrotic factor TGFβ1, finding several up- and downregulated genes [66]. Furthermore, valproic acid (VPA), known to inhibit histone deacetylase 1 (HDAC1) activity, was administered, resulting in partial reversal of the downregulation, especially in *CDH1* (E-cadherin; epithelium-specific gene) and *GATSL2* (GATS protein-like 2) genes. The H3K27 histone in the *CDH1* loci has been shown to be deacetylated by TGFβ1 and restored under VPA stimulation, which strongly corresponds with expression changes. In this study, there was almost no such effect on TGFβ1-upregulated genes, with the exception of *COL1A1* [66].

Ota et al. also examined A549 cells exposed to TGFβ1 and EMT reversibility via HDACi—trichostatin A (TSA). The histological phenotype did not alter after the intervention, but TSA treatment increased mRNA expression of surfactant protein C (*Sftpc*), histone H4 acetylation in its promoter region, and propeptide of surfactant C protein (pro-Sp-C) in both control and TGFβ1-induced cells in vitro. Surfactant protein C (Sp-C) is necessary for alveolar homeostasis and was reported to be reduced in the

lungs of bleomycin-treated mice. In the lung tissue from these mouse models, TSA treatment elevated the overall histone H3 and H4 acetylation levels and, especially, pro-SP-C expression. Among magnetically separated alveolar type II cells from BILF mice, the Sftpc and Cdh-1 mRNA levels were restored after TSA treatment [67]. TSA also inhibited TGFβ-induced expression of α-SMA and collagen type 1 and blocked contractile function of normal human lung fibroblasts [68].

The expression of one of the best known transcription factors that mediates EMT, zinc finger E-box-binding homeobox 1 (ZEB1), is highly dependent on H3K4me3 and H3K9me3 balance [69]. ZEB1 has a known effect of suppressing epithelial marker expression, E-cadherin [70], thus allowing EMT, cell migration, and invasion. Challenging A549 cells with TGFβ1 results in a loss of H3K9me3, increased ZEB1 transcription, and switching to the mesenchymal phenotype. The addition of Schisandrin B, a substance obtained from a fruit used in Chinese medicine, restored H3K9me3 and reverted EMT of A549 cells, revealing a strong epigenetic influence [69].

HDACi have also been tested for a direct in vitro effect on fibroblasts. Davies et al. examined the effect of spiruchostatin A (SpA), an HDACi specific for class I histone deacetylase, on IPF fibroblasts. The presence of SpA resulted in significant inhibition of proliferation after 144 h of incubation, irrespective of the presence of TGFβ1, with an effect that was still evident after 96 h post-treatment, with little cytotoxicity at effective doses. Histone H3 acetylation was accelerated after treatment and P21WAF1 (cell-cycle regulator) expression level increased, thus revealing the possible antiproliferative effect of SpA. Treatment with SpA suppressed α-SMA (mRNA and protein), collagen 1 and 1 overexpression caused by TGFβ, as well as the formation of actin filaments—characteristic for myofibroblast differentiation and fibrosis [71].

Lysine-specific demethylase 1 (LSD1), known to mediate tumor cell behavior via epigenetic mechanisms, was recently investigated in a BILF mouse model. Its expression is increased in BILF mice lungs, while its knockdown alleviates lung fibrosis in vivo [72]. Lung coefficient and histological scores, as well as ECM accumulation measured with COL1α levels, were significantly in favor of the tested group.

When Pan et al. tried to achieve similar results in vitro, they found that TGFβ-incubated primary mouse lung fibroblasts slowed their migration and were less likely to differentiate into α-SMA positive cells after LSD1 knockdown. The effects are dependent on the TGFβ/Smad3 signaling pathway, which was probably downregulated due to high repressive H3K9me1/2 expression [72].

3.2.2 Thy-1

Mouse lung fibroblasts challenged with lipopolysaccharide (LPS) resulted in a significant increase in proliferation in comparison to the control group. Further investigation revealed a rapid decrease in Thy-1 (CD90) mRNA and protein expression, which indicates a profibrotic transformation phenotype. Global histone H3 and H4 acetylation levels were lowered in the LPS-challenged cells, as well as Ace-H4 level in the *Thy-1* promoter region, suggesting epigenetic silencing of the *Thy-1* gene. Furthermore, pre-challenge depletion of TLR4 (receptor for LPS) via the effect of specific siRNA resulted in a lack of Thy-1 expression inhibition and significantly higher Ace-H3 and Ace-H4 levels [73].

In another experiment, the researchers managed to partially restore Thy-1 expression in *Thy-1* (−) rat lung fibroblasts using TSA, while open chromatin markers associated with *Thy-1* promoter raised their levels. The HDACi also partially increased CpG island methylation in the *Thy-1* promoter region, consistently raising overall DNMT levels. Thy-1 re-expression was associated with decreased expression of α-SMA, suggesting the cells were undergoing a phenotypic alteration [74].

3.2.3 Apoptosis

Huang et al. have shown resistance to Fas-mediated apoptosis and impaired Fas expression in fibroblasts acquired from lungs of bleomycin-injured mice [75]. Decreased histone H3 and H4 acetylation in the Fas promoter region and increased overall HDAC2/4 expression were detected, attributing histone deacetylation to phenotypic changes. Treatment with HDACi (TSA and suberoylanilide hydroxamic acid [SAHA]) increased Fas mRNA and surface expression levels significantly, partially restoring the apoptotic activity of fibroblasts. SAHA treatment restored histone H3 acetylation levels in BILF fibroblasts, while TSA treatment elevated the susceptibility to Fas-mediated apoptosis in this cell group, with no such effect in control cells [75]. Increased trimethylation of H3K9 histone was an additional finding of no known significance. SAHA, also known as vorinostat, is a non-specific histone deacetylase inhibitor (HDI) registered for treatment of cutaneous T-cell lymphoma.

Reduced histone H3 acetylation and increased histone H3K9 methylation at the Fas promoter region, along with restoration of Fas mRNA and protein expression after TSA treatment, has been confirmed in human IPF fibroblasts in a subsequent experiment [75].

Cav-1 is a tumor suppression gene. Reduced levels of caveolin 1 (Cav-1) have been reported in IPF lung fibroblasts. In the mouse model, Cav-1 is experimentally suppressed along with elevated collagen 1α1 in response to bleomycin lung injury. Sanders et al. have found that it is

possibly a result of decreased association of *Cav-1* with H3K4Me3 (an active histone mark), as this differed significantly when compared to control cells. Additionally, the inhibition of the p38 MAPK pathway prevented downregulation of *Cav-1* gene associated with H3K4Me3 [76].

In contrast, no significant differences in DNA methylation were proven between IPF and control fibroblasts, as well as between normal fibroblasts with and without TGFβ1 stimulation, suggesting that DNA methylation is irrelevant to *Cav-1* regulation.

Wang et al. assessed the antifibrotic effect of SAHA on human fibroblasts and its anti-inflammatory effect on peripheral blood morphonuclear cells (PBMCs) and lymphocytes. It emerged that SAHA induces acetylation of histone 3 and α-tubulin in both *TGFβ1* (+) and *TGFβ1* (−) conditions. It mitigates the myofibroblast differentiation and collagen I deposition and reduces both the elevated matrix metalloproteinase-1 (MMP1) and tissue inhibitor of metalloproteinases 1 (TIMP1) levels in IPF lung fibroblasts. SAHA also inhibited cell proliferation with no effect on apoptosis. HDACi affected cytokine release in PBMCs and lymphocytes, with TNFα, IL-8, IL-13, and IL-10 being decreased, while IL-6 and IL-10 levels, respectively, were diminished in lymphocytes and monocytes alone [77].

Another research team reported decreased *COL3A1* gene expression and collagen 3 protein after the SAHA treatment. It reduced the high acetylation rate of histone H3 and H4 observed in the IPF group [78]. Moreover, SAHA promoted apoptosis of IPF myofibroblasts by stimulating apoptotic function of Bcl2-antagonist/killer (Bak), while inhibiting Bcl-xl's (one of the B-cell lymphoma 2, Bcl2, protein family) anti-apoptotic activity [79].

A recent study by Bai et al. showed that IPF fibroblasts cultured without glutamine (Gln) exhibit an increased apoptosis rate. A similar result of increased apoptosis was observed after glutaminase (GLS1), Gln-glutamate conversion enzyme, depletion [80]. GLS1 inhibition in BILF mice attenuates pulmonary fibrosis in vivo [81]. The effect seems to be associated with the lack of Gln metabolites. Molecules from the inhibitor of apoptosis protein (IAP) family such as X-linked inhibitor of apoptosis (XIAP) and survivin exhibit increased expression in IPF lung fibroblasts, but their levels are highly dependent on Gln-glutamate balance [80]. It also appears that H3K27me3 rate increases during GLS1 gene knockdown and when incubating IPF fibroblasts in a medium deficient in Gln. To sum up, Gln emerges as a key factor that prevents apoptosis and mediates survival of fibroblasts, possibly via epigenetic silencing of IAPs.

3.2.4 PGE_2 Regulation

As discussed in Sect. 2.1.2., low COX-2 expression has been linked to hypoacetylation of histone H3 and H4 caused by decreased activity of HATs [82]. The same research group revealed histone H3 and H4 deacetylation, as well as histone H3 hypermethylation of gamma interferon induced protein 10 gene (*IP-10/CXCL10*). These abnormalities were caused by reduced recruitment of HATs, increased activity of HDAC-containing repressor complexes (HMTs G9a and SUV39H1), and the presence of heterochromatin protein 1 at the *IP-10* promoter region [83].

When inhibiting or knocking out *G9a* or *EZH2* (H3K9 and H3K27-specific HMT, respectively) in IPF fibroblasts, the histone H3 and H4 acetylation at the *IP-10* promoter region increases in IL-1β-stimulated cells, and the previously reduced expression of IP-10 seems to be restored [84]. H3K9me3 and H3K27me3 levels are lowered under similar conditions, which means less repressive chromatin in this region after the intervention.

On the other hand, the knockdown of either G9a or EZH2 in normal lung fibroblasts reverses the excessive (TGFβ-induced) association of EZH2 to the *IP-10* promoter [84]. It appears that they physically interact with each other and both are required for gene expression regulation. The intervention mentioned above also affects H3K9me3 and H3K27me3 levels. Moreover, histone and DNMTs cooperate in the case of epigenetic changes, as inhibition of either G9a or EZH2 alone significantly reduces DNA methylation in the COX-2 promoter region [85]. Taken together, results are suggestive for a complex epigenetic regulatory mechanism.

What is the association between G9a, EZH2, and COX-2? Coward et al. investigated G9a- and EZH2-associated histone and DNA methylation at the *COX-2* promoter in IPF fibroblasts [85]. The reduction of repressive histone H3 methylation and DNA methylation using G9a, EZH2, and DNMT1 inhibitors, as well as specific siRNAs, has been confirmed; the expected variability between experiments that is also partially dependent on stimulation from IL-β1 was also shown. Both inhibition and disruption of these factors increased COX-2 mRNA and PGE_2 levels [85], including cells previously stimulated with IL-β1.

Nevertheless, the study on fibroblasts from non-fibrotic lung tissue did not show the same effect on COX-2 mRNA levels via G9a and EZH2 inhibition. In this study, Pasini et al. identified a significant increase in COX-2 protein expression without an increase in COX-2 mRNA levels in response to SAHA, which may be mediated via other factors, e.g., the suppression of a translational silencer TIA-1 or via changes in miRNA expression [86], which requires validation by further experiments.

3.2.5 Other

While H3K9 dimethylation is overrepresented in fibrotic regions of both BILF mice and IPF lungs [87], G9a specifically methylates H3K9, allowing binding of chromobox

homolog 5 (CBX5) and a consequent assembly of a transcriptional repressor complex [88]. Consequent chromatin modifications inhibit transcription of PPARγ coactivator 1α (*PPARGC1A*—peroxisome proliferator-activated receptor gamma coactivator 1-alpha) [87] that encodes peroxysmal protein PGC1α. PGC1α acts as a regulator of mitochondrial biogenesis and fatty acid oxidation [89], probably playing an important fibrosis-inhibiting role in BILF [90] and IPF.

This study was first to identify the roles of the G9a/CBX5 pathway that both directly and via H3K9 methylation depresses PGC1α expression in its gene promoter region and leads to an uncontrolled IPF fibroblast activation. Administration of BIX01294, a G9a inhibitor, prevents PGC1α depression and mitigates profibrotic effects of TGFβ on IPF fibroblasts; knockdown of either CBX5 or G9a reduces the profibrotic *ACTA2* gene expression in IPF lung fibroblasts [87].

Recent research by Jones et al. presents the effects of another HDAC inhibitor, pracinostat, on IPF primary lung fibroblasts [91]. The significant reduction of α-SMA, fibronectin, and collagen 1 levels after TGFβ stimulation was proved, which means alleviation of myofibroblast differentiation and ECM production. Intervention altered fibroblast contractile functions as well. Pracinostat increased global histone acetylation rate; it prevented TGFβ from repressing various antifibrotic genes [91]. The authors investigated the effects of HDAC knockdown on lung fibroblast activation, and HDAC7 emerges as the most important HDAC that mediates TGFβ-induced fibroblast activation [91].

3.3 miRNA

miRNAs have been extensively studied in the pathogenesis of pulmonary fibrosis. The comprehensive study conducted by Xie et al. revealed different expression of 161 miRNAs in BILF. The altered miRNAs were involved in a number of pathways and regulatory processes, including cell apoptosis and the TGFβ and Wnt signaling pathway [92]. Abnormal miRNA expression in BILF may also be associated with insulin-like growth factor signaling according to increased expression of Igf1 in lungs of these mice [93].

One paper has identified circulating miRNAs in patients' sera [94]. Forty-seven miRNAs differed between IPF and a control group (21 upregulated vs 26 downregulated). In the second cohort, authors reported enhanced expression of miR-21, miR-199a-5p, and miR-200c, accompanied by reduced expression of miR-31, let-7a, and let-7d in IPF patients. miRNAs in this case have been assigned to disrupted molecular pathways such as TGFβ, MAPK, PI3K-Akt, Wnt, HIF-1, Jak-STAT [94].

Regarding the high variability of disease progression, the study conducted by Oak et al. showed different expression of miRNAs in patients with progressive IPF [95]. miRNAs were assessed in surgical lung biopsy specimens, and overexpression of five miRNAs (miR-302c, miR-423-5p, miR-210, miR-376c, and miR-185) has been linked to rapid progression, while only one miRNA level (miR-423-3p) was decreased when compared to more stable disease. Intriguingly, miR-302c was downregulated in lung tissue in sarcoidosis, opposite to that seen in IPF, a fact possibly useful for establishing the final diagnosis [96]. mRNA profiling revealed promotion of EMT via downregulation of specific miRNAs. The vital components of the miRISC complex that process miRNA, ARO1 and ARO2, were also shown to have lower expression in subjects with IPF [95]. The results strongly relate miRNA abnormalities to IPF.

3.3.1 Profibrotic miRNA

miR-21 presumably plays a key role in the pathogenesis of IPF. Enhanced production of TGFβ stimulates miR-21 expression. It is supposed that miR-21 induces EMT and promotes the fibrotic processes via inhibition of Smad7 [97]. High expression levels of miR-21 were found in myofibroblasts from patients with IPF and also in an experimental model of pulmonary fibrosis [98]. Yang and colleagues found that the presence of miR-145 leads to α-SMA expression, which is a characteristic of fibroblast–myofibroblast transition. Additionally, miR-145 activates the latent TGFβ, inducing fibrosis [99]. miR-424 seems to promote myofibroblast differentiation during EMT through targeting Smurf2 (TGFβ pathway inhibitor) and thereby enhancing TGFβ production [100]. Another group found that increased expression of miR-96 in response to a collagen-rich environment suppressed forkhead box O3a (FoxO3a), which made IPF fibroblasts capable of abnormal expansion in the diseased lung. This action can be explained by impaired tumor suppressor factors p27, p21, and Bim activity [101].

Relaxin-deficient mice develop age-related lung fibrosis, and treatment with relaxin reverses collagen deposition in their lungs [102]. The expression of relaxin receptor, relaxin/insulin-like family peptide receptor 1 (RXFP1), is downregulated in lungs from IPF and SSc patients, which limits the antifibrotic effect of endogenous relaxin and its potential therapeutic use [103]. Additionally, lowest RXFP1 levels are associated with highest predicted mortality. miR-144-3p is an miRNA upregulated over 70-fold in IPF fibroblasts, and its mimic has been recently proven to downregulate RXFP1 in IPF lung fibroblasts [104]. Decrease in RXFP1 accompanies increased α-SMA levels and implies a direct profibrotic effect of miR-144-3p.

Milosevic et al. revealed the profibrotic function of miR-154 in IPF through activation of the WNT pathway. miR-154 regulates fibroblast migration and proliferation, additionally inhibiting p15 expression [105]. Another molecule, the miR-23a cluster, acts together with ZEB1 transcription

factor to reinforce the presence of mesenchymal cells in pulmonary fibrosis [106], in opposition to miR-200c. Lino Cardenas et al. found increased expression of miR-199a-5p in IPF samples, particularly in injured murine myofibroblasts and fibroblastic foci. The synthesis of miR-199a-5p was enhanced with TGFβ, but addition of TGFβ was not required to activate pulmonary fibroblasts. The authors suggest that miR-199a-5p regulates Cav-1 and has an important role in the mouse model of liver and renal fibrosis [107]. Bodempudi and co-workers reported that hypoxia stimulates fibroblast proliferation via miR-210 expression. HIF-2α, a protein induced by hypoxia, targeted miR-210 and upregulated its expression [108]. Abnormal expression of miR-21, miR-101-3p, and miR-155 were matched with impaired forced vital capacity and radiological patterns in IPF [109].

3.3.2 Antifibrotic miRNA

Liang et al. described low expression of miR-26a in a mouse model of pulmonary fibrosis and consequently sought the antifibrotic effect of miR-26. TGFβ1 inhibits expression of miR-26a by phosphorylation of Smad3 in both a mouse model of pulmonary fibrosis and IPF. Admission of the exogenous miR-26 reduces collagen and connective tissue growth factor (CTGF) expression, as well as the overall level of lung fibrosis in BILF mice [110] to a degree dependent on the time from symptom onset. Additionally, Li et al. revealed that miR-26a targets TGFβ fibrotic response via regulation of cyclin D2, TGFβ2, and the receptor for TGFβ1 [111]. miR-26a targets high-mobility group AT-hook 2 (HMGA2), a transcriptional factor which plays a significant role in EMT, and downregulation of miR-26a boosts this process [112]. A number of other miRNAs are suppressed in pulmonary fibrosis via the influence of TGFβ. Graham and colleagues found that low expression of miR-27b is associated with increased collagen expression. miR-27b binds directly to the 3′-UTR of Gremlin 1 and blocks the synthesis of its mRNA; decrease in its function causes enhanced Gremlin 1 expression [113]. The miR-200 family was markedly decreased in IPF patients and BILF mice, while its overexpression plays a crucial role in the inhibition of EMT [114]. miR-326 inhibits profibrotic genes such as *Ets1*, *Smad3*, and matrix metalloproteinase 9 (*MMP9*) and stimulates the antifibrotic Smad7 [115]. miR-375's function is the regulation of AEC type II trans-differentiation into AEC type I cells, which occurs after lung injury, via the WNT/b-catenin pathway [116]. miR-30a and miR-92a levels were predicted to target and mitigate WNT1-inducible signaling pathway protein 1 (WISP1) expression, which is upregulated in response to TGFβ1 and is probably associated with de novo collagen production in BILF [117]. One of the targets of let-7d is HMGA2, which becomes overexpressed in fibrotic lung. The loss of let-7d in IPF fibroblasts impairs epigenetic gene silencing via the MiCEE complex required for normal lung repair; it also interferes with HDAC1 and HDAC2 that are part of this complex [118]. The inhibition of let-7d leads to EMT and promotes collagen deposition in mouse lungs [119]. Let-7d interacts during the EMT process; its administration diminishes mesenchymal transition by reducing α-SMA and other markers involved in this process [120].

ECM deposition is highly dependent on miR-29 regulation. Cushing et al. suggest that miR-29 is a controller of many genes associated with complex fibrotic response such as laminins, integrins, ADAM metallopeptidases, MMPs, and collagen and that its downregulation enhances fibrosis and sensitizes cultured lung fibroblasts to TGFβ effects [121, 122]. In addition, miR-29 reduces expression of COL1α via phosphorylation of PI3K-Akt [123]; however, its expression is downregulated by TGFβ and SMAD3 [124]. miR-29c also mitigates the abnormal mRNA levels of *LOXL2*, *COL3A1*, and *SPARC*, which are associated with extracellular matrix; at the same time, it accelerates the expression of Fas mRNA and protein, increasing the Fas-mediated apoptosis in human lung fibroblasts (HFL1 cells) [125]. It also reverses the apoptotic resistance in cells treated with TGFβ. To sum up, overexpression of miR-29 limits ECM production and deposition [126]. Inhibition of miR-29c had the opposite biological effects in the abovementioned studies, and expression of miR-29 is known to be reduced in lung fibrosis. There is evidence that its biological effects are associated with the regulation of DNA methylation, as miR-29c directly decreases DNMT3A and 3B levels and there was no change in Fas levels after treatment with miR-29c inhibitor in the environment containing 5-aza [125].

The experiment performed by Xiao et al. deserves even more attention. Firstly, the researchers confirmed, using the mouse model, that bleomycin-induced pulmonary fibrosis, as well as the loss of the miR-29 family, is associated with Smad3 expression, possibly due to induction from TGFβ1. Secondly, they showed that transfection of pre-miR-29b to the lung tissue is possible, efficient, and—most importantly—that it restores miR-29b to the normal levels and blocks collagen 1, collagen 3, and fibronectin expression, as well as macrophage infiltration [124]. When similar tests were performed after 14 consecutive days, the miR-29 transfection similarly inhibited progressive disease. It did not modify the established lung fibrosis, which is consistent with expectations. Fibrogenetic factors, such as TGFβ1, CTGF, and phospho-Smad3, were also negatively affected by the intervention described above.

Other data considering miR-29b efficacy in vivo come from the paper from Montgomery et al. [127]. Histological specimens of BILF mice lungs after miR-29b administration presented diminished fibrotic and inflammatory reaction. IL-12, IL-4, and granulocyte colony-stimulating factor (G-CSF) levels dropped in broncho-alveolar lavage fluids (BALF) from

miR-29b-treated BILF mice, along with neutrophil, lymphocyte, and macrophage counts. Once more, the alleviation of ECM production has been confirmed to some extent, even when miR-29b administration started 10 days after bleomycin supply. The researchers also confirmed the effect on Col1α1 and Col3α1 expression in vitro on human IPF fibroblasts and human AEC type II cells (A459 line) [127].

miR-155 levels in normal lung fibroblasts change with cytokine stimulation. Treatment with TNFα, interferon-γ (IFNγ), IFNβ, and poly (I:C) inducts [128] and TGFβ [129] represses its levels. The instillation of bleomycin that provokes lung fibrosis elevates miR-155 in mice lung fibroblasts, in a scale dependent on the mice strain [129]. To test the functional consequences, HLF1 cells (human lung fibroblasts) have been transfected with pre-miR-155 and control pre-miRNA, and pangenomic assays revealed changes in expression of 474 and 1404 genes at 24/48 h respectively; all of them associated with cell signaling, death, and movement. These findings were somehow confirmed via testing for the increased levels of caspase 3—possibly indicating an enhanced apoptosis—and for the elevated mobility of these cells, especially on the base of collagen 1. Last but not least, it was proved to directly alleviate IL-1β-induced keratinocyte growth factor/fibroblast growth factor 7 (KGF/FGF7) expression, which is an EMT-involved mitogen produced by mesenchymal cells [129].

miR-200c interferes with ZEB1 transcription factor to inhibit the EMT of AECs. Downregulation of miR-200c was revealed in BILF and IPF lungs [114], which is possibly via a novel lnc-RNA-ATB mechanism [23]. In recent research, miR-200c agomir attenuated silica-induced lung fibrosis in mice [23].

3.3.3 Hybrid Approach

Another study has investigated the role of the miR-17–92 cluster in the regulation of DNA methylation in IPF. The miR-17–92 cluster targets specific genes involved in fibrosis, including collagen, TGFβ, and metalloproteinases. The authors found that the miR-17–92 cluster has an overall reduced expression in IPF. Moreover, the promoter CpG islands of this cluster were severely methylated and there was evidence of DNMT1 overexpression, which may explain excessive methylation [130]. Mice with bleomycin-induced fibrosis were treated with 5-aza-2′-deoxycytidine and exhibited raised expression of miR-17–92, presumably via demethylation of its promoter. Treatment had not induced a clearance of collagen, but inhibited its further deposition [130].

There is evidence that miRNA biological effects are associated with the regulation of DNA methylation, e.g., miR-29c directly decreases DNMT3A and 3B levels and there was no change in Fas levels after treatment with miR-29c inhibitor in the environment containing 5-aza [125].

Another molecule, miR-30a, is decreased in lung fibrosis animal models, HFL fibroblasts after H_2O_2 stimulation, and IPF patients' blood [131]. In the search for its target gene, tet methylcytosine dioxygenase 1 (TET1) seems most promising. It is a protein that converts 5-methylcytosine to 5-hydroxymethylcytosine, an epigenetic mark for actively expressed genes, and a DNA demethylation/activation promoter. In this study, an miR-30a designed agomir was sprayed across BILF mice lungs, resulting in fewer histological changes compared to the control BILF group, with fewer collagen fibers; which is suggestive of an antifibrotic effect in vivo [131]. The expression of hydroxyproline, α-SMA, and vimentin was reduced, while E-cadherin levels increased, indicating alleviation of EMT and enhancement of apoptosis. The miR-30a level was raised, while the TET1 level dropped—reversing the toxic effect of bleomycin. Other experiments suggest it can also alleviate the bleomycin-induced levels of dynamin-related protein 1 (Drp-1) that lead to epithelial cell apoptosis and which is correlated with higher p53 rates [132].

3.3.4 Drugs and miRNA

Arsenic trioxide was used by Chinese investigators to study its role in BILF rats. The treatment prevented pulmonary fibrosis via high expression of miR-98 and decreased the signal transducer and activator of transcription 3 (STAT3) signaling pathway, inhibiting the production of collagen 1 and hydroxyproline [133]. Another group has explored a sulindac (a nonsteroidal anti-inflammatory drug [NSAID]) mechanism in BILF. Sulindac diminished the severity of fibrosis measured by collagen deposition, thickening of the alveolar interval, and wet lung to body weight ratios. Moreover, sulindac inhibits EMT by restoring E-cadherin and SMA levels. Authors suggest that sulindac might decrease STAT3 expression with IFNγ and miR-21 involvement [134]. Wang et al. found that administration of paclitaxel induces miR-140 expression and ameliorates pulmonary fibrosis in BILF rats via EMT reduction [135]. Tectorigenin, a type of isoflavone, reduces fibroblasts' ability to proliferate in BILF rats via increased miR-338 expression and, in turn, via downregulation of its plausible target gene—lysophosphatidic acid receptor 1 (*LPA1*) [136]. Another flavone that showed the antifibrotic effect is baicalein; lowered expression of miR-21 in BILF rats treated with this agent was proven [137].

4 Conclusions

There is a continuous deficiency of therapeutic options in fibrotic lung diseases. With regard to countering IPF, the fatal disease this paper primarily discusses, there are only two antifibrotic medications that slow down progression, but

Table 1 List of the substances used in experimental studies cited in the review and their discovered in vitro and in vivo effects

Substance	Phenotypic effect	Association	Model
DNA methylation modifiers			
5-Aza-2′-deoxycytidine and zebularine	Inhibition of collagen synthesis and cell proliferation	PGE_2	Murine BILF and human IPF fibroblasts
5-Aza-2′-deoxycytidine	Reduced collagen 1α expression; increased sensitivity to FasL apoptosis	PGE_2	Human IPF and SSc fibroblasts
5-Aza-2′-deoxycytidine	Alleviation of stress fiber formation and EMT in response to TGFβ1	Thy-1	Murine primary lung fibroblasts
5-Azacytidine	Alleviation of matrix production and migration of fibroblasts	BMPER	Human IPF fibroblasts
5-Azacytidine	Attenuation of matrix accumulation and lung fibrosis	BMPER	Murine BILF lungs in vivo
5-Aza-2′-deoxycytidine	Reduction of collagen 1α1 and α-SMA levels; attenuation of histological alveolitis and fibrosis scores, as well as radiological density and fibrosis scores	SFRPs and Wnt pathway	Murine BILF lungs in vivo
AMI1 and sinefungin	Disruption of fibroblast-derived ECM architecture	Snail1	Human IPF fibroblasts
Histone structure modifiers			
Trichostatin A	No changes of histological phenotype	–	Human AEC type II A549
Trichostatin A	Mitigation of α-SMA production in response TGFβ; less cell contraction	Akt/SMAD	Normal human lung fibroblasts
Spiruchostatin A	Inhibition of fibroblast proliferation	P21WAF1	Human IPF fibroblasts
Spiruchostatin A	Reduced expression of α-SMA and actin filament formation	TGFβ	Human IPF fibroblasts
Trichostatin A	Mitigation of α-SMA production and Thy-1 expression restoration	Thy-1	Primary rat lung fibroblasts
Trichostatin A	Increased susceptibility to Fas-mediated apoptosis	Fas	Murine BILF fibroblasts
Trichostatin A	Restoration of Fas expression	Fas	Human IPF fibroblasts
SAHA	Lower expression of α-SMA and collagen 1 deposition; reduced cell proliferation	TGFβ1	Human IPF, adult and fetal fibroblasts
SAHA	Reduced collagen 3α1 expression	–	Human IPF fibroblasts
SAHA	Reduced collagen 3α1 expression, less aberrated lung structure	–	Murine BILF lungs in vivo
BIX01294	Mitigation of profibrotic TGFβ actions	G9a, PGC1α	Human IPF fibroblasts
Schisandrin B	Reversion of EMT among lung epithelial cells	ZEB1	Human AEC type II A549
Pracinostat	Inhibition of α-SMA, fibronectin and collagen 1 production in response to TGFβ	HDAC7	Human IPF fibroblasts
Engineered miRNA			
agomiR-26a	Attenuation of collagen, CTGF expression and lung fibrosis	p-SMAD3	Murine BILF lungs in vivo
let-7d	EMT alleviation (α-SMA and N-CAD reduction); slowing of fibroblast migration and proliferation	HMGA2, Myc, cyclin D2	Human FLF and NHLF fetal and normal lung fibroblasts
Pre-miR-29c	Mitigation of ECM-related gene expression (*LOXL2, COL3A1, SPARC*) and Fas-related apoptosis resistance	–	Human HFL1 fetal lung fibroblasts
Pre-miR-29b	Alleviation of matrix production and macrophage infiltration	Smad3, CTGF	Murine BILF lungs in vivo
miR-29b mimic	Mitigation of lung fibrosis and tissue inflammation (reduced collagen 1α1, hydroxyproline, IL-12, IL-4, G-CSF)	IGF1	Murine IPF fibroblasts in vivo
miR-29b mimic	Mitigation of collagen induction	–	Human IPF fibroblasts, A549 AEC II

Table 1 (continued)

Substance	Phenotypic effect	Association	Model
Has-miR29c	Mitigation of ECM-related gene expression, especially *COL12A1*	–	Human IPF fibroblasts
Pre-miR-155	Increased apoptosis (caspase-3) and motility	–	Human HFL1 fetal lung fibroblasts
agomiR-30a	Fibrosis and EMT alleviation; apoptosis enhancement	TET1, Drp-1	Murine BILF fibroblasts
miR-200c agomir	Decrease in α-SMA expression and EMT	ZEB1, lnc-RNA-ATB	Murine silica-induced pulmonary fibrosis fibroblasts
Other antifibrotic factors with known miRNA associations			
5-Aza-2′-deoxycytidine	Inhibition of collagen deposition ($P > 0.05$) and expression of VEGF, CTGF, DNMT1	miR17–92	Murine BILF lungs in vivo
Arsenic trioxide	Mitigation of lung fibroblasts, collagen deposition and lung density	STAT3 and miR-98	Rats' BILF lungs in vivo
Sulindac	Diminished severity of fibrosis—reduced collagen deposition and alveolar interval thickening	STAT3/IFNγ; miR-21	Rats' BILF lungs in vivo
Sulindac	EMT alleviation	STAT3/IFNγ; miR-21	A459 AEC II
Paclitaxel	EMT alleviation	TGFβ1/SMAD3 and miR-140	A459; RLE-6TN AEC II cells
Paclitaxel	Less interalveolar collagen 1 deposition and alveolar disruption	TGFβ1/SMAD3 and miR-140	Rats' BILF lungs in vivo
Tectorigenin	Reduction of fibroblast proliferation	LPA1; mir-338	Rats' BILF fibroblasts

α-SMA α-smooth muscle actin, *AEC* alveolar epithelial cell, *AMI1* arginine N-methyltransferase inhibitor-1, *BILF* bleomycin-induced lung fibrosis, *BMP* bone morphogenetic protein, *BMPER* BMP endothelial cell precursor-derived regulator, *COL3A1* collagen 3α1, *COL12A1* collagen 12α1, *CTGF* connective tissue growth factor, *DNMT* DNA methyltransferase, *Drp-1* dynamin-related protein, *ECM* extra-cellular matrix, *EMT* epithelial-mesenchymal transition, *FLF* fetal lung fibroblasts, *G-CSF* granulocyte colony-stimulating factor, *HDAC* histone deacetylase, *HFL1* human lung fibroblasts 1, *HMGA2* high-mobility group AT-hook 2, *IFNγ* interferon-γ, *IGF1* insulin-like growth factor 1, *IL* interleukin, *LOXL2* lysyl oxidase homolog 2, *LPA1* lysophosphatidic acid receptor 1, *IPF* idiopathic pulmonary fibrosis, *lncRNA* long non-coding RNA, *N-CAD* N-cadherin, *NHLF* normal human lung fibroblasts, *PGC1α* peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGE_2 prostaglandin E_2, *SAHA* suberoylanilide hydroxamic acid, *SFRP* secreted fizzled protein, *SPARC* secreted protein acidic and rich in cysteine, *SSc* systemic sclerosis, *STAT3* signal transducer and activator of transcription 3, *TET1* tet methylcytosine dioxygenase 1, *TGF* transforming growth factor, *Thy-1* thymocyte differentiation antigen 1, *VEGF* vascular endothelial growth factor, *ZEB1* zinc finger E-box-binding homeobox 1

adverse effects limit their use [138]. Better understanding of the epigenetics of ILDs may potentially increase our knowledge about their natural history and, eventually, contribute to the development of new therapeutic strategies (Table 1). The use of drugs that alter oligonucleotide activity, methylation pattern, or histone structure is a tempting possibility yet to be verified in clinical trials. Several substances are already registered for malignant lesions, and they seem to be obvious candidates for further experiments, especially considering their efficacy in alleviating BILF in mice. However, the heterogeneity of methodologies used in particular studies should be considered as a major limitation and indicates a strong need for further, intensive research in this field.

Declarations

Funding The Medical University of Lodz. Open access funding was provided through an agreement between Springer Nature and the Polish Ministry of Science and Higher Education.

Conflict of Interest The authors declare no conflict of interest.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Availability of Data and Material Not applicable.

Code Availability Not applicable.

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