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Epigenetics of Idiopathic Pulmonary Fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a complex lung disease of unknown etiology. Development of IPF is influenced by both genetic and environmental factors. Recent work by our and other groups has identified strong genetic predisposition factors for the development of pulmonary fibrosis while cigarette smoke remains the most strongly associated environmental exposure risk factor. Gene expression profiling studies of IPF lung have taught us quite a bit about the biology of this fatal disease and those in peripheral blood have provided important biomarkers. However, epigenetic marks may be the missing link that connects the environmental exposure in genetically predisposed individuals to transcriptional changes associated with disease development. Moreover, epigenetic marks represent a promising therapeutic target for IPF. In this review, we will introduce the disease, summarize genetic and gene expression studies in IPF, discuss exposures relevant to IPF and known epigenetic changes associated with cigarette smoke exposure, and summarize epigenetic studies conducted so far in IPF. We will end by discussing limitations, challenges and future opportunities in this field.

Keywords

idiopathic pulmonary fibrosis; interstitial pneumonia; cigarette smoke exposure; DNA methylation; histone modifications; miRNAs; gene expression

Introduction to Idiopathic Pulmonary Fibrosis

Pulmonary fibrosis defines a group of fibrosing interstitial lung diseases that can result from environmental exposures (asbestos or silica), connective tissue disease, drug toxicity, or occur as idiopathic pulmonary fibrosis (IPF), in which case the cause is unknown [1]. IPF is a fatal lung disease with a median survival of only 3 years that is characterized by

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progressive scarring of the pulmonary parenchyma, which leads to progressive loss of lung function with dyspnea and hypoxemia, and ultimately respiratory failure and death. The prevalence of IPF is currently estimated at 14 to 43 individuals in 100,000 [2]. IPF increases in prevalence with age [2, 3] and as our population ages, IPF will affect a larger proportion of individuals. Histologically, IPF is characterized by usual interstitial pneumonia (UIP), a fibrosing interstitial pneumonia with a pattern of heterogeneous, subpleural regions of fibrotic and remodeled lung [4]. Development of fibroblastic foci, areas of active fibroproliferation, is a hallmark feature of IPF. Fibroblastic foci consist largely of myofibroblasts, cells that have features of both fibroblasts and smooth muscle cells, are involved in wound healing, and whose differentiation is induced by TGF- β 1. Myofibroblasts appear to be the main source of excessive extracellular matrix (ECM) production in IPF. Another hallmark pathological feature of IPF is microscopic honeycombing, 1- to 2-mm dilated bronchioles surrounded by airless fibrotic lung on a pathologic specimen [5].

It is currently believed that IPF results from aberrant activation of injured alveolar epithelial cells which produce mediators that lead to proliferation of resident fibroblasts, fibrocyte recruitment and epithelial mesenchymal transition (EMT) resulting in the formation of myofibroblastic foci, followed by accumulation of extracellular matrix (ECM), dysregulated wound repair, and lung remodeling [6]. Fibroblasts from these three sources are thought to be the key cell type in the disease pathophysiology [6]; however, the extensive work that has been performed on fibroblast biology has not resulted in any promising therapeutic strategies. It is becoming increasingly clear that the disease process underlying the IPF phenotype is heterogeneous and many different molecular processes may be involved (Table 1).

The disease is likely the result of complex interactions between genetic [7, 8] and environmental factors with exposures such as cigarette smoking [9, 10], or comorbidities such as gastroesophageal reflux and type 2 diabetes [11] being risk factors for development of IPF. In animals, the bleomycin model of lung injury and subsequent fibrosis in mice has been the most commonly used model to decipher the role of specific genetic factors in the development of the disease. However, animal models of pulmonary fibrosis recapitulate some but not all pathological features of disease [12]. For example, microscopic honeycombing is not present in any animal model, and all models lead to resolution of fibrosis whereas lung fibrosis is an irreversible process in humans.

Genetic Basis of Pulmonary Fibrosis

Evidence for a genetic basis to pulmonary fibrosis is substantial with familial aggregation confirmed through a variety of studies in twins, siblings raised apart, and multigenerational families [13]. Pulmonary fibrosis has been associated with private mutations in surfactant protein C [14] and surfactant protein A2 [15], and genes that maintain telomere length (*TERT* and *TERC*) [16]. Based on resequencing across a linkage candidate region, we identified a promoter polymorphism (rs35705950) in the *MUC5B* gene that is strongly associated with familial and sporadic forms of IPF [17]. This finding has been validated in five independent cohorts [18–22]. In lung tissue from subjects with IPF and unaffected controls, *MUC5B* gene expression was upregulated 14.1-fold among IPF subjects compared

to unaffected subjects ($P=0.0001$) [17]. In more recent and extensive gene expression studies, comparing subjects with the variant allele to WT subjects, we observed a 34.1-fold increase in *MUC5B* expression among unaffected subjects and a 5.3-fold increase in *MUC5B* expression among IPF subjects [23]. *MUC5B* immunohistochemical staining in lung tissue showed dense accumulation of *MUC5B* in the terminal bronchioles and areas of microscopic honeycombing [17, 24]. Additional studies by our group demonstrated that the *MUC5B* promoter SNP appears to be predictive [25] and prognostic [26] in IPF. However, the *MUC5B* variant is absent in ~40% of cases, suggesting that other genetic variants and/or environmental exposures contribute to disease risk. Thus, we conducted a genome-wide association study of non-Hispanic, white individuals with fibrotic idiopathic interstitial pneumonias (IIPs; $n = 1,616$) and controls ($n = 4,683$), with follow-up replication analyses in 876 cases and 1,890 controls [20] and confirmed association with *TERT* at 5p15, *MUC5B* at 11p15 and the 3q26 region near *TERC*, and identified seven newly associated loci ($P_{meta} = 2.4 \times 10^{-8}$ to 1.1×10^{-19}), including *FAM13A* (4q22), *DSP* (6p24), *OBFC1* (10q24), *ATP11A* (13q34), *DPP9* (19p13) and chromosomal regions 7q22 and 15q14–15. These results suggest that genes involved in host defense, cell-cell adhesion and DNA repair contribute to risk of fibrotic idiopathic interstitial pneumonias (IIPs). Interestingly, these variants are similarly associated with the development of sporadic and familial forms of disease pointing to the common genetic basis for pulmonary fibrosis.

Role of Environmental Exposures in Pathophysiology of IPF

Epidemiological studies have demonstrated associations of exposures to inhaled environmental agents and the development of IPF; these include exposure to cigarette smoke, wood dust, metal dust, silica, textile dust, and possibly agriculture, farming and livestock [9, 11]. Cigarette smoke is the most prevalent exposure that has been linked to the development of IPF. Ever having smoked cigarettes remains a risk factor for the development of IPF [27] even after smoking cessation, suggesting that the fibroproliferative process or response (e.g. gene expression profile) induced by cigarette smoke-related lung injury may at some point become self-sustaining[28]. Smoking history has also been associated with poorer survival among patients with IPF [29–31]. For the familial form of pulmonary fibrosis, we have shown that cigarette smoking was the strongest risk factor for the development of disease[13]. After adjusting for age and sex, patients who had ever smoked cigarettes were at greater risk for development of familial interstitial pneumonia (FIP) ($OR=3.6$; 95% $CI=1.3-9.8$). This finding suggests that although a certain genotype places an individual at risk to develop pulmonary fibrosis, cigarette smoking contributes significantly to the development of disease.

Gene Expression Studies in IPF

Gene expression profiling studies have demonstrated that transcriptional changes are present in the lung parenchyma of individuals with IPF [32–38]. Gene expression changes are quite dramatic and involve large numbers of genes, generally on the order of a few thousand differentially expressed genes. In aggregate, these studies have consistently identified similar genes and pathways that are differentially expressed in fibrotic lungs, namely, genes associated with extracellular matrix formation, degradation, and signaling, smooth muscle

markers, growth factors, and genes encoding immunoglobulins, complement, and chemokines. Another prominent change in IPF lung is in expression of developmental pathways - Sonic Hedgehog (SHH) [39, 40], Fibroblast Growth Factor (FGF) [41–43], Platelet-derived Growth Factor (PDGF) [44, 45], and canonical/non-canonical WNT [46–50].

Some expression studies have successfully identified transcriptional profiles associated with rapid disease progression and acute exacerbations in IPF [33, 34, 37]. More recent work from our laboratory identified two molecular subtypes of IPF based on a strong gene expression signature that contains a number of genes that have been previously shown to be upregulated in IPF but is most strongly enriched for cilium genes (Gene Ontology (GO) category 0005929; Benjamini corrected p value 3.7×10^{-11}) and their structural components (axoneme, 3.9×10^{-11} , dynein, 9.4×10^{-7}) [51]. The cilium gene signature was validated in multiple lung specimens from the same subjects and in an independent cohort of subjects with IPF. In addition to being associated with higher expression of *MUC5B* and matrix metalloproteinase 7 (*MMP7*), higher cilium gene expression is also associated with the presence of microscopic honeycombing but not fibroblastic foci.

In addition to gene expression changes in the lung parenchyma, several biomarkers have emerged from peripheral blood studies. Rosas and colleagues studied the peripheral blood in IPF [52] using a targeted proteomic approach and identified a protein signature consisting of MMP1, MMP7, MMP8, IGFBP1 and TNFRSA1F. They established that this signature was able to distinguish IPF from healthy controls with a sensitivity of 98.6% and specificity of 98.1%. A more recent study demonstrated that high concentrations of MMP7, ICAM1, IL8, VCAM1, and S100A12 in the plasma predict poor survival among patients with IPF [53]. Other studies have used microarrays to identify transcriptional signatures in peripheral blood associated with the presence and the extent of disease [54] and transplant-free survival [55].

Epigenetic Regulation of Gene Expression

Epigenetic processes translate environmental exposures associated with disease risk into regulation of chromatin, which shapes the identity, gene expression profile, and activity of specific cell types that participate in disease pathophysiology [56]. Epigenetic mechanisms are emerging as key mediators of the effects of both genetics and the environment on gene expression and disease [57, 58]. In addition to a set of inherited epigenetic marks, there are likely non-heritable epigenetic marks that are more dynamic and change in response to environmental stimuli.

Traditionally epigenetic processes refer to DNA methylation and histone modifications although noncoding RNAs are often considered a part of the epigenome. Methylation of cytosine residues in CpG dinucleotides within the context of CpG islands is the simplest form of epigenetic regulation; hypermethylation of CpG islands in gene promoters leads to gene silencing while hypomethylation leads to active transcription [59, 60]. More recent studies have demonstrated that methylation of less CpG dense regions near islands ('CpG island shores') [61, 62] and within gene bodies [63, 64] is also important in regulation of

gene transcription and alternative splicing. Methylation, acetylation, phosphorylation, and ubiquitylation[56] of histone tails occur at specific sites and residues, and control gene expression by regulating DNA accessibility to RNA polymerase II and transcription factors (Table 2). H3K4 trimethylation (H3K4me3), for example, is strongly associated with transcriptional activation whereas H3K27 trimethylation (H3K27me3) is frequently associated with gene silencing[65]. Similarly, histone tail acetylation leads to active gene transcription while deacetylation is a repressive mark and leads to gene silencing. Histone acetyltransferases (HATs) are enzymes that acetylate histone tails while histone deacetylases (HDACs) remove acetyl groups from histone tails. Bromodomain (Brd) proteins are chromatin readers that recognize and bind acetylated histones and play a key role in transmission of epigenetic memory across cell divisions and transcription regulation[66]

MicroRNAs (miRNAs) are ~22 nucleotide long regulatory RNAs that control gene expression by binding to the 3' untranslated regions (UTRs) of messenger RNA (mRNA), which leads to either mRNA degradation or inhibition of protein translation [67]. miRNAs, which were first identified in 1993 [68], are evolutionarily conserved and are the most extensively studied family of small non-coding RNAs. Other ncRNAs, such as PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), and large intergenic non-coding RNAs (lincRNAs) are emerging as key elements that regulate gene expression both in normal and diseased cell states [69].

Unlike an individual's genetic make-up, epigenetic marks can be influenced much more easily by environmental exposures, diet, and ageing [70]. Randy Jirtle's seminal experiments showed that maternal diet supplemented with methyl donors (folic acid, vitamin B12, choline and betaine) shifts coat color distribution of progeny towards the brown pseudoagouti phenotype, and that this shift in coat color resulted from an increase in DNA methylation in a transposon adjacent to the agouti gene [70, 71]. These studies also revealed that mice with yellow coat color are obese and are more prone to develop cancer, suggesting for the first time that changes in DNA methylation caused by diet may be linked to disease development. Other studies have since shown that exposures such as pesticides and fungicides [72] and PM2.5 particles [73] could alter the methylome, and that ageing is also associated with changes in DNA methylation, histone modifications, and gene expression [74, 75]. The association of ageing and epigenetics is especially important for IPF. Genome-wide studies in aging cells and tissues have revealed the occurrence of stochastic changes in DNA methylation, also referred to as drift, that creates epigenetic mosaicism in aging stem cells. This could potentially restrict their plasticity and worsen phenotypes that lead to the development of ageing related diseases such as IPF[76].

Moreover, an individual's genetic background influences epigenetic marks in two ways – by direct inheritance (imprinted loci) [77] and by genetic variants that segregate with disease exerting their effects through epigenetic modifications, such as the case of haplotype-specific methylation. In addition to investigation at specific loci, genomewide studies demonstrate a strong genetic component to inter-individual variation in methylation [78–80] and histone modification profiles [81–83].

Modulation of Epigenetic Marks by Exposure to Cigarette Smoke

There is extensive evidence for the influence of cigarette smoke exposure on the epigenome. Early studies demonstrated the link between exposure to tobacco smoke and lung cancer via methylation of CpG islands in cancer genes such as p16 [84]. Other studies have shown that cigarette smoke has an influence on the methylome [85–87] and on methylation of specific promoters in genes involved in pathogenesis of IPF such as *WNT7A* [88]. Importantly, recent work identified extensive genomic changes in DNA methylation in small airway epithelium (SAE) of smokers compared to smokers with corresponding modulation of gene expression [89]. Other recent studies have shown how cigarette smoke influences histone modifications and chromatic accessibility. Acrolein, a component of cigarette smoke and a potential major carcinogen forms adducts with histone proteins and preferentially reacts with free histones rather than with nucleosomal histones in BEAS-2B and A549 cell lines [90]. Furthermore, acrolein exposure significantly reduces total H3 acetylation as well as specific H3K9 and H3K14 acetylation at several genes/loci, as well as H3K4 trimethylation. Exposure of NHBE cells to cigarette smoke condensate (CSC) resulted in dose- and time dependent decrease of H4K16Ac and H4K20Me3, and increase of H3K27Me3, decreased DNA methyltransferase 1 (DNMT1) and increased DNMT3b expression as well as time-dependent hypomethylation of LINE-1 repeats and hypermethylation of specific tumor suppressor genes that are frequently silenced in human lung cancers [91]. Finally, CSC represses expression of E-cadherin in A549 and BEAS-2B cell lines by regulating transcription factors LEF-1 and SLUG, which leads to epithelial-mesenchymal transition (EMT) but HDAC inhibitor MS-275 can restore E-cadherin expression and reduce EMT [92]. These studies collectively demonstrate a strong influence of cigarette smoke exposure on epigenetic marks.

Cigarette smoke exposure has also been shown to have a significant influence on expression of miRNAs [93–95]. Comparing current to never smokers, 28 miRNAs were differentially expressed, mostly downregulated in human bronchial airway epithelium of smokers [93]. miR-218 was found to be one of the strongly associated miRNA with cigarette smoke exposure and it was further shown that a change in miR-218 expression in primary bronchial epithelial cells and H1299 cell line resulted in a corresponding negatively correlated change in expression of predicted mRNA targets for miR-218. Two recent studies also demonstrated changes in miRNA expression in lungs of mice [95] and rats [94] exposed to cigarette smoke. All three studies showed the predominant effect of smoke exposure is down-regulation of miRNAs, with substantial overlap between mice and rats and some overlap of rodent miRNA expression changes in the lung with those observed in human airway epithelium. However, the mechanisms linking cigarette smoke to any of these epigenetic changes have not been clearly defined; thus, raising uncertainty about the cause and effect relationship between cigarette smoke and epigenetic marks. Finally, *in utero* exposure to cigarette smoke results in differential methylation [96–98] and downregulation of miRNAs [99] in the placenta, cord blood or peripheral blood of children, suggesting transgenerational effects of smoke exposure.

Epigenetic studies in IPF

Epigenetic mechanisms are likely to be involved in the control of gene expression in IPF, especially given the association of IPF with cigarette smoking and the relationship between cigarette smoke and changes in DNA methylation, histone modifications, and miRNAs. Moreover, these epigenetic changes are likely to be important factors in determining transcriptional profiles that directly contribute to pathogenic features of this disease (Figure 1).

Targeted Studies

Several targeted studies have shown that epigenetic modulation regulates expression of genes involved in pathogenesis of IPF. Defective histone acetylation is responsible for the repression of expression of two antifibrotic genes, cyclooxygenase-2 (COX2) [100] and chemokine IP-10 [101]. Similarly, Thy-1 (CD90) is an important regulator of cell–cell and cell–matrix interactions that is expressed on normal lung fibroblasts but its expression is absent in myofibroblasts within fibroblastic foci (FF) in IPF. Thy-1 downregulation in rat lung fibroblasts is controlled by both promoter DNA hypermethylation [102] and histone modifications [103]. Thy-1 hypermethylation and downregulation of expression have also been observed in human lung fibroblasts as a result of hypoxia [104]. Moreover, hypermethylation-mediated silencing of *p14(ARF)* expression [105] and decreased Fas expression by histone modifications (methylation and acetylation) [106] have also been proposed as a mechanism of IPF lung fibroblast resistance to apoptosis.

Different levels of methylation of three CpG islands in the promoter of the α -smooth muscle actin (α -SMA) in fibroblasts, myofibroblasts, and alveolar epithelial type II cells were shown to correlate with expression of the α -SMA gene in these different cell types [107]. This study also demonstrated that pharmacological and siRNA mediated inhibition of DNA methyltransferase activity induced expression of α -SMA in fibroblasts while overexpression of DNA methyltransferase suppressed α -SMA gene expression. Inhibition or overexpression of DNA methyltransferase also affected TGF- β 1-induced myofibroblast differentiation. A more recent study from the same group showed that methyl CpG binding protein 2 (MeCP2) binds to the α -SMA gene [108], and that suppression or overexpression of MeCP2 leads to changes α -SMA gene expression in fibroblasts. Furthermore, MeCP2-deficient mice exhibited significantly decreased alveolar wall thickness, inflammatory cell infiltration, interstitial collagen deposition, and myofibroblast differentiation in response to bleomycin. Taken together, these data strongly suggest DNA methylation as an important mechanism that regulates expression of the α -SMA and fibroproliferation.

Dakhlallah *et al.* identified an epigenetically controlled feedback loop that contributes to the IPF fibroblast phenotype and ECM deposition [109]. Specifically, they demonstrated that increased DNA methylation in the promoter of the miR-17~92 cluster abolishes expression of this cluster which in turn results in upregulation of genes that are a hallmark of fibroproliferative response in IPF, more fibrotic phenotypes of lung fibroblasts, and upregulation of DNA methyltransferase 1 (DNMT1), which in turn can methylate the CpG motifs in the promoter of miR-17~92. Collectively, these targeted studies demonstrate that

DNA methylation and histone modifications regulate expression of mRNAs and miRNAs that are crucial in the development of fibroproliferative lung disease.

Epigenomic Profiles

Epigenomic studies of DNA methylation profiles in IPF lung are emerging. Two published studies have examined methylation patterns within CpG islands. Rabinowitz *et al.* showed that 625 CpG islands are differentially methylated between IPF (n=12) and control (n=10) lungs [110] using methylated DNA immunoprecipitation (MeDIP) and Agilent CpG island arrays. Comparison of IPF methylation patterns to lung cancer (n=10) revealed that IPF lungs display an intermediate methylation profile, partly similar to lung cancer with 402 differentially methylated CpG islands overlapping between IPF and cancer. Yan and colleagues profiled bisulfite-converted DNA from lung tissue of 12 IPF patients and 7 controls on the Illumina 27k chip [111]. While they identified 835 differentially methylated CpG motifs, only 35 of them are associated with differential expression and 16 have inverse relationship with expression. These results are somewhat surprising but are likely reflective of the limitations of only examining CpG islands and the small sample size. The Lung Genomics Research Consortium (LGR; lung-genomics.org) has profiled lung tissue DNA from 100 individuals with IPF and 79 controls on Comprehensive High-Throughput Arrays for Relative Methylation (CHARM) [112, 113]. Our results revealed that the majority of the 2130 differentially methylated regions (DMRs) are located within CpG island 'shores', areas of lower CpG density near CpG islands and are contained within genes [114]. Our analysis demonstrates more extensive overlap in methylation and expression and a statistically significant enrichment for inverse relationships of methylation and expression. While these early studies of genomewide profiles are uncovering important information on regulation of expression by methylation in IPF lung, no genomewide histone modification studies have been done in IPF. This is largely due to the fact that it is difficult to obtain fresh cells to perform chromatin immunoprecipitation (ChIP) on specific cell types.

A number of very recent publications studied genomic miRNA profiles in lung tissue from patients with IPF [115–119] and identified several miRNAs with role in fibroproliferation, EMT, and the TGF- β 1 signaling pathway. Similar to extensive transcriptional changes in IPF lung, 10% of miRNAs are differentially expressed in lung tissue from subjects with IPF compared to nondisease controls. Among downregulated miRNAs in IPF are let-7d and miR-29 while miR-155 and miR-21 are upregulated in IPF.

Given the prominent role of TGF- β 1 signaling in fibroproliferation, Pandit and colleagues scanned promoters of differentially expressed miRNAs in IPF for SMAD binding elements (SBE) and focused on one of the miRNAs whose promoter contains SBE, let-7d [115]. They showed that TGF- β 1 down-regulated let-7d expression, and SMAD3 binding to the let-7d promoter was demonstrated. Inhibition of let-7d *in vitro* and *in vivo* by an antagomir for the let-7 family resulted in upregulation of mesenchymal and downregulation of epithelial markers, suggesting a role for the let-7 family of miRNAs in prevention of EMT and profibrotic phenotype.

Cushing *et al* examined expression of miRNAs in lungs of bleomycin-treated mice and demonstrated reduced expression of miR-29 in response to bleomycin [117]. Inhibition of

miR-29 in human fetal lung fibroblasts led to upregulation of a number of genes associated with fibrotic phenotype including its predicted targets, genes up-regulated by TGF- β 1 as well as genes independent of TGF- β 1, including laminins and integrins. Although the authors did not examine expression of miR-29 in human lung tissue, this miRNA is downregulated in IPF lung in the dataset from Pandit et al [115]. These data suggest a prominent role for miR-29 in lung fibrosis by regulation of expression of TGF- β 1-inducible or other fibrotic genes.

The study by Liu et al [116] established up-regulation of miR-21 in the lungs of mice treated with bleomycin and in the lungs of patients with IPF with expression primarily localized to myofibroblasts. Inhibition of miR-21 expression diminished the severity of bleomycin-induced lung fibrosis in mice while TGF- β 1 enhanced miR-21 expression in primary lung fibroblasts. Overexpression of miR-21 promoted whereas inhibition of miR-21 attenuated the pro-fibrogenic activity of TGF- β 1 in fibroblasts, suggesting a feed-forward loop in which miR-21 amplifies TGF- β 1 signaling and fibrosis [120].

Pottier et al examined expression profiles of miR-155 in human lung fibroblasts stimulated with different cytokines and showed that upregulation of miR-155 correlated with downregulation of a number of its target genes and that transfection of miR-155 led to fibroblast migration [118]. Among fibroblast-selective targets was keratinocyte growth factor (KGF, FGF-7F) and functional *in vitro* assays experimentally validated that miR-155 can efficiently target KGF 3'-UTR. The authors also demonstrated increased expression of miR-155 in lungs of mice with bleomycin-induced fibrosis, a finding that is also supported by the fact that miR-155 is upregulated in IPF lungs in the study by Pandit et al. [115]. A recent re-analysis of published miRNA data and TargetScan-predicted targets identified a network of dysregulated miRNAs and their direct targets that belong to pathways associated with IPF (Figure 2; [120]).

miRNA expression in IPF lung has also been correlated with disease severity; five miRNA (miR-302c, miR-423, miR-210, miR-376c, and miR-185) are increased in lung biopsies of rapidly versus slowly progressing IPF patients [119]. Further studies are needed to provide a functional link between differential expression of these miRNAs and IPF phenotypes.

Conclusions, Future Opportunities, and Challenges

Evidence for the role of epigenetic regulation of gene expression in the development of IPF is based on studies demonstrating association of epigenetic marks with exposures such as cigarette smoke and targeted studies of epigenetic marks in specific genes relevant to the pro-fibrotic phenotype. These studies provide strong support for studies of the IPF epigenome. Genomic studies of miRNA expression patterns have identified a number of miRNA that regulate expression of key genes involved in pathogenesis of IPF. Genomic studies of DNA methylation are just emerging and those examining histone modifications are lacking at this time.

Given the strength of association of recently identified genetic variants to IPF combined with the evidence for the genetic component to methylation [78–80] and histone

modification profiles [81–83], one of the most interesting and potentially fruitful area of investigation in the near future will be integration of genetic and epigenetic data to identify regulatory modules that lead to extensive changes in gene expression profiles in IPF lung. In addition to common variants that have been uncovered using high-density genotyping chips, sequencing studies will likely identify rare variants that may also interact with epigenetic marks.

Another important area of investigation will be to fully elucidate the role that cigarette smoke exposure plays in modifying epigenetic marks that lead to transcriptional changes in IPF lung. While there is substantial evidence for the influence of cigarette smoke on epigenetic marks in cell and animal studies, there are significant challenges associated with linking cigarette smoke exposure to changes in the IPF lung epigenome. One of them is the fact that self-reported smoking histories are often inaccurate. Another challenge is that lung tissue we use for genomic profiling is obtained late in the disease and that many different processes contribute to the extensive transcriptional changes that are observed. A related challenge is the dynamic nature of epigenetic marks; the epigenome needs to be considered in the context of other diseases, exposures, diet, and age. Unraveling relative contributions of the different processes is a significant challenge and will require a combination of cell and animal studies together with human samples collected earlier in the disease process, to the extent that this is feasible. Another interesting and challenging aspect of understanding the role of the epigenome in mediating the effects of cigarette smoke exposure will be to delineate the effect of cigarette smoke on the development of pulmonary fibrosis, COPD, and lung cancer. While there is some overlap among these three cigarette smoke related diseases, there are many more differences in pathologic and molecular features [121].

Similar to studies that have uncovered changes in gene and protein expression in peripheral blood of patients with IPF, there is an opportunity to identify epigenetic signatures of exposure and disease. Given that DNA is more stable and easier to collect than RNA, and that DNA methylation is easily measured, DNA methylation marks in peripheral blood may prove to be important biomarkers that could be used to identify the presence and the extent of disease as well as outcome.

All epigenomic studies published to date in IPF have used array technologies to assess genomewide methylation or miRNA patterns. Technologies for collecting epigenomic profiles are either array- or next-generation-sequencing-based and have been reviewed elsewhere [57, 122, 123]. Next generation sequencing technologies are rapidly becoming more affordable and are likely to be employed in the next wave of studies of epigenetic mark in IPF. They will provide not only better coverage and more accurate data for known epigenetic marks but they will allow for discovery of novel marks; this is especially true in the area of noncoding RNAs where many short and long noncoding RNAs are yet to be identified.

The major challenge that plagues all epigenetic studies is cell specificity. Epigenetic marks are cell type specific yet much of the research in this area has been done on the whole lung tissue because isolation of enough material for specific cell types is often not feasible in human subjects. One approach to address this concern may be to identify epigenetic marks

in the whole lung and then attribute them to specific types using immunohistochemistry or confocal microscopy with antibodies to specific epigenetic marks. The disadvantage of this approach is that many important epigenetic marks may be missed in the initial screen because the change in the whole lung may be below detection limits of assays utilized. Another approach is to isolate specific cell types from fresh lung biopsies, which presents feasibility challenges for large scale studies. This same issue arises in gene expression data and methods to decompose whole tissue expression into cell-specific components [124] have been developed. Similarly, methodology for decomposition of DNA methylation data in peripheral blood has been developed [125, 126] and could be useful in the future for decomposition of cell specific data in lung tissue.

Another major challenge with epigenomics is to integrate the epigenetic mechanisms that affect transcription and translation. For example, evidence for cross-talk between DNA methylation and histone modifications has been rapidly accumulating [127–130]. Each of the three epigenetic mechanisms is independently complex but when combined, the complexity of these interactions presents unique experimental and analytic challenges. Together with the complexity of exposures and genetic factors that contribute to the disease process both through epigenetic marks and independently, understating the mechanisms that regulate IPF lung transcriptome will require sophisticated computational approaches. Expression quantitative trait loci (eQTL) mapping approaches [131] can be applied to identify genetic variants that underlie methylation status (methyl-QTL) or methylation marks that control expression changes (methyl-expression QTL). Similarly, co-expression network analysis strategies that have been developed for expression analysis can be applied to epigenomic analysis [132, 133].

Despite these many challenges, using epigenomic profiling to understand the dynamic biology in IPF lung, and applying this knowledge to the development of novel diagnostic and therapeutic approaches represent promising approaches for patients with this fatal disease. The discovery of key epigenetic marks is likely to lead to a new way of treating pulmonary fibrosis as changes to a gene's methylation or histone modifications can be reversed, and reversal of epigenetic marks can lead to changes in expression. Drugs targeting DNA methylation such as 5-aza-cytidine (AZA) and decitabine (DAC) used in low doses improved survival and led the FDA to approve AZA (Vidaza) and DAC (Dacogen) for the treatment of myelodysplastic syndrome [134–136]. Clinical trials of DNA methyltransferase (DNMT) inhibitors have been extended from leukemias to solid organ cancers [137]. Furthermore DNMT inhibitors have now been combined with histone deacetylase (HDAC) inhibitors for treatment of lung cancer [138]. While current DNA methylation therapies are not locus specific, we expect such locus-specific therapies to become available in the near future. One early example of a targeting approach relies on recently discovered RNA transcripts that interact with DNMT1 [139] and others that rely on various genome editing technologies [140, 141] are being developed. Moreover, current FDA-approved and in development histone mark modifying drugs are effective in targeting specific pathways [66] and treating diseases such as lung cancer [142]. First generation drugs affect multiple pathways simultaneously (ie. SAHA), while more recently developed treatments target specific enzymes or pathways (ie. GSK-J4).

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Figure 1.

An overview of epigenetic regulation of gene expression in IPF lung. Environmental exposures such as cigarette smoke influence epigenetic marks which in turn regulate gene expression. It is feasible that exposures influence gene expression by other mechanisms. Similarly, underlying genetic variation (asterisk) can regulate gene expression by affecting epigenetic marks or by other mechanisms (alteration of transcription factor binding sites, for example). Alterations in epigenetic marks have consequences on expression of key genes and pathways (Table 1) that lead to the clinical presentation, radiological (top panel) and pathologic (middle and bottom panels; fibroblastic foci [ff] and microscopic honeycombing [m]) features of IPF.

Table 1

Summary of cellular and molecular processes that have been observed in the IPF lung.

Process	Reference
Epithelial-mesenchymal transition (EMT) of type II alveolar cells	[143–145]
Growth factor (GF) regulation	[146]
Apoptosis	[147, 148]
Oxidative stress	[149]
Endoplasmic reticulum (ER) stress	[150, 151]
Cellular senescence associated with aging/telomere shortening	[16, 152, 153]
Epithelial stem cell exhaustion	[154]
Intra-alveolar coagulation	[155]
Aberrant recapitulation of developmental pathways -Sonic Hedgehog (SHH), Fibroblast Growth Factor (FGF), Platelet-derived Growth Factor (PDGF), canonical/non-canonical WNT	[39–45, 156] [46–50]
Impaired mucociliary clearance (MCC) and host defense	[17, 157]

Table 2

Most commonly studied histone modifications that regulate gene expression by controlling chromatin accessibility at different genomic elements.

Histone Mark	Putative Functions (from the ENCODE project)[156]
H3K4me1	Mark of regulatory elements associated with enhancers and other distal elements, but also enriched downstream of transcription starts
H3K4me2	Promoters and enhancers
H3K4me3	Promoters/transcription starts
H3K9ac	Mark of active regulatory elements with preference for promoters
H3K9me1	Preference for the 5' end of genes
H3K9me3	Repressive mark associated with constitutive heterochromatin and repetitive elements
H3K27ac	Mark of active regulatory elements; may distinguish active enhancers and promoters from their inactive counterparts
H3K27me3	Repressive mark established by polycomb complex activity associated with repressive domains and silent developmental genes
H3K36me3	Elongation mark associated with transcribed portions of genes, with preference for 3' regions after intron 1
H3K79me2	Transcription-associated mark, with preference for 5' end of genes
H4K20me1	Preference for 5' end of genes