

*EZH2 and G9a regulate CXCL10 repression in IPF***Interplay between EZH2 and G9a Regulates CXCL10 Gene Repression in Idiopathic Pulmonary Fibrosis**

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Abstract

Selective repression of the antifibrotic gene CXCL10 contributes to tissue remodelling in idiopathic pulmonary fibrosis (IPF). We have previously reported that histone deacetylation and histone H3 lysine 9 (H3K9) methylation are involved in CXCL10 repression. This study explored the role of H3K27 methylation and the interplay between the two histone lysine methyltransferases, Enhancer of Zest Homolog 2 (EZH2) and G9a, in CXCL10 repression in IPF. By applying chromatin immunoprecipitation (ChIP), Re-ChIP and proximity ligation assays, we demonstrated that, like G9a-mediated H3K9 methylation, EZH2-mediated H3K27me3 was significantly enriched at the CXCL10 promoter in fibroblasts from IPF lungs (F-IPF) compared with fibroblasts from non-fibrotic lungs (F-NL) and that EZH2 and G9a physically interacted with each other. EZH2 knockdown reduced not only EZH2 and H3K27me3 but also G9a and H3K9me3 and G9a knockdown reduced not only G9 and H3K9me3 but also EZH2 and H3K27me3. Depletion and inhibition of EZH2 and G9a also reversed histone deacetylation and restored CXCL10 expression in F-IPF. Furthermore, treatment of F-NL with the profibrotic cytokine TGF- β 1 increased EZH2, G9a, H3K27me3, H3K9me3 and histone deacetylation at the CXCL10 promoter, similar to that observed in F-IPF, which was correlated with CXCL10 repression and was prevented by EZH2 and G9a knockdown. These findings suggest that a novel and functionally interdependent interplay between EZH2 and G9a regulates histone methylation-mediated epigenetic repression of the antifibrotic CXCL10 gene in IPF. This interdependent interplay may prove to be a target for epigenetic intervention to restore the expression of CXCL10 and other anti-fibrotic genes in IPF.

Keywords: histone methylation; CXCL10; gene expression; lung fibroblast; pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a deadly respiratory disease of unknown aetiology with a median survival of 3–4 years and a lack of effective therapy (1). IPF is characterized by the accumulation of activated lung fibroblasts (myofibroblasts) that are ultimately responsible for the excessive deposition of collagen, leading to distortion of the alveolar architecture, loss of lung function, and ultimately death (2). Fibroblasts isolated from IPF lungs (F-IPF, activated lung fibroblasts) are phenotypically different from those from non-fibrotic lungs (F-NL) as they express higher levels of α -smooth muscle actin (α -SMA) (3) and lower levels of antifibrotic genes such as cyclooxygenase-2 (COX-2) (4-6), Thy-1 (7, 8), Caveolin-1 (9) and CXCL10 (or interferon- γ -inducible protein of 10 kDa (IP-10)) (10, 11). Recent studies strongly suggest that epigenetic dysregulation plays a key role in the targeted repression of these antifibrotic genes (4, 5, 7, 10, 12).

CXCL10 is a strong inhibitor of angiogenesis (13) and can be induced by IL-1 β and IFN- γ (10), but not by the profibrotic cytokine TGF- β 1 (14). It has been shown that lung fibroblasts and tissues from IPF patients and the murine model of bleomycin-induced pulmonary fibrosis express significantly less CXCL10 than normal cells and tissues and that CXCL10 inhibits angiogenesis, lung fibroblast migration and proliferation and subsequent lung fibrosis (11, 15, 16, 17). In contrast, blockade of CXCL10 increases pulmonary fibrosis (15, 16) and genetically targeting the CXCL10 receptor CXCR3 results in increased mortality with progressive interstitial fibrosis in bleomycin-induced pulmonary fibrosis (18). These observations strongly suggest that CXCL10 deficiency, as a result of gene repression, plays a key role in the aberrant tissue remodeling and the development of pulmonary fibrosis.

TGF- β 1 is a major activator of lung fibroblasts both *in vitro* (19) and *in vivo* (20). Although TGF- β 1 treatment reduces the expression of antifibrotic genes (e.g. Caveolin-1) in human lung fibroblasts (9), whether it leads to alterations of epigenetic modifications of antifibrotic genes similar to those observed in F-IPF has not been explored.

Methylation of histone proteins at specific lysine residues plays a major role in the regulation of gene expression and repression. The most well-characterized histone methylations are histone H3 lysine 27 trimethylation (H3K27me3) and H3K9me2/3, which are generally correlated with gene repression (21).

H3K27me₃ is catalyzed by the histone lysine methyltransferase (KMT) Enhancer of Zest Homolog 2 (EZH2), the catalytic subunit of polycomb repressive complex 2 (PRC2) (22). In contrast, lysine-specific demethylase (KDMs), such as KDM6A and KDM6B (also known as jumonji domain-containing protein 3 (JMJD3)), are enzymes capable of specifically demethylating H3K27me₃ at gene promoters to de-repress gene transcription (23, 24). H3K9me_{2/3} is catalyzed by the H3K9 specific KMTs, such as G9a and G9a-Like Protein (GLP) (25). It has been shown that G9a can methylate H3K27 *in vitro* and *in vivo* (26, 27), suggesting a crosstalk between H3K9 and H3K27 methylations. A recent study has also demonstrated that G9a enzymatic activity can mediate EZH2 recruitment to regulate the repression of a subset of genes that are common targets of both enzymes (28), thus providing a direct proof of a functional interplay between G9a and EZH2. We have previously demonstrated that histone deacetylation and H3K9 methylation, but not DNA methylation, are involved in CXCL10 repression in IPF (10). However, whether H3K27 methylation by EZH2 impacts on CXCL10 repression in IPF and whether EZH2 enzymatic activity is required for G9a-mediated gene repression remains unknown. In this study we explored the role of EZH2-mediated H3K27 methylation and the interplay between EZH2 and G9a in CXCL10 epigenetic repression in F-IPF. We found that EZH2 and G9a physically interact with each other at the CXCL10 promoter in F-IPF and that the interplay between EZH2 and G9a plays a key role in the epigenetic repression of CXCL10 in IPF. Some of the results of these studies have been previously reported in the form of an abstract (29).

Materials and Methods

Cell Culture

Fibroblasts from IPF lungs (F-IPF) and fibroblasts from non-fibrotic lungs (F-NL) were supplied by Dr Feghali-Bostwick (University of Pittsburgh) and were described before (10, 30). IL-1 β (R&D Systems, Abingdon, Oxfordshire, UK) was used to induce CXCL10 expression (10). For TGF- β 1 treatment, F-NL were incubated with TGF- β 1 (R&D Systems) for 3 days before analyses or treatment with IL-1 β and siRNAs.

Chromatin Immunoprecipitation (ChIP) and Re-ChIP

ChIP was performed using ChIP-IP express kit and Re-ChIP-IT express kit (Active Motif, La Hulpe, Belgium) as described (5, 10) with antibodies against H3K27me3, H3K9me3, EZH2, acetylated and total H3 and H4 (Merck Millipore, Billerica, MA), and G9a (Santa Cruz, Dallas, TX). Re-ChIP was conducted with chromatin from the first ChIP using antibodies against the secondary targets.

JMJD3 Overexpression

80% confluent F-IPF were incubated with pMSCV vectors encoding human JMJD3 (pMSCV-JMJD3) or a mutant variant lacking catalytic activity (pMSCV-mJMJD3) (Adgene, Cambridge, MA) (31) or empty vectors (1 μ g), pre-mixed 2:1 with TransFast (Promega, Madison, WI) for 72 h before being treated with IL-1 β for 4 and 24 h. mJMJD3 and JMJD3 overexpression was assessed by Western blotting with an antibody recognizing both JMJD3 and mJMJD3 (Santa Cruz).

Inhibitor Study and siRNA Transfection

F-IPF were pre-treated with the EZH2 inhibitor 3-Deazaneplanocin A (DZNep) (32) (Cayman Chemical, Ann Arbor, MI) (10 nM) for 3 days before being treated with IL-1 β for 4 and 24 h for epigenetic analyses and for CXCL10 mRNA and protein expression by real-time RT-PCR and ELISA, respectively (10).

F-IPF and F-NL were transfected with predesigned siRNAs against human EZH2 (Hs_EZH2_3 FlexiTube siRNA, target sequence: CAGACGAGCTGATGAAGTAAA) and G9a (Hs_BAT8_3 FlexiTube siRNA, target sequence: CACCATGAACATCGATCGCAA), and ALLStars negative control siRNA (Qiagen, Hilden, Germany) using HiPerFect transfection reagent (Qiagen) (5). Cells were then serum-starved for 24 h before treatment with TGF- β 1 and IL-1 β for further analyses.

Proximity Ligation Assay (PLA)

Serum-starved F-IPF on glass coverslips were fixed in 4% paraformaldehyde and treated with 0.3% Triton X-100. EZH2 and G9a interaction was detected with Duolink *In Situ* Red Starter Kit Mouse/Rabbit (Olink Bioscience, Uppsala, Sweden) following the manufacturer's instructions. Briefly, after blocking, slides were incubated with primary mouse anti-EZH2 antibody (1:200, clone AC22, Merck Millipore) and rabbit anti-G9a antibody (1:200, ab133482, Abcam, Cambridge, UK) overnight at 4°C. Slides were washed and then incubated with PLA probes anti-Mouse MINUS and anti-Rabbit PLUS, respectively, for 1 hour at 37°C. After removal of unbound probes, slides were incubated with Duolink Ligase for 30 min at 37 °C followed by rolling circle amplification. Amplified DNA was detected by Duolink *In Situ* Detection Reagent Red. Nuclei were stained with Dapi. Images were taken with Nikon 90i fluorescence imaging microscope.

Statistical Analysis

Data were presented as mean \pm SEM from experiments with 3-6 separate cell lines. Data were examined for normality with Shapiro-Wilk and Pearson Chi Squared tests. ANOVA was used to evaluate statistical significance of the mean values between F-NL and F-IPF, this was followed by Student's t-test for comparisons at specific time points. $P < 0.05$ was regarded as statistically significant.

Results

H3K27me3 is Increased at the CXCL10 Promoter in F-IPF and is Reduced by EZH2 Inhibition and JMJD3 Overexpression

We have previously shown that H3K9me3 is increased at the CXCL10 promoter in F-IPF compared with F-NL (10). As H3K27me3 is another typical histone methylation mark linked to repressive chromatin, we went on to analyze its presence at the CXCL10 promoter by ChIP assay. It was revealed that under both unstimulated and IL-1 β -stimulated conditions H3K27me3 at the CXCL10 promoter was markedly elevated in F-IPF compared with F-NL (Figure 1A). In addition, the association of EZH2, the KMT responsible for H3K27me3, with the CXCL10 promoter was also consistently increased in F-IPF compared with F-NL with or without IL-1 β treatment (Figure 1B). The data suggest that H3K27, like H3K9, is also hypermethylated at the CXCL10 promoter in F-IPF as a result of increased recruitment of EZH2. To determine whether EZH2 inhibitors and the specific H3K27me3 demethylase JMJD3 could modulate H3K27me3 at the CXCL10 promoter we examined the effect of the EZH2 inhibitor DZNep and overexpression of JMJD3 in F-IPF cells. Treatment of the cells with DZNep, alone or with IL-1 β , reduced H3K27me3 at the CXCL10 promoter by 45% and 70%, respectively (Figure 1C) compared with untreated cells. Transfection of F-IPF with the pMSCV-mJMJD3 and the pMSCV-JMJD3 showed increased expression of mutated and wild-type JMJD3, respectively, whereas transfection with empty vector and treatment with IL-1 β , either alone or with the vectors, had no effect on JMJD3 expression (Figure 1D). Furthermore, overexpression of wild-type JMJD3, but not the mutated mJMJD3 lacking JMJD3 enzymatic activity, in both control and IL-1 β -treated cells markedly reduced H3K27me3 association with the CXCL10 promoter (Figure 1E). The results thus suggest that EZH2 is critically involved in H3K27me3 at the CXCL10 promoter in F-IPF and that inhibition of EZH2 and overexpression of JMJD3 can reduce H3K27me3 and impact CXCL10 repression.

H3K27me3 and H3K9me3 at the CXCL10 Promoter are Reduced by EZH2 and G9a siRNAs

Figure 1C and our previous study (10) show that inhibitors of EZH2 and G9a reduce H3K27me3 and H3K9me3, respectively, at the CXCL10 promoter. To further validate the role of EZH2 and G9a in mediating CXCL10 repression in F-IPF, EZH2 and G9a siRNAs were applied. As shown in Figure 2A, EZH2 and G9a siRNAs, but not the control siRNA, completely abolished their respective target gene expression. Like the EZH2 inhibitor DZNep, EZH2 siRNA, but not the control siRNA, either alone or with IL-1 β , reduced H3K27me3 at the CXCL10 promoter by 73% and 44%, respectively, compared with untreated cells (Figure 2B). Similarly, like the G9a inhibitor BIX-01294 (10), G9a siRNA, but not the control siRNA, either alone or with IL-1 β , reduced H3K9me3 at the CXCL10 promoter by 85% and 83%, respectively, compared with untreated cells (Figure 2D). The data confirm the effect of the EZH2 and G9a inhibitors in Figure 1C and in our previous study (10) and suggest a key role for EZH2 and G9a in mediating H3K27me3 and H3K9me3, respectively, at the CXCL10 promoter in F-IPF.

Crosstalk between EZH2 and G9a at the CXCL10 Promoter in F-IPF

To explore the crosstalk between EZH2 and G9a, we first applied ChIP to assess whether G9a knockdown with siRNA could affect EZH2-induced H3K27me3, and vice versa. We found that G9a knockdown significantly reduced EZH2 and H3K27me3 at the CXCL10 promoter (Figure 3A and B); similarly, EZH2 knockdown with siRNA also led to a significant reduction of G9a and H3K9me3 at the CXCL10 promoter (Figure 3C and D). In addition, Re-ChIP assay revealed EZH2 presence in the immunoprecipitate (IP) of G9a (Figure 3E) and G9a presence in the IP of EZH2 (Figure 3F) at the CXCL10 promoter in unstimulated F-IPF, suggesting physical interactions between the two KMTs. To further confirm physical interaction between EZH2 and G9a proteins, PLA was conducted in unstimulated F-IPF. As shown in Figure 3G, strong red PLA signals were detected in untreated and control siRNA-transfected F-IPF, however, the signals were markedly reduced when G9a was knocked down. As PLA signals were produced when the two PLA probes bound, which only took place if both EZH2 and G9a were closer than 40 nm, these observations strongly suggest that EZH2 and G9a physically interact with each other and regulate repressive histone methylation at the CXCL10 promoter in F-IPF in an interdependent manner.

Histone Acetylation at the CXCL10 Promoter in F-IPF is Increased by EZH2 and G9a Inhibition

To further validate the role of EZH2 and G9a in mediating CXCL10 epigenetic repression in F-IPF, the effect of EZH2 and G9a inhibition on histone acetylation was assessed. As shown in Figure 4, treatment of F-IPF cells with either DZNep or IL-1 β alone did not significantly alter histone H3 and H4 acetylation at the CXCL10 promoter. However, like G9a inhibition by BIX-01294 (10), EZH2 inhibition markedly increased histone H3 and H4 acetylation in cells treated with both DZNep and IL-1 β compared with cells treated with IL-1 β alone (Figure 4A and B). Similarly, treatment of F-IPF with G9a siRNA or EZH2 siRNA alone had no significant effect on histone H3 and H4 acetylation without cytokine stimulation, however, marked increases were observed with IL-1 β stimulation compared with untreated cells (Figure 4C and D). The data strongly suggest that G9a and EZH2 play a key role in mediating not only repressive histone methylation but also histone deacetylation at the CXCL10 promoter in F-IPF.

CXCL10 Expression in F-IPF is Restored by EZH2 and G9a Inhibition

To investigate whether EZH2 and G9a inhibition could lead to the CXCL10 gene derepression, F-IPF cells were treated with DZNep or JMJD3 overexpression, either alone or with the potent CXCL10 inducer IL-1 β . DZNep and JMJD3 overexpression alone had no effect on CXCL10 mRNA expression, as analyzed by real-time RT-PCR, whereas marked increases in CXCL10 mRNA were observed when cells were treated with either DZNep or JMJD3 overexpression together with IL-1 β compared with IL-1 β alone (Figure 5A and C). ELISA analysis revealed no increase in CXCL10 protein expression when cells were treated with DZNep and slight increases when cells were treated with empty vector or overexpression of mJMJD3 and JMJD3 compared with untreated control cells. However, when cells were treated with DZNep or JMJD3 overexpression together with IL-1 β , marked increases in CXCL10 protein expression were observed compared with cells treated with IL-1 β alone (Figure 5B and D). Furthermore, knocking down EZH2 and G9a with siRNAs alone did not markedly increase CXCL10 mRNA and protein

expression. However, when cells were then stimulated with IL-1 β , significant increases in CXCL10 mRNA and protein were observed compared with cells treated with IL-1 β alone (Figure 5E and F). The results thus show that EZH2 and G9a inhibition not only removes repressive histone modifications at the CXCL10 promoter but also restores the capacity of F-IPF cells to express CXCL10 and therefore strongly suggest a central role for EZH2 and G9a interplay in mediating CXCL10 epigenetic repression in IPF.

TGF- β 1-induced Epigenetic Repression of CXCL10 in F-NL is Mediated by an Interdependent Crosstalk between EZH2 and G9a

Since TGF- β 1 is a potent profibrotic cytokine that may play a key role in the phenotype changes observed in F-IPF compared with F-NL, we went on to explore whether TGF- β 1 treatment of F-NL could lead to repressive histone modifications at the CXCL10 promoter and CXCL10 repression, similar to those observed in F-IPF, and to assess the role of the crosstalk between EZH2 and G9a in this process. As shown in Figure 6, TGF- β 1 treatment significantly increased the association of EZH2 and G9a to the CXCL10 promoter in F-NL (Figure 6A and C). This was mirrored by corresponding increases of H3K27me3 and H3K9me3 (Figure 6B and D). Knocking down EZH2 with siRNA markedly reduced TGF- β 1-induced association of not only its target protein EZH2 (Figure 6A) but also G9a to the CXCL10 promoter (Figure 6C). Likewise, knocking down G9a with siRNA markedly reduced TGF- β 1-induced association of not only its target protein G9a (Figure 6C) but also EZH2 to the CXCL10 promoter (Fig. 6A). Similar cross inhibition was also observed for both H3K27me3 and H3K9me3 with either EZH2 siRNA or G9a siRNA (Figure 6B and D). In addition, TGF- β 1 treatment also led to significant reduction of active histone H3 acetylation (Figure 6E) and H4 acetylation (Figure 6F) at the CXCL10 promoter in F-NL, however, this was largely prevented by siRNA knockdown of either EZH2 or G9a (Figure 6E and F). Furthermore, TGF- β 1 treatment also induced a significant reduction of IL-1 β -induced CXCL10 production in F-NL compared with cells treated with IL-1 β alone, and this downregulation was also prevented by EZH2 or G9a siRNA knockdown (Figure 6G). These observations suggest that the interdependent crosstalk between EZH2 and G9a also plays a key role in TGF- β 1-induced epigenetic

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repression of CXCL10 in F-NL and thereby further support the importance of EZH2 and G9a in mediating CXCL10 epigenetic repression in IPF.

Discussion

We have previously demonstrated that H3K9 methylation is involved in CXCL10 repression in IPF (10). The main novel findings of the current study are that EZH2-mediated H3K27me₃ was also significantly increased at the CXCL10 promoter in F-IPF and that EZH2 and G9a interacted with each other at the CXCL10 promoter as demonstrated by Re-ChIP assay. More importantly, we found that EZH2 siRNA markedly reduced not only EZH2 and H3K27me₃ but also G9a and H3K9me₃ and that G9a siRNA markedly reduced not only G9a and H3K9me₃ but also EZH2 and H3K27me₃ at the CXCL10 promoter. EZH2 and G9a siRNAs also led to increased histone acetylation and restored CXCL10 expression in F-IPF. Furthermore, treatment of F-NL with TGF- β 1 largely recapitulated EZH2- and G9a-mediated repressive histone modifications and CXCL10 repression observed in F-IPF. To the best of our knowledge, this is the first report to suggest that a functionally interdependent crosstalk between EZH2 and G9a regulates the epigenetic repression of antifibrotic genes in any fibrotic condition.

It is well established that EZH2- and G9a-mediated histone hypermethylation contributes to the epigenetic silencing of tumor suppressor genes. We have previously shown that the G9a specific inhibitor BIX-01294 reduces H3K9 methylation and the recruitment of additional chromatin modifiers, but increases histone H3 and H4 acetylation at the CXCL10 promoter in F-IPF (10). In this study we reported that the EZH2 inhibitor DZNep not only reduced H3K27me₃ as expected, but also increased histone H3 and H4 acetylation at the CXCL10 promoter. Similarly, H3K27me₃ was also reduced by the overexpression of the H3K27me₃ specific demethylase JMJD3 (Figure 1 and 4). The data suggest that EZH2, like G9a, also plays a role in CXCL10 epigenetic repression in IPF. However, epigenetic inhibitors may display off-target effects, as demonstrated by a recent study showing inhibition of both EZH2 and the H3K9 KMT SETDB1 by DZNep (32). It is therefore necessary to apply siRNA knockdown to validate the findings with epigenetic inhibitors. We also revealed that successful siRNA knockdown of EZH2 and G9a recapitulated the effects of EZH2 and G9a inhibitors in this (Figure 2 and 4) and previous (10) studies. This is consistent with recent findings that EZH2 and G9a knockdown reduces H3K27me₃ and H3K9me₃

at the COX-2 promoter and restores COX-2 expression in F-IPF cells (5) and that G9a knockdown reduces H3K9 methylation at the promoters of the cell adhesion molecule Ep-CAM and E-cadherin and restores their expression in lung cancer cells (33) and human breast cancer cells (34). As a result of the removal of epigenetic repression by inhibition and knockdown of EZH2 and G9a, active epigenetic modifications, such as histone acetylation, and the capability of F-IPF to express CXCL10 in response to IL-1 β were restored. These observations thus suggest that EZH2- and G9a-mediated histone methylation is closely associated with the epigenetic repression of a group of antifibrotic genes including COX-2 and CXCL10 in IPF and that inhibition and knockdown of these two KMTs switch local epigenetic status from repressive to active in response to IL-1 β . However, although G9a and EZH2 are important in the epigenetic repression of antifibrotic genes in IPF and G9a and EZH2 are upregulated in a number of different cancers to maintain the malignant phenotype (35, 36), our observations suggest that increased expression is not necessarily a prerequisite for their repressive effect. In fact, we have previously reported that there is no difference in the global expression of EZH2 and G9a between F-IPF and F-NL (5, 10). The increase of H3K27me3 and H3K9me3 at the CXCL10 promoters in F-IPF is therefore likely due to gene promoter specific recruitment of EZH2 and G9a.

Although H3K27 and H3K9 methylation is catalyzed by different lysine-specific KMTs, they share common functions in gene silencing and have similar roles in the control of many cellular processes. Crosstalk between the two methylation marks has also been suggested. For instance, G9a has been shown to methylate H3K27 *in vitro* and *in vivo* (26, 27). However, the underlying mechanisms were unclear until a recent study demonstrated that G9a interacts physically and functionally with PRC2 and that G9a enzymatic activity mediates the recruitment of EZH2 to specific target genes to enable H3K27me3, thus providing a direct proof of an interplay between the two main histone lysine methylation mechanisms to ensure epigenetic gene silencing (28). To find out whether this interplay is involved in the epigenetic repression of antifibrotic genes in IPF and whether EZH2 can regulate G9a-mediated gene repression through H3K9me3, we applied Re-ChIP assay to explore the physical interaction between EZH2 and G9a at the CXCL10 promoter in F-IPF. The findings that EZH2 was detected in the IP of G9a and G9a was

detected in the IP of EZH2 suggest that the two KMTs co-localize at the CXCL10 promoter (Figure 3). This was further confirmed by the Proximity Ligation Assay (PLA) assay demonstrating that EZH2 and G9a physically interact with each other in the nuclei of F-IPF (Figure 3). We also demonstrated that EZH2 siRNA markedly reduced not only EZH2 and H3K27me₃, but also G9a and H3K9me₃, at the CXCL10 promoter and *vice versa* with G9a siRNA. Since the EZH2 and G9a siRNAs we used only knocked down the expression of their respective specific genes, but not the other (5) and the expression of PRC2 core members, including EZH2, was not affected by the absence of G9a (28), our data therefore suggest that the local presence of EZH2 and G9a is required for the recruitment, not the expression, of each other to the CXCL10 promoter and that the two KMTs act cooperatively and interdependently to regulate CXCL10 epigenetic repression in IPF. This is further supported by the observations that histone acetylation was increased at the CXCL10 promoter and CXCL10 expression was restored in response to IL-1 β by the knockdown of either EZH2 or G9a with siRNA.

We noticed that EZH2 siRNA- and G9a siRNA-induced reduction in H3K27 and H3K9 methylation was not compensated by other H3K27 and H3K9 KMTs. This is unlikely due to the off-target effects of the siRNAs on other KMTs. Several lines of evidence indicate that the depletion of one subunit of the H3K9 KMT complex or the PRC2 complex can lead to the destabilization of other subunits at the protein, but not mRNA level. For instance, depletion of G9a leads to the destabilization of other H3K9 KMTs such as GLP, SETDB1 and SUV39H1 and results in reduced H3K9me₂/me₃ on G9a target genes, indicating that the integrity of these HKMTs is interdependent (37). Similarly, depletion of EZH2 leads to the destabilization of other core components of the PRC2 complex such as SUZ12 and EED and results in reduced H3K27me₃ on PRC2 target genes (38). It is therefore possible that the destabilization of the other KMTs, as a result of EZH2 and G9a depletion, may also contribute to the reduction in H3K27 and H3K9 methylation in siRNA transfected cells. Further investigation is required to confirm this.

It is increasingly recognized that F-IPF represent a persistently activated phenotype of lung fibroblasts and play a key role in the development and progression of IPF and that these cells are epigenetically reprogramed so that a group of antifibrotic genes, including CXCL10, COX-2, and Thy-1,

are epigenetically repressed, in a way similar to the epigenetic silencing of tumor suppressor genes in cancers. We provide here further evidence that dysregulated histone methylation contributes to CXCL10 epigenetic repression in F-IPF by comparing the cells with F-NL. However, one clear limitation of this comparative study is that it explored the epigenetic events at the end of the diseases process. Although the results are novel and valuable in understanding the epigenetic mechanisms involved in the epigenetic repression of antifibrotic genes in IPF, little is known about whether similar repressive histone modifications and mechanisms can be established *in vitro* with factors that promote persistent fibroblast activation and pulmonary fibrogenesis. We report that treatment of F-NL with the profibrotic cytokine TGF- β 1 increased EZH2, G9a, H3K27me3, H3K9me3 and histone deacetylation at the CXCL10 promoter, similar to that observed in F-IPF. These epigenetic modifications were correlated with the repression of IL-1 β -induced CXCL10 and were prevented by EZH2 and G9a siRNAs. These findings strongly suggest that TGF- β 1 treatment of lung fibroblasts can recapitulate the epigenetic events associated with CXCL10 repression in F-IPF and further support an important role of the interdependent crosstalk between EZH2 and G9a in mediating CXCL10 epigenetic repression in IPF. It is worth noting that although IL-1 β is regarded as a profibrotic cytokine (39), it exhibits antifibrotic effects such as induction of antifibrotic genes COX-2 (4, 5) and CXCL10 (10) in normal lung fibroblasts and inhibition of TGF- β 1-induced myofibroblast formation and collagen synthesis (40). However, in TGF- β 1-treated F-NL the capability to express antifibrotic genes COX-2 (41) and CXCL10 (this study) in response to IL-1 β is markedly reduced, similar to that observed in F-IPF (4, 5, 10), resulting in unopposed profibrotic effects of both TGF- β 1 and IL-1 β . Our observations provide early evidence that repressive epigenetic modifications can be introduced, at least to some extent, by TGF- β 1 treatment, leading to the repression of IL-1 β -induced antifibrotic genes in IPF. However, further studies are needed to understand the order of epigenetic events and the mechanisms responsible for the maintenance of these epigenetic modifications and the profibrotic phenotype of the cells. Since antifibrotic genes are important to maintain the non-fibrotic phenotype of lung fibroblasts, it is plausible that epigenetic inhibitors, by preventing antifibrotic gene repression, could contribute to the maintenance of the phenotype of F-NL. Preliminary data from our

on-going studies indeed show that the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA, Vorinostat) and LBH589 and the G9a inhibitor BIX-01294 reduce TGF- β 1-induced expression of collagen 1 and α -SMA in F-NL and that depletion of G9a and EZH2 reduces basal level expression of collagen 1 and α -SMA in F-IPF (unpublished observations), but the role of antifibrotic genes, such as CXCL10 and COX-2, plays in this process remains to be investigated.

In conclusion, our data strongly suggest that a novel interdependent interplay between EZH2 and G9a plays a key role in the regulation of the epigenetic repression of CXCL10 and potentially other antifibrotic genes in IPF (Figure 7). Our data also suggest that the machinery for the expression of these genes in IPF remains intact and functional and can mediate de-repression once the repressive epigenetic modifications are removed. Thus, the interplay between EZH2 and G9a may represent a viable target to reactivate silenced antifibrotic genes for the therapeutic development of this fatal disease.

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Figure Legends

Figure 1. H3K27me3 is increased and modulated at the CXCL10 promoter in F-IPF. Confluent and serum-starved F-NL and F-IPF cells were treated with IL-1 β (1 ng/ml) for the times indicated (**A** and **B**). F-IPF cells were incubated with DZNep (10 nM) (**C**) or transfected with empty vector, pMSCV-mJMJD3 or pMSCV-JMJD3 (**D** and **E**) before being treated with IL-1 β (1 ng/ml) for a further 4 h (**C** and **E**) or 24 h (**D**). ChIP was conducted with specific antibodies against H3K27me3 (**A**, **C** and **E**), EZH2 (**B**), and total histone H3 (**A**, **B**, **C** and **E**). The associated CXCL10 promoter DNA was amplified by real-time PCR and the amount was calculated and normalized to total histone H3 (**A**, **C** and **E**) or to input control (**B**). Data are expressed as mean \pm SEM from experiments with six separate F-NL and/or F-IPF cell lines performed in duplicate. *, $P < 0.05$, **, $P < 0.01$ compared with corresponding F-NL or untreated F-IPF cells. Total cell lysates were collected for Western blotting analysis of mJMJD3 and JMJD3 overexpression with β 2-microglobulin (β 2M) as the loading control (**D**). This is representative of three separate experiments with different F-IPF cell lines.

Figure 2. EZH2 and G9a knockdown alters repressive histone methylation at the CXCL10 promoter in F-IPF. F-IPF cells were transfected with control siRNA, EZH2 siRNA, or G9a siRNA before being treated with IL-1 β (1 ng/ml) for a further 24 h (**A**) or 4 h (**B** and **C**). Total cell lysates were collected for Western blotting analysis of EZH2 and G9a with β 2-microglobulin (β 2M) as the loading control (**A**). This is representative of three separate experiments with different F-IPF cell lines. Chromatin immunoprecipitation (ChIP) was conducted with specific antibodies against H3K27me3 (**B**), H3K9me3 (**C**) and total histone H3 (**B** and **C**). The associated CXCL10 promoter DNA was amplified by real-time PCR and the amount was calculated and normalized to total histone H3 (**B** and **C**). Data are expressed as mean \pm SEM from experiments with six separate F-IPF cell lines performed in duplicate. *, $P < 0.05$, **, $P < 0.01$ compared with corresponding untreated cells.

Figure 3. EZH2 and G9a interact with each other to regulate histone methylation at the CXCL10 promoter in F-IPF. F-IPF cells were transfected with control siRNA, G9a siRNA, or EZH2 siRNA (**A to D**) or untreated (**E and F**). Chromatin pellets were extracted (**A to F**) and immunoprecipitated with specific antibodies against EZH2 (**A and F**), G9a (**C and E**), H3K27me3 (**B**), H3K9me3 (**D**), and total histone H3 (**B and D**). The immunoprecipitates (IP) of G9a (**E**) and EZH2 (**F**) were immunoprecipitated again with antibodies against EZH2 (**E**) and G9a (**F**). The associated CXCL10 promoter DNA was amplified by real-time PCR and the amount was calculated and normalized to total histone H3 (**B and D**) or to input control (**A, C, E, F**). Data are expressed as mean \pm SEM from experiments with six separate F-IPF cell lines performed in duplicate. **, $P < 0.01$ compared with untreated cells. Serum-starved F-IPF cells grown on sterile glass coverslips were fixed in 4% paraformaldehyde. *In situ* interaction between EZH2 and G9a was detected by proximity ligation assay (PLA) with primary mouse anti-EZH2 antibody and rabbit anti-G9a antibody and PLA probes anti-Mouse MINUS and anti-Rabbit PLUS. Amplified DNA was detected by Duolink In Situ Detection Reagents Red and Dapi was used to identify the nuclei (**G**). Images are representative of three separate experiments with different F-IPF cell lines.

FIGURE 4. EZH2 and G9a inhibition and knockdown promote histone acetylation at the CXCL10 promoter in F-IPF. F-IPF cells were incubated with DZNep (10 nM) (**A and B**) or transfected with control siRNA, G9a siRNA, or EZH2 siRNA (**C and D**) in culture medium for 48 h and in serum free medium for 24 h before being treated with IL-1 β (1 ng/ml) for a further 4 h. The protein-DNA complexes were then cross-linked by formaldehyde treatment and chromatin pellets were extracted. Acetylated and total histone H3 (**A and C**) and H4 (**B and D**) were immunoprecipitated with specific antibodies. The associated CXCL10 promoter DNA was amplified by real-time PCR and the amount was calculated and normalized to total histone H3 and H4. Data are expressed as mean \pm SEM from experiments with six separate F-IPF cell lines performed in duplicate. *, $P < 0.05$, compared with corresponding untreated cells.

FIGURE 5. EZH2 and G9a inhibition and knockdown restore CXCL10 expression in F-IPF. F-IPF cells

were incubated with DZNep (10 nM) (**A** and **B**) or transfected with empty vector, pMSCV-mJMJD3 or pMSCV-JMJD3 (**C** and **D**) or transfected with control siRNA, EZH2 siRNA, or G9a siRNA (**E** and **F**) in culture medium for 48 h and in serum free medium for 24 h before being treated with IL-1 β (1 ng/ml) for a further 4 h (**A**, **C** and **E**) or 24 h (**B**, **D** and **F**). Total RNA was isolated and mRNA levels of CXCL10 and the internal control β 2-microglobulin (β 2M) were determined by real-time RT-PCR. Results are calculated as the ratio of CXCL10 mRNA and β -2M mRNA (**A**, **C** and **E**). Medium was collected and CXCL10 concentration was analyzed by ELISA (**B**, **D** and **F**). Data are expressed as mean \pm SEM of six separate experiments performed in duplicate. *, $P < 0.05$, **, $P < 0.01$ compared with corresponding untreated cells.

FIGURE 6. TGF- β 1-induced epigenetic repression of CXCL10 in F-NL is mediated by an interdependent crosstalk between EZH2 and G9a. F-NL cells were transfected with control siRNA, EZH2 siRNA, or G9a siRNA in culture medium for 72 h prior to incubation with TGF- β 1 (2 ng/ml) in culture medium for 48 h and in serum free medium for 24 h (**A** to **F**) before being treated with IL-1 β (1 ng/ml) in the presence of TGF- β 1 for a further 24 h (**G**). The protein-DNA complexes were then cross-linked by formaldehyde treatment and chromatin pellets were extracted. EZH2 (**A**), H3K27me3 (**B**), G9a (**C**), H3K9me3 (**D**), acetylated histone H3 (**E**) and H4 (**F**), and total histone H3 (**B**, **D**, and **E**) and H4 (**F**) were immunoprecipitated with specific antibodies. The associated CXCL10 promoter DNA was amplified by real-time PCR and the amount was calculated and normalized to input control (**A** and **C**) or total histone H3 and H4 (**B**, **D**, **E**, and **F**). CXCL10 concentration in the medium was analyzed by ELISA (**G**). Data are expressed as mean \pm SEM of six separate experiments performed in duplicate. *, $P < 0.05$, **, $P < 0.01$ compared with control cells; +, $P < 0.05$, ++, $P < 0.01$ compared with TGF- β 1 alone.

FIGURE 7. A hypothetical model depicting the role of the interplay between EZH2 and G9a in the regulation of CXCL10 gene repression in IPF. EZH2 and G9a physically and interdependently interact with each other in F-IPF. EZH2- and G9a-mediated H3K27me3 and H3K9me3 result in the recruitment of

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HDACs to the CXCL10 promoter. This then leads to histone deacetylation, causing reinforced epigenetic silencing of CXCL10 gene in F-IPF. Inhibition or depletion of either EZH2 or G9a leads to the removal of repressive H3K27me3, H3K9me3 and histone deacetylation, resulting in chromatin derepression and CXCL10 gene re-expression in F-IPF.