Histone deacetylase inhibition promotes fibroblast apoptosis and ameliorates pulmonary fibrosis in mice

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ABSTRACT Idiopathic pulmonary fibrosis (IPF) is a fatal disease, and therapeutic agents have shown only modest efficacy. Epigenetic alterations contribute to the pathogenesis of IPF. The histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), has been approved for clinical use in cancer; however, its potential efficacy in modulating fibroblast survival and lung fibrosis has not been extensively investigated.

We investigated the effects of SAHA on apoptosis of primary IPF myofibroblasts and on injury-induced lung fibrosis in a murine model. SAHA-induced apoptosis of IPF myofibroblasts, an effect that was mediated, at least in part, by upregulation of the pro-apoptotic gene *Bak* and downregulation of the anti-apoptotic gene *Bcl-xL*.

Alterations in the expression of these apoptosis-related genes were associated with histone modifications and changes in DNA methylation. In addition to the expected higher levels of histone acetylation in treated cells, we also detected changes in other histone modifications, such as histone methylation. In a murine model of bleomycin-induced pulmonary fibrosis, SAHA-treated mice displayed decreased lung fibrosis and improved lung function compared to the bleomycin only group.

These results suggest that histone deacetylase inhibitors may offer a new therapeutic strategy in IPF by modulating myofibroblast susceptibility to apoptosis.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal disease characterised by irreversible distortion of lung architecture resulting, presumably, from aberrant wound healing after alveolar injury [1]. In normal tissue repair, myofibroblastic cells disappear through mechanisms such as apoptosis. In pathological fibrosis, myofibroblasts persist in fibrotic lesions, fail to undergo apoptosis and continue to elaborate and remodel extracellular matrix, resulting in fibrosis [2]. Primary cultures of IPF fibroblasts demonstrate myofibroblastic differentiation and resistance to apoptosis [3]. These apoptosis-resistant cells may contribute to the pathogenesis of IPF [2], as these α-smooth muscle actin (SMA)-expressing myofibroblasts are shown to persist in locations of excessive scarring and fibrosis. Therefore, interventions that induce myofibroblast apoptosis may be an effective therapeutic strategy. A number of factors have been shown to induce apoptosis in lung fibroblasts *in vitro*, including acidic fibroblast growth factor [4] and hepatocyte growth factor [5]. However, the potential role of epigenetic modulators in (myo)fibroblast apoptosis and animal models of lung fibrosis are not well defined.

Recently, histone modifying drugs have shown promising results in cancer clinical trials and have demonstrated anti-fibrotic effects *via* unknown mechanisms [6]. In general, histone deacetylase inhibitors (HDACi) favour histone acetylation and chromatin relaxation, thus allowing transcription of genes. Trichostatin A (TSA) was the first naturally occurring HDACi, and is reported to induce apoptosis and decrease Bcl-2 in small cell lung cancer [7]. However, TSA has been used in few, if any, clinical trials. However, a TSA structural analogue, suberoylanilide hydroxamic acid (SAHA) (Vorinostat; Merck, Whitestation, NJ, USA), is currently being investigated in a number of clinical trials, and is the first US Food and Drug Administration approved HDACi to treat cutaneous T-cell lymphoma [8]. Previously, studies have indicated that TSA and SAHA have anti-inflammatory [9] and anti-fibrotic properties [10–12] in fibroblasts, such as inhibition of cell proliferation, collagen production and myofibroblastic differentiation. However, few studies have explored the mechanisms and effects of SAHA on apoptosis and their mechanisms in IPF fibroblasts.

In this study, we examined whether SAHA can induce apoptosis in IPF primary lung fibroblasts and explored mechanisms by which SAHA might regulate expression of apoptosis-related genes. We identified the apoptotic genes *Bak* and *Bcl-xL* as targets for this drug, and explored the histone modifications and DNA methylation changes in these genes associated with SAHA treatment. In a murine model of bleomycin-induced pulmonary fibrosis, SAHA improved lung function in the treatment group. Our data demonstrate that SAHA induces apoptosis in IPF fibroblasts *via* epigenetic mechanisms, supporting a new anti-fibrotic therapeutic strategy for IPF.

Materials and methods

For a full detailed protocol refer to the online supplementary material.

Cell culture and treatments

This study was approved by the University of Alabama at Birmingham (UAB; Birmingham, AL, USA) Institutional Review Board. Human primary IPF lung fibroblasts were a generous gift from C. Feghali-Bostwick (University of Pittsburgh, Pittsburgh, PA, USA) or derived from tissues from the UAB Tissue Procurement Facility. At 80% confluence, cells were made quiescent and treated with dimethyl sulfoxide only (vehicle control) or SAHA (Sigma-Aldrich, St Louis, MO, USA) at 100 nM, 200 nM or as indicated in fresh 1% fetal bovine serum medium for 60 h.

Apoptosis assays: Annexin-V FITC and caspase-3 assay

Cells near confluence were treated with SAHA and were analysed using an Annexin-V FITC kit (MBL International Corporation, Woburn, WA, USA) as previously described [13] or with an active caspase-3 kit (MBL International Corporation) to measure apoptosis.

DNA/RNA/protein/nuclear extraction and real-time RT-PCR

Allprep (Qiagen, Germantown, MD, USA) or EpiQuick Nuclear extraction kits (Epigentek, Brooklyn, NY, USA) were used. Real-time RT-PCR was performed in triplicate and normalised to 18S using the ΔΔCt method [14]. Primers are listed in table 1.

Antibodies and immunoblotting

Anti-α-SMA was from Biocarta US (San Diego, CA, USA). Anti-Bak (#12105), Bcl-xL (#2764), β-tubulin (#2128), H3Ac (#9671), H3 (#9715), H4Ac (#2591) and H4 (#2935) were obtained from Cell Signaling (Beverly, MA, USA). Anti-H3K9Ac (#61251) and H3K9Me3 (#61013) were obtained from Active Motif (Carlsbad, CA, USA). Western blots were performed as previously reported [15].

TABLE 1 Primer sequences

Primer	Gene information	Sequence
RT-PCR		
Bak	NM_001188	F: 5′-GACGACATCAACCGACGCTATG-3′ R: 5′-GCTGGTGGCAATCTTGGTGAAG-3′
Bcl-xL	NM_138578	F: 5′-ACTGTGCGTGGAAAGCGTAGAC-3′ R: 5′GATCCAAGGCTCTAGGTGGTCATTC-3′
DNMT1	NM_001379	F: 5′-CCGTCTCTTGAAGGTGGTGTTAATG-3′ R: 5′-ATGAGGTGCTGAAGCCGATGAG-3′
DNMT3a	NM_175630	F: 5′-GCACCACGGCACGGAAGG-3′ R: 5′-GGACTTGGAGATCACCGCAGG-3′
18S	NR_003278	F: 5′-GTCTGCCCTATCAACTTTCG-3′ R:5′-ATGTGGTAGCCGTTTCTCA-3′
ChIP and methylation		
Bak	ENSG00000030110	F: 5′-ACTGGAGTCTCGCGGGTCCC-3′ R: 5′-CAACCCGGGTGGCTCAGCAG-3′
Bcl-xL	ENSG00000171552	F: 5′-GGCTTGTTCGGGAGAGACGGC-3′ R: 5′-CCCCCTTCATCGGCCCGGTAG-3′

DNMT1: DNA methyltransferase 1: DNMT3a: DNA methyltransferase 3a; ChIP: chromatin immunoprecipitation.

Immunofluorescence staining

Cells were cultured on coverslips as described previously with or without 100 nM SAHA for 60 h. Antibodies to H3K9Ac or H3K9Me3 were used (1:800) with fluorescein isothiocyanate (FITC)-conjugated secondary antibody [13]. The slides were examined with a Zeiss Axiovert 200 M fluorescence/phase microscope with axiovision LE software (Carl Zeiss International, Oberkochen, Germany).

ChIP assays

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's protocol (Epigentek), with minor modifications [15]. ChIPed-DNA was amplified by real-time PCR with the primers list in table 1.

DNA methylation changes in apoptosis-related genes

Quantitative methylation analysis was carried out as described previously [16] using OneStep qMethyl Kit (Zymo Research, Irvine, CA, USA) with some modifications.

Animal models, lung function analysis and immunohistochemistry

All animal studies were performed in accordance with UAB Institutional Animal Care and Use Committee approved protocols. 6–8 week-old healthy C57BL mice were used. A single dose of normal saline or bleomycin sulfate at 3 $U \cdot kg^{-1}$ body weight was instilled intra-tracheally. SAHA in 2-hydroxypropyl-β-cyclodextrin (HOP-β-CD; Sigma-Aldrich) solution [17] was orally administered every other day at 20 $mg \cdot kg^{-1}$, starting at day 10 post-bleomycin injury, to a group of mice subjected to intra-tracheal saline and another group of mice subjected to bleomycin. Before sacrifice on day 28, lung function was measured using flexiVent apparatus (SCIREQ, Montreal, Canada). Mice were then euthanised and the lungs prepared for histology. Images were obtained with a Nikon TE2000U microscope equipped with a QiCam Fast Cooled high-resolution CCD camera (Nikon Inc., Melville, NY, USA) with MetaMorph software (v.6.2r4; Universal Imaging, West Chester, PA, USA).

Statistical analysis

Data are presented as mean±SD. t-test or one-way ANOVA were performed for comparisons involving three or more groups, and the Holm–Sidak method for all pairwise multiple comparison procedures. A p-value $\leqslant 0.05$ was considered statistically significant.

Results

SAHA induces apoptosis of primary IPF lung fibroblasts

IPF primary fibroblasts showed increased α-SMA, collagen I A1 and Bcl-xL, and decreased Bak (fig. S1). We evaluated the responses of IPF lung fibroblasts to SAHA. After 60 h, at concentrations between 100 nM and 1 μM, SAHA induced apoptosis in ~40% of IPF cells; while at 2 μM SAHA induced apoptosis in ~60%

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(fig. 1a). We also measured active caspase-3 in IPF primary fibroblasts. 100 nM of SAHA significantly increased active caspase-3 in IPF cells (fig. 1b). SAHA also induces apoptosis of normal lung fibroblasts, although this requires relatively higher doses (fig. S1). These data demonstrate that SAHA potently induces apoptosis of IPF lung fibroblasts.

SAHA alters the expression of apoptosis-related genes Bak and Bcl-xL in IPF fibroblasts

As SAHA induced apoptosis in IPF fibroblasts, we then examined whether this was associated with changes in apoptosis-related genes. Based on previous studies implicating altered expression of Bcl-2 family proteins in IPF [18], we focused on members of this gene family. The pro-apoptosis gene *Bak* demonstrated a more than two-fold increase at the mRNA level in response to 100–200 nM SAHA (by real-time RT-PCR) (fig. 2a), while expression of the anti-apoptosis gene *Bcl-xL* was downregulated (fig. 2b). The expression changes of Bak and Bcl-xL were confirmed at the protein level by Western blotting (fig. 2c and d). Altered expression of other members of the Bcl-2 family, in addition to Bak and Bcl-xL, were also observed (fig. S2a). HDACi is also reported to regulate cell cycle and cell proliferation [19]. We observed decreased proliferation following SAHA treatment in association with increased p16 expression (fig. S2c and d). The expression of the myofibroblast marker, α-SMA (fig. 2c), and collagen (fig. S2b) were decreased by SAHA exposure, similar to previously published studies [11, 12, 20]. These data indicate that SAHA alters expression of apoptosis-related genes in IPF fibroblasts.

SAHA modulates global histone acetylation and specific histone modifications associated with apoptosis-related genes in IPF lung fibroblasts

Core histones, particularly H3 and H4, have multiple residues such as lysine (K) that are subject to post-translational modifications, including acetylation and methylation, which modify regulation of gene expression [21]. We examined acetylation of H3K9 (H3K9Ac) (fig. 3a–c), and total acetylation of histones H3 and H4 (fig. 3a and 3d), which all demonstrated increased acetylation. Due to the crosstalk between histone acetylation and methylation [21], we examined tri-methylation of H3K9 (H3K9Me3). SAHA induced increased expression of H3K9Me3 in IPF fibroblasts (fig. 3). Immunofluorescent cell staining for H3K9Ac and H3K9Me3 revealed increases in acetylated and trimethylated histone H3K9 in the nuclei of IPF fibroblasts in response to SAHA (fig. 3b).

H3K9Ac is associated with active chromatin structure, while H3K9Me3 is a repressive chromatin mark [21]. We explored the association of the apoptosis-related genes, *Bak* and *Bcl-xL*, with these chromatin marks by ChIP assays. SAHA treatment resulted in an enrichment of Bak with H3K9Ac, and depletion of Bak with H3K9Me3 (fig. 4a), corresponding to increased Bak expression. We found the opposite effect with the anti-apoptosis gene, Bcl-xL, which was enriched with the repressive mark H3K9Me3 by SAHA treatment, while

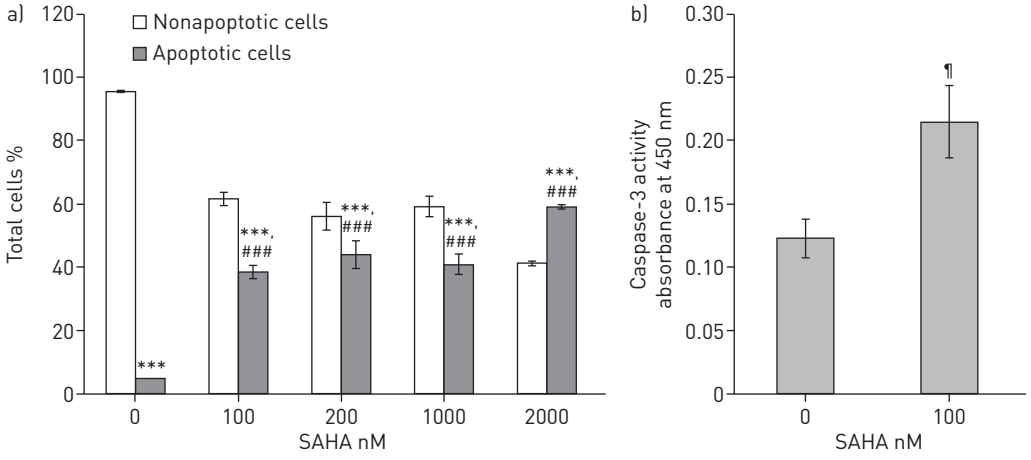

FIGURE 1 Suberoylanilide hydroxamic acid (SAHA)-induced apoptosis in primary idiopathic pulmonary fibrosis (IPF) fibroblasts. Cells were cultured in 6-well dishes; when 80% confluent, the medium was changed to 1% fetal bovine serum containing medium. SAHA was added for 60 h at the concentrations indicated. The cells were then collected for a) Annexin-V FITC apoptosis assay (MBL International Corporation, Woburn, WA, USA) or b) caspase-3 assay. a) SAHA at concentrations of 100 nM to 2 μM induces apoptosis in IPF primary fibroblasts. b) Caspase-3 activity was measured in IPF primary fibroblasts without SAHA (0 μM, control; dimethyl sulfoxide vehicle only) or with 100 nM SAHA. Results are averages of at least three independent experiments (n=3 for IPF primary cell lines). Data are presented as mean±SD. ***: $p<0.001$, apoptotic cells *versus* nonapoptotic cells in the same group; ###: $p<0.001$, apoptotic cells in the SAHA-treated *versus* untreated (SAHA 0 nM) group; ¶: $p<0.05$, caspase-3 activity in the SAHA-treated (100 nM) *versus* untreated (SAHA 0 nM) group.

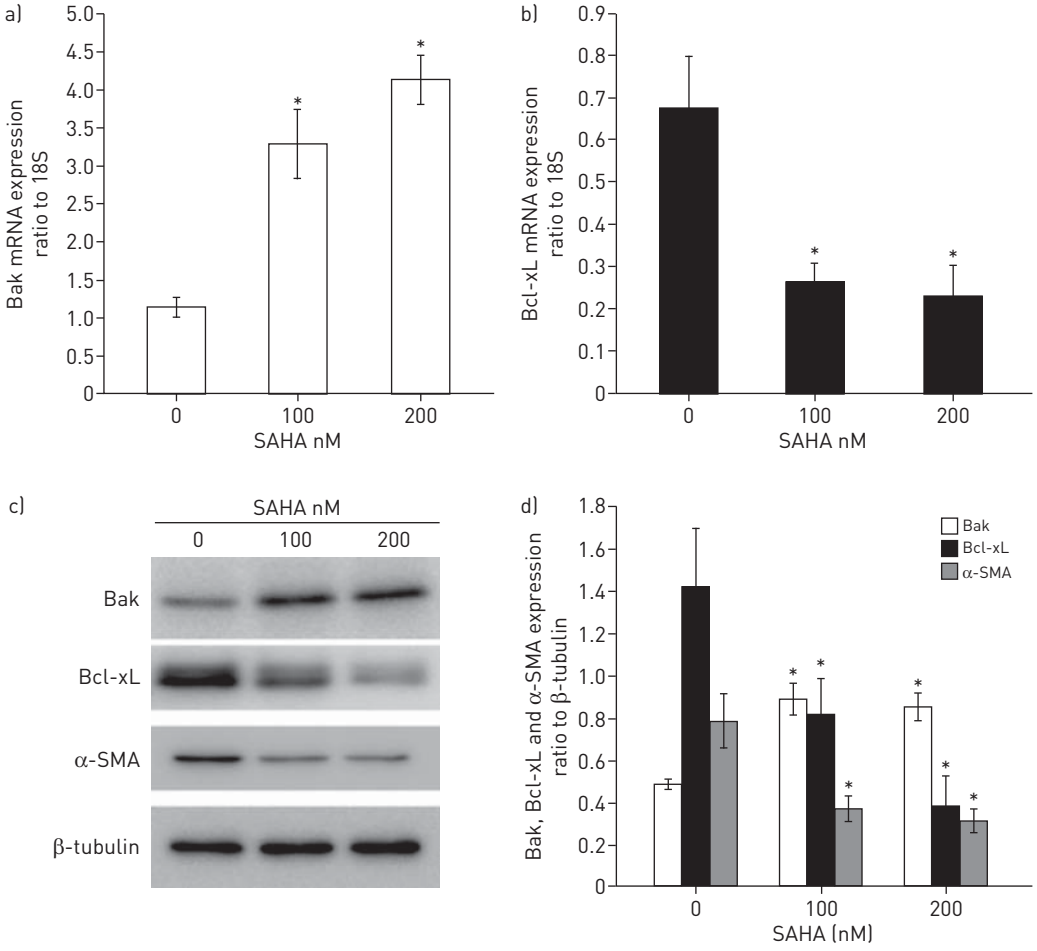

FIGURE 2 Altered expression of the apoptosis-related genes Bak and Bcl-xL after treatment with suberoylanilide hydroxamic acid (SAHA). Idiopathic pulmonary fibrosis (IPF) fibroblasts were treated with SAHA at 0 nM (untreated control), 100 nM and 200 nM for 60 h. Real-time RT-PCR of a) Bak and b) Bcl-xL using the $2^{-\Delta\Delta Ct}$ method, normalised to 18S. c) Western blots for Bak, Bcl-xL and α-smooth muscle actin (SMA) without (SAHA 0 nM) or with SAHA treatment at 100 nM or 200 nM. Protein lysates were subjected to immunoblots and β-tubulin was used as a loading control. d) Relative protein levels of Bak, Bcl-xL and α-SMA from Western blots were determined by scanning densitometry and normalised to β-tubulin. Results are averages of at least three independent experiments. Data are representative of one primary IPF fibroblast line. Similar results were obtained in three different primary IPF fibroblast cell lines (see online supplementary material). Data are presented as mean±SD. *: $p<0.05$, SAHA-treated (100 nM or 200 nM) *versus* untreated (SAHA 0 nM) group.

being depleted of the active mark H3K9Ac, corresponding to a decrease in expression (fig. 4b). These data indicate that the HDACi SAHA not only increases histone acetylation globally but also modulates other specific histone modifications associated with the expression of Bak and Bcl-xL. Epigenetic regulation of these apoptosis-related genes may confer apoptosis susceptibility of IPF lung fibroblasts in response to SAHA treatment.

SAHA affects DNA methyltransferases expression and the DNA methylation status of Bak and Bcl-xL

Epigenetic modifications such as DNA methylation and histone modifications interact with each other to control gene expression. Prior studies have shown that HDAC inhibition affects DNA methylation status [15, 22]. We examined the effect of SAHA on DNA methyltransferase gene expression in IPF lung fibroblasts. DNA methyltransferase (DNMT)1 and DNMT3a were significantly downregulated at the mRNA level following treatment with SAHA, at both 100 nM and 200 nM (fig. 5a and b). At the protein level, DNMT1 and DNMT3a were downregulated by SAHA at 200 nM, while DNMT3a had a nonsignificant downregulation trend at 100 nM (fig. 5c and d). We did not observe significant changes in DNMT3b mRNA or protein (data not shown).

Changes in DNA methyltransferase expression can alter DNA methylation status of specific genes, including the apoptosis-related genes *Bak* and *Bcl-xL*. We quantified DNA methylation changes at the promoter

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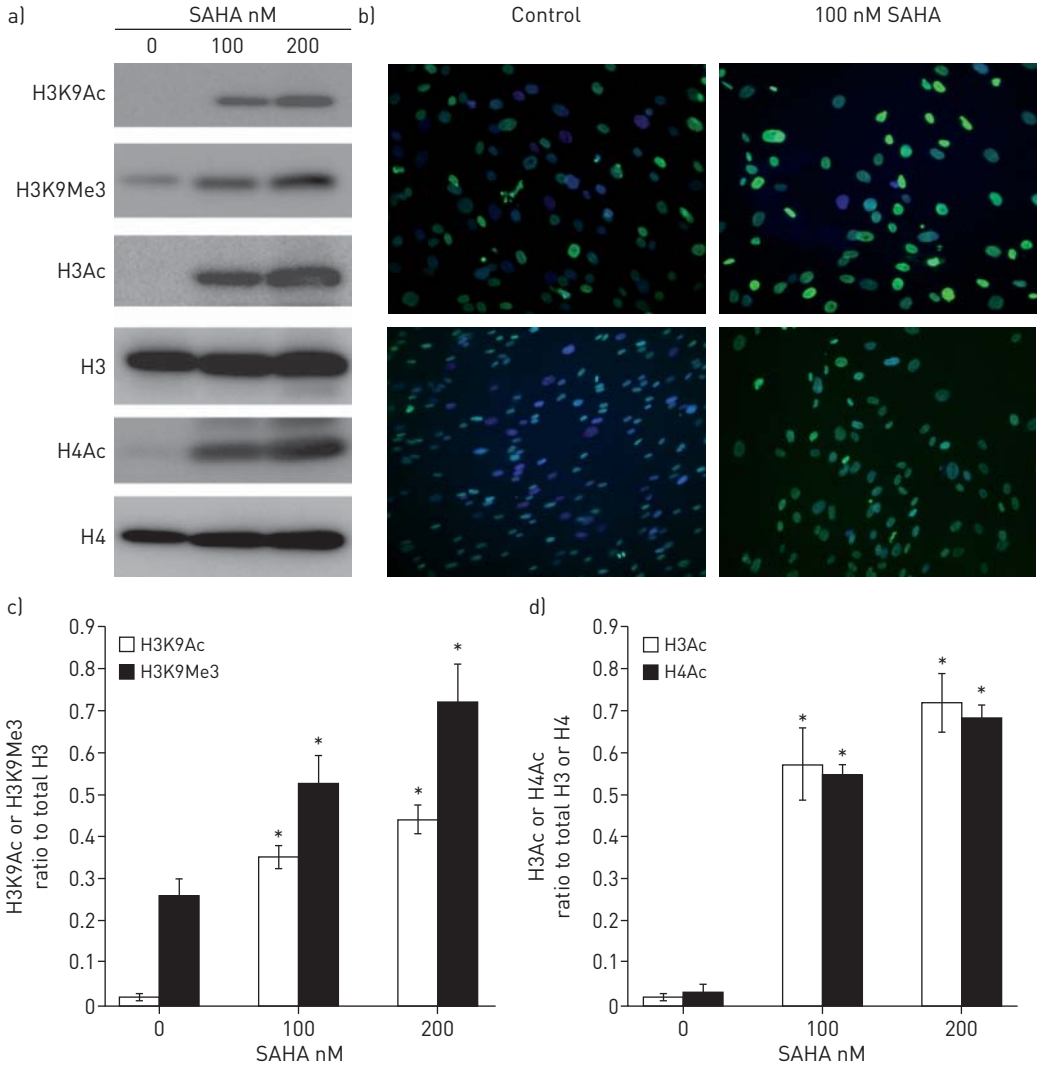

FIGURE 3 Histone-related changes in suberoylanilide hydroxamic acid (SAHA)-treated idiopathic pulmonary fibrosis (IPF) fibroblasts. a) Histone modifications change following treatment with SAHA at 100 nM or 200 nM for 60 h. Nuclear extracts were subjected to immunoblots. Total H3 and H4 were used as controls for H3 or H4 histone modifications, respectively. b) Immunofluorescence of control (SAHA 0 nM) or 100 nM SAHA-treated IPF fibroblasts for 60 h. H3K9Ac (top panels) or H3K9Me3 (bottom panels) were stained green and nuclei were stained blue (4′,6-diamidino-2-phenylindole). For more information refer to the Methods section. c, d) Relative band densitometries from blots of specific histone modifications were determined by scanning densitometry and normalised to H3 or H4. Results are averages of at least three independent experiments. Data are presented as mean±SD. *: $p<0.05$, SAHA-treated (100 nM or 200 nM) *versus* untreated (SAHA 0 nM) group.

region of both genes using the OneStep qMethyl kit (Zymo Research), which quantifies locus-specific DNA methylation [16, 23]. Following treatment with SAHA, Bak showed increases in unmethylated DNA, while Bcl-xL demonstrated decreases that correlate with the observed increased expression of Bak and decreased expression of Bcl-xL, respectively (fig. 6). These data indicate that SAHA regulates the expression of DNA methyltransferases and alters the DNA methylation status of the apoptosis-related genes *Bak* and *Bcl-xL*.

SAHA improves lung function and histopathology in bleomycin-induced pulmonary fibrosis in mice

To examine whether SAHA mediates anti-fibrotic effects in experimental lung fibrosis, we evaluated SAHA treatment in the murine model of bleomycin-induced pulmonary fibrosis. In this model, fibrosis develops from 7–10 days and peaks at 14–21 days after bleomycin injury [24]. We started SAHA treatment during the post-inflammatory phase at day 10 with therapy on alternate days until day 28. Animals were euthanised on day 28 in order to evaluate the resolution phase of fibrosis.

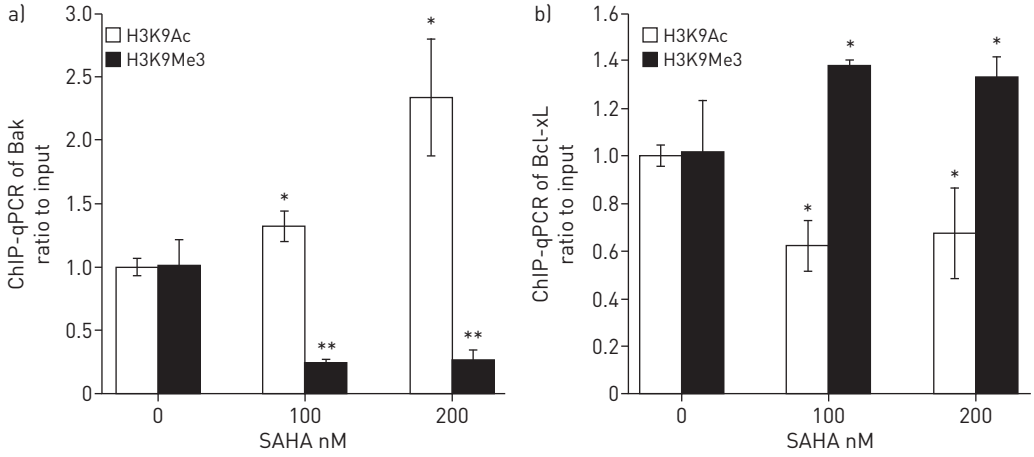

FIGURE 4 Chromatin immunoprecipitation (ChIP) assays of histone modifications H3K9Ac and H3K9Me3 with Bak and Bcl-xL. The quantitative ChIP assays were performed to analyse the association of a) Bak and b) Bcl-xL with the histone modifications H3K9Ac or H3K9Me3 (active and repressive marks, respectively). DNA was immunoprecipitated with the specific antibodies as indicated. Quantitative (q)PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method. Results normalised to input DNA were expressed as fold over untreated control cells. Data are presented as mean±SD and represent relative levels of the PCR product of the Bak or Bcl-xL region associated with this histone modification state at baseline (control, 0 nM suberoylanilide hydroxamic acid (SAHA)) and after treatment with 100 nM and 200 nM SAHA. Results are averages of at least three independent experiments. *: $p<0.05$; **: $p<0.001$, SAHA-treated (100 nM or 200 nM) *versus* untreated (SAHA 0 nM) idiopathic pulmonary fibrosis fibroblasts.

The SAHA-treated bleomycin group demonstrated improved lung function (increased compliance and reduced airway resistance) when compared to the bleomycin group (fig. 7a and b). We did not observe any significant differences in the histopathology or lung function between the saline and the saline/SAHA groups, or the bleomycin and the bleomycin/vehicle groups. Histological examination of haematoxylin and eosin-stained sections demonstrated decreased fibrosis and more normal lung histology in the SAHA-treated bleomycin group compared to the bleomycin group on day 28 (figs 7c and S3). We also examined the changes in H3K9Ac by immunohistochemistry in mouse lung and expression of Bak and Bcl-xL in whole lung homogenates on day 28 (figs S3e and S4). We observed that most cells were stained with H3K9Ac in the bleomycin/SAHA group, while many nuclei were absent in H3K9Ac staining in the bleomycin group. Increased Bak and decreased Bcl-xL were observed in whole lung homogenates in the bleomycin/SAHA group (fig. S4). Together, these data provide support for the anti-fibrotic effects of SAHA in an animal model of lung fibrosis which, at least in part, is mediated by modulating apoptosis-related pathways.

Discussion

In this study, we demonstrate that SAHA induces apoptosis of IPF lung (myo)fibroblasts and modulates the expression of apoptosis-related genes. Through specific histone modifications and DNA methylation changes, SAHA upregulates the pro-apoptotic gene *Bak* while downregulating the anti-apoptotic gene *Bcl-xL*. This has important therapeutic implications as myofibroblasts in fibroblastic foci of IPF lung tissues are resistant to apoptosis [25]. A therapeutic strategy that induces apoptosis of myofibroblasts could have significant benefit for IPF patients.

Previous studies demonstrate that SAHA mediates anti-inflammatory properties and affects myofibroblast differentiation [9, 10]; however, few studies have reported its effect on myofibroblast apoptosis. A recent report indicated that histone modifications are responsible for Fas-mediated apoptosis resistance in fibrotic lung fibroblasts [26]. In the current report, we focused on the apoptosis susceptibility of IPF myofibroblasts in response to SAHA, and examined the changes of the pro-apoptotic gene *Bak* and the anti-apoptotic gene *Bcl-xL* at DNA, RNA and protein levels in association with specific histone modifications. We observed increased expression of the pro-apoptotic *Bak* gene and decreased expression of the anti-apoptotic *Bcl-xL* gene in response to SAHA treatment. Bcl-xL and Bak are known to interact [27], and it is currently unknown if the pro-apoptotic effect of SAHA is related to the effect on one or both genes. We should point out that there are changes in other apoptosis-related genes as well. For example, we also detected upregulation of the pro-apoptotic genes, *Bid* and *Bok*, in SAHA-treated IPF fibroblasts by Western blot analyses (online supplementary material). Bcl-2 expression was also examined, but we did not detect statistically significant changes in its expression after treatment with SAHA in these cells (online supplementary material). The apoptosis-inducing effects of SAHA on fibroblasts are probably related to the

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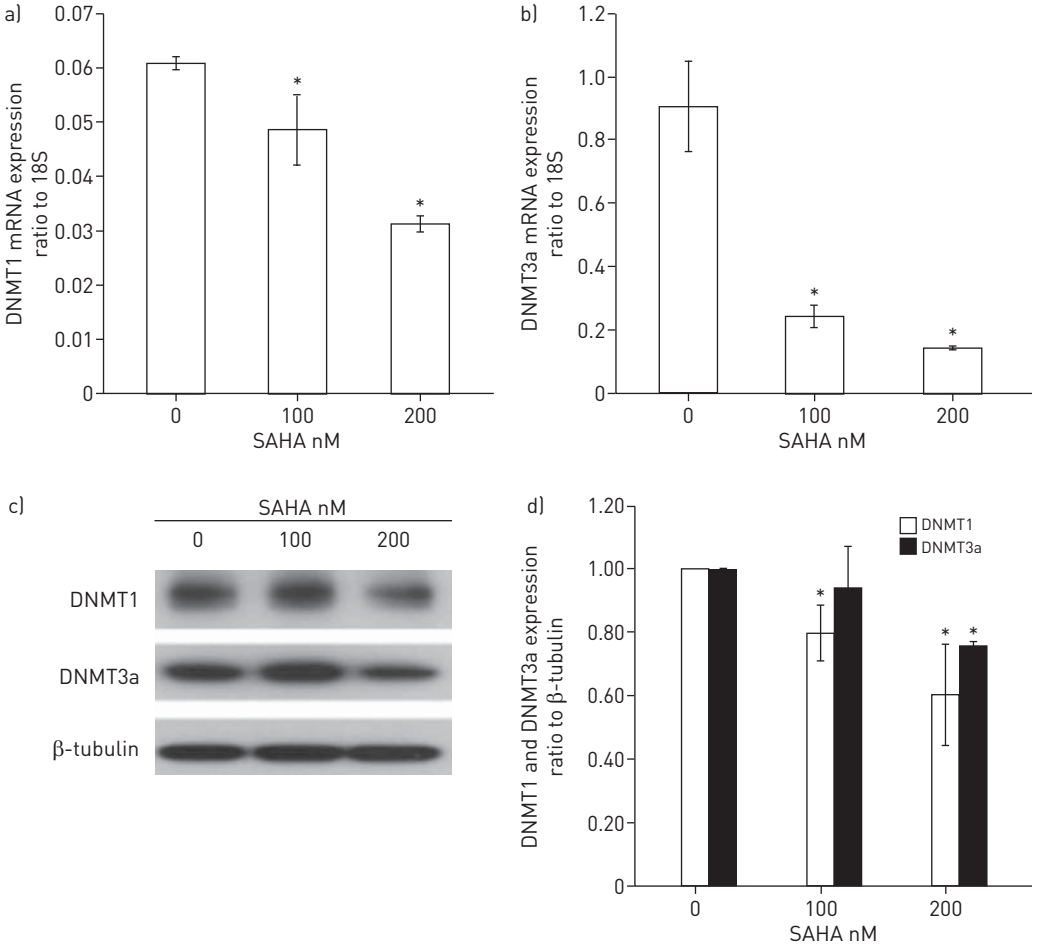

FIGURE 5 DNA methyltransferase (DNMT) expression changes in idiopathic pulmonary fibrosis fibroblasts after treatment with 100 nM or 200 nM suberoylanilide hydroxamic acid (SAHA). a, b) Real-time RT-PCR of DNMT1 and DNMT3a, respectively, using the $2^{-\Delta\Delta Ct}$ method, normalised to 18S. c) Western blots for DNMT1 and DNMT3a. Protein lysates were subjected to immunoblots and β-tubulin was used as a loading control (for more information refer to the Methods section). d) Relative protein levels of DNMT1 and DNMT3a from Western blots were determined by scanning densitometry and normalised to β-tubulin. Results are averages of at least three independent experiments (for more results see online supplementary material). Data are presented as mean±SD. *: $p<0.05$, SAHA-treated (100 nM or 200 nM) *versus* untreated (SAHA 0 nM) group.

combined effects of inducing pro-apoptotic gene expression, while suppressing anti-apoptotic genes. While the regulation of these Bcl-2 family genes is a plausible mechanism for SAHA-induced apoptosis of IPF fibroblasts, other apoptosis-regulating mechanisms are not excluded [26].

SAHA is a broad-spectrum HDACi that has been approved for cancer treatment [8]. Interestingly, we observed markedly increased apoptosis in fibrotic lung fibroblasts compared to non-IPF control cells under the same conditions in response to SAHA treatment (online supplementary material). This is similar to previously published studies showing that SAHA selectively induces apoptosis of malignant T-cells [28]. Another previous study showed that SAHA abrogates transforming growth factor-β effects by inhibiting α-SMA expression and collagen production, although they were unable to detect effects on apoptosis [11]. This may be related to a shorter duration of SAHA treatment (24 h) than we report here (60 h); indeed, we detected minimally higher apoptosis rates in SAHA-treated IPF myofibroblasts at 24 h (data not shown).

SAHA, as with other HDACi, may influence the acetylation status of non-histone proteins, some of which are critical for cell growth and differentiation, including the tumour suppressor p53 [29]. In a previous report, the HDACi CG-1521 was found to stabilise p53 in prostate cancer cells and translocate Bax to the mitochondria to induce apoptosis [29]. HDACi have also been reported to affect other cellular functions, such as inhibiting cell proliferation and inducing cell cycle arrest [19]. In a study of synovial fibroblasts, the HDACi desipeptide inhibited cell proliferation by inducing p16 expression [30]. Similarly, we observed decreased proliferation and increased p16 expression by SAHA treatment of IPF lung fibroblasts (online

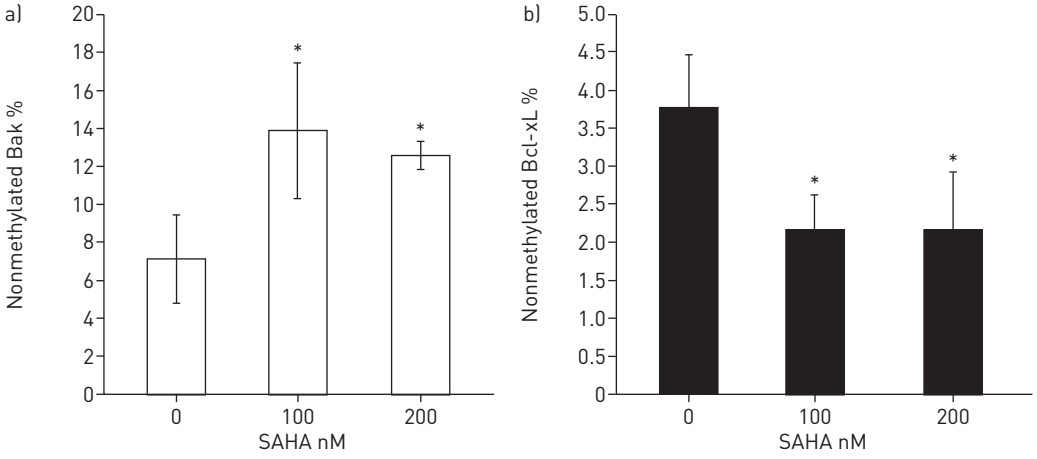

FIGURE 6 Nonmethylated DNA changes at the promoter region of a) Bak and b) Bcl-xL after treatment with 100 nM or 200 nM suberoylanilide hydroxamic acid (SAHA) for 60 h. Relative DNA nonmethylation level by quantitative PCR of Bak or Bcl-xL amplicons after digestion with methylation-sensitive restriction enzymes against its undigested control. Results are averages of at least three independent experiments. Data are presented as mean±SD. *: $p<0.05$, SAHA-treated (100 nM or 200 nM) *versus* untreated (SAHA 0 nM) group.

supplementary material). In our studies, changes in the expression of the apoptosis-related genes were related to alterations of histone acetylation in addition to changes in histone methylation and DNA methylation status. These data indicate that synergistic and combinatorial mechanisms may be involved in the altered gene expression mediated by HDACi. The therapeutic efficacy of SAHA, in addition to its apoptosis-inducing effects reported here, may be related to other beneficial effects on pathways unrelated to apoptosis.

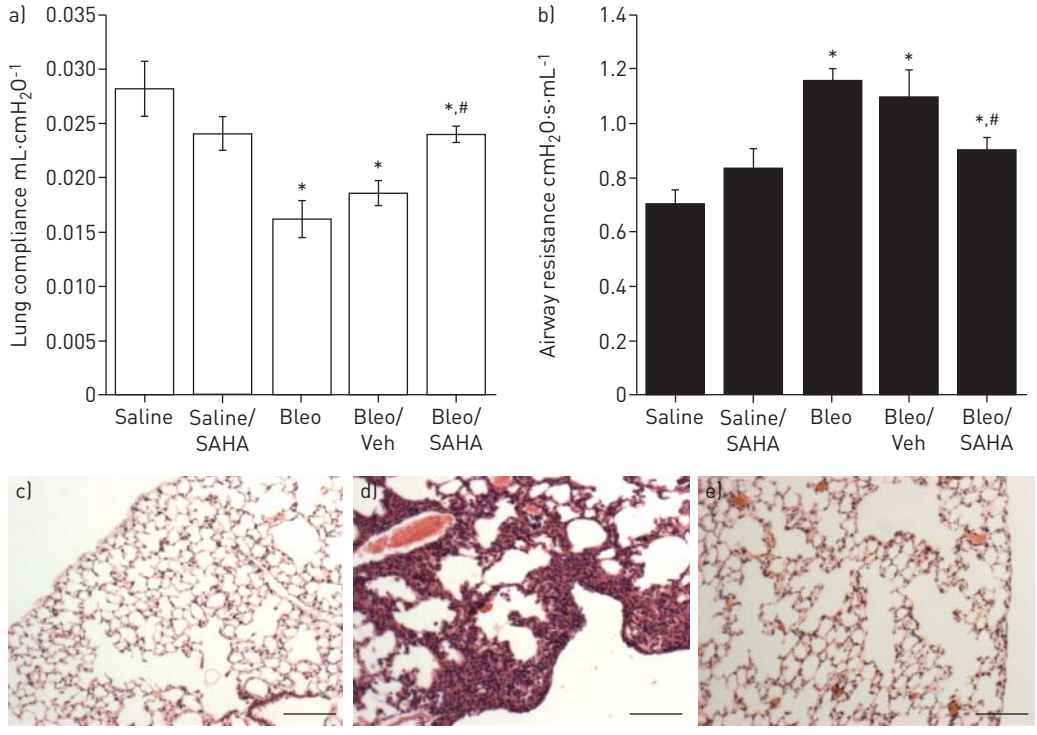

FIGURE 7 Suberoylanilide hydroxamic acid (SAHA) improves bleomycin-induced pulmonary fibrosis in mice. a) Lung compliance and b) airway resistance in normal saline (saline), saline with SAHA (saline/SAHA), bleomycin only (Bleo), bleomycin with vehicle (Bleo/Veh) and bleomycin with SAHA (Bleo/SAHA) groups of 6–8-week-old mice 28 days post-bleomycin injury. Data are presented as mean±SD ($n\geqslant 3$ per group). *: $p<0.05$, compared to saline; #: $p<0.05$, Bleo/SAHA compared to Bleo. c–e) Representative haematoxylin and eosin-stained 5-μm sections of formalin-fixed, paraffin-embedded right lung of 6–8-week-old C57BL mice 28 days after c) intra-tracheal normal saline, d) bleomycin or e) bleomycin treated with SAHA every other day from day 10 to day 28 post-bleomycin injury. Scale bars: 100 μM.

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Some genes can be governed by multiple epigenetic mechanisms. For example, we previously demonstrated that *Thy-1* is epigenetically repressed in IPF by both DNA methylation and histone modifications [14, 15]. Re-expression of such genes by epigenetic modifiers may require dual effects on both histone modifications and DNA methylation for maximal effect. There are other genes that are regulated primarily by one epigenetic mechanism. For example, the pro-apoptotic gene *p14*, which has decreased expression in IPF fibroblasts, could be re-expressed with the DNMT inhibitor 5′-aza-2′-deoxycytidine, but not by the HDACi TSA [31]. DNA methylation interacts with other epigenetic mechanisms, such as microRNA and histone modifications. A study indicated that 5′-aza-2′-deoxycytidine can reduce DNMT1 and fibrotic gene expression, enhance miR-17-92 cluster expression and attenuate pulmonary fibrosis in a murine model [32].

Previously, we reported that another HDACi, TSA, demethylates hypermethylated CpG sites in the *Thy-1* promoter region in Thy-1(-) lung fibroblasts [15]. In the present study, we showed that SAHA alters the expression of DNMT1 and DNMT3a, and modulates the DNA methylation status of Bak and Bcl-xL. DNMT1 is the major maintenance methyltransferase. DNMT3a and DNMT3b are *de novo* methyltransferases; however, they display distinct functions which suggest that DNMT3a and DNMT3b might methylate different sets of genes in the genome. DNMT3b may methylate a broader spectrum of genes in early development, while DNMT3a methylates genes that are critical during later development or in postnatal life [33]. DNA methylation and histone modifications can be interdependent, with crosstalk mediated by DNMTs and histone modification enzymes [34]. For example, H3K9 methyltransferase Suv39h interacts directly with DNMTs [35]. Similarly, one histone modification can recruit or activate chromatin modifying complexes to generate a different histone modification [21]. We showed SAHA not only mediates histone acetylation but also modulates histone methylation. In this study, we demonstrate that both histone modifications and DNA methylation regulate gene expression of the apoptosis-regulating genes, *Bak* and *Bcl-xL*, in response to SAHA. It is currently unknown which of these epigenetic mechanisms are critical for the maintenance of the apoptosis-resistant myofibroblast phenotype. In general, histone modifications are more rapidly altered, while DNA methylation mediates more long-term effects [34].

SAHA (Vorinostat) is approved by the US Food and Drug Administration for T-cell lymphoma treatment and is being investigated in several cancer clinical trials [36]. Because of its anti-fibrotic and apoptosis-inducing properties, and no obvious toxicity in healthy mice [37], we evaluated the efficacy of SAHA to ameliorate pulmonary fibrosis in an animal model. We chose to treat mice from day 10 to 28 following injury to evaluate effects of SAHA on established fibrosis. One of the limitations of this model is that we were unable to evaluate some of the early events such as inflammation, which peaks in the first 2 weeks. However, the design of our studies to initiate treatment in the post-inflammatory phase of injury suggests that potential effects of SAHA on early inflammation are not likely to explain the observed anti-fibrotic effects. We observed marked improvement in lung function with improved compliance and reduced resistance in mice treated with SAHA. We were unable to detect obvious changes in large airway remodelling, suggesting that these physiological improvements are related to amelioration of lung fibrosis. This indicates that SAHA may have the capacity to accelerate resolution or even reverse established fibrosis. This is important from a therapeutic standpoint and deserves further exploration in future studies. In our animal model of lung fibrosis, we also examined some histone mark changes with SAHA treatment. H3K9Ac is an active mark associated with open chromatin structure. We observed a large number of cells that did not have H3K9Ac nuclear staining in fibrotic regions of bleomycin-treated animals, whereas most cells in the relatively normal regions of lung parenchyma in the bleomycin/SAHA and saline groups stained positively for H3K9Ac. Since our protein expression analyses were from whole cell lysates, we are unable to conclusively ascertain the specific cell types in which Bak and Bcl-xL expression are altered. Nevertheless, our finding of altered apoptosis-related gene expression by SAHA in lung fibroblasts *in vitro* in combination with the observed therapeutic efficacy of SAHA administered during the post-inflammatory phase of lung injury *in vivo* support an anti-fibrotic effect of this agent.

In summary, this study demonstrates that the HDACi, SAHA, induces apoptosis of primary IPF myofibroblasts in association with increased expression of pro-apoptotic and decreased expression of anti-apoptotic Bcl-2 family genes. Alterations in the expression of these genes are mediated through histone acetylation changes and other epigenetic mechanisms involving histone methylation and DNA methylation. The *in vivo* study in a murine model of pulmonary fibrosis demonstrates proof-of-concept for the therapeutic usage of HDACi in fibrosis. Since SAHA and related HDACi are already in clinical use or undergoing clinical trials, this approach may quickly translate into new therapeutic options for IPF and perhaps other fibrotic disorders.

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