BET proteins are associated with the induction of small airway fibrosis in COPD

Razia Zakarya[1,2], Yik Lung Chan[1,2], Sandra Rutting[3,4], Karosham Reddy[1,2], Jack Bozier[1,2], Roy R. Woldhuis[1,2,6], Dikaia Xenaki[1,2], David Van Ly[5], Hui Chen[2], Corry-Anke Brandsma[6], Ian M. Adcock[7], Brian G. G. Oliver[1,2]

[1]Respiratory Cell and Molecular Biology, Woolcock Institute of Medical Research, Sydney, Australia

[2]School of Life Sciences, University of Technology Sydney, Sydney, Australia

[3]Airway Physiology & Imaging Group, Woolcock Institute of Medical Research, The University of Sydney, NSW, Australia

[4]The Department of Respiratory Medicine, Royal North Shore Hospital, NSW, Australia

[5]Genome Integrity Unit, Children's Medical Research Institute, University of Sydney, Westmead, NSW, Australia

[6]Pathology, University Medical Center Groningen, Groningen, Netherlands

[7]Airways Disease Section, National Heart & Lung Inst, Imperial College, London, United Kingdom

Corresponding author

Razia Zakarya, Respiratory Cell and Molecular Biology

Woolcock Institute of Medical Research, The University of Sydney, NSW, Australia

Email: razia.zakarya@sydney.edu.au

Authorship statement

R.Z., S.R., K.R., Y.L.C, J.B., R.R.W. and D.X. performed experiments; R.Z. analysed data; R.Z., B.G.O., and I.M.A. interpreted results of experiments; R.Z. prepared figures and drafted manuscript; D.V., C.B., I.M.A., and B.G.O. edited and revised manuscript;

R.Z., S.R., K.R., Y.L.C., J.B., R.R.W., D.V., H.C., C.B., I.M.A., and B.G.O. approved final version of manuscript; R.Z., C.B., I.M.A., and B.G.O. conceived and designed research.

Key messages:

What is the key question?

Is augmented extracellular matrix (ECM) deposition by small airway smooth muscle cells in COPD underpinned by a unique epigenetic mechanism?

What is the bottom line?

Increased expression of ECM genes *COL15A1* and *TNC* in COPD is modulated by histone H4 acetylation.

Why read on?

Further investigations demonstrate the link between BET proteins and the propagation of aforementioned histone H4 acetylation in COPD cells.

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Abstract

Rationale: In COPD, small airway fibrosis occurs due to increased extracellular matrix (ECM) deposition in and around the airway smooth muscle (ASM) layer. Studies of immune cells and peripheral lung tissue have shown that epigenetic changes occur in COPD but it is unknown whether airway mesenchymal cells are reprogrammed.

Objectives: Determine if COPD ASM cells have a unique epigenetic response to pro-fibrotic cytokine TGF-β1.

Methods: Primary human ASM cells from COPD and non-COPD smoking patients were stimulated with TGF-β1. Gene array analysis performed to identify differences in ECM expression. Airway accumulation of collagen 15α1 and tenascin-C proteins were assessed. COPD and non-COPD patients' ASM cells were stimulated with TGF-β1 ± epigenetic inhibitors with qPCR carried out for *COL15A1* and *TNC*. Global HAT and HDAC activity were assessed. ChIP-qPCR for histone H3 and H4 acetylation at *COL15A1* and *TNC* promoters was carried out. Effects of BET inhibitor JQ1(+) on expression and acetylation of ECM target genes was assessed.

Measurements and Main Results: COPD ASM show significantly higher *COL15A1* and *TNC* expression *in vitro* and ~~significantly~~ higher levels of collagen 15α1 deposited in COPD airways *in vivo*. Epigenetic screening indicated differential response to HDAC inhibition. ChIP-qPCR revealed histone H4 acetylation at *COL15A1* and *TNC* promoters in COPD ASM only. ChIP-qPCR found JQ1(+) pre-treatment significantly abrogated TGF-β1 induced histone H4 acetylation at *COL15A1* and *TNC*.

Conclusions: BET protein binding to acetylated histones is important in TGF-β1 induced expression of *COL15A1* and *TNC* and maintenance of TGF-β1 induced histone H4 acetylation in cell progeny.

Abstract word count: 250

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Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the world and as the ageing population grows, has been projected to increase to the third by 2030[1]. COPD is characterised by a persistent loss in lung function precipitating as shortness of breath in patients, with small airways disease and emphysema being the pathological manifestations of the disease. Small airways disease is the predominant site of obstruction in COPD[2] and describes changes in the airway walls of 4th – 16th generation bronchioles which are <2mm in diameter. These changes include heightened inflammation[3, 4] and increased ECM deposition in close proximity to the airway smooth muscle (ASM) layer[5] within the airway wall[6]. The overall effect of which being a thickened, more rigid, fibrotic airway wall wrapped around a lumen; subsequently decreasing lumen size and causing airway obstruction. Small airways disease has been shown to precede emphysematous changes[7], and thereby considered the primary pathological insult in COPD. Using primary mesenchymal cells, we have shown that cells from COPD smokers are hyperresponsive to cigarette smoke stimulation *in vitro*, depositing more ECM than those cells from non-COPD smokers[8, 9].

Although cigarette smoking is an established risk factor for COPD, not all smokers develop COPD. The reasoning behind the same stimuli having variable outcomes on disease is the result of both genetic and epigenetic susceptibility. Epigenetic aberrancies in COPD have been described in epigenome-wide association studies (EWAS) describing differentially methylated CpG sites in leukocytes[10] and small airway epithelial cells[11], and we previously found that peripheral lung tissue and immune cells from COPD patients had greater levels of histone H4 acetylation at the CXCL8

promoter and decreased HDAC activity[12]. However, no studies to date have investigated if epigenetic dysregulation also occurs in genes involved in the pathogenesis of small airway fibrosis, namely ECM encoding genes.

Epigenetics is a wide-encompassing term that is best described by Riggs[13] as mitotically or meiotically heritable changes to genetic function not attributable to gene sequence. The field encapsulates modifications made directly to DNA and post-translational modification of histones. In this study, we will explore DNA methylation and histone acetylation, in the context of ECM gene expression, in ASM cells. We posit that epigenetic factors are critical in small airways disease in COPD. The aim of this study is to identify and investigate the underlying epigenetic mechanisms of an aberrant ECM gene response to TGF-β1 from primary human ASM cells from smokers with and without COPD. The study further delineates the role of epigenetic readers from the bromo- and extra-terminal domain (BET) protein family such as Brd2 and Brd4, which recognise and interact with acetylated histones to initiate and maintain transcriptional elongation[14].

Methods

Additional detail for all noted methods is provided in the online data supplement.

Study subjects

All human lung tissue obtained following written informed consent. Human ASM cells were obtained from bronchial airways of subjects with COPD or no obstructive lung disease. Patient demographics are provided in Supplement Table E1.

Cell isolation & culture

Airways were isolated from the lung and human ASM cells were isolated from the small airways (<2mm) of patients undergoing lung transplantation or lung resection for thoracic malignancies and cells were isolated as previously described[15]. These cells were used between passages 3-7.

Focused microarray analysis

cDNA from basal and TGF-β1 treated cells from n=7 non-COPD and n=7 COPD subjects was pooled and a focused microarray analysis was performed for each group using Taqman® Fast Array Plates targeting Human ECM Matrix & Adhesion Molecules (Life Technologies, Carlsbad, CA, USA) at baseline and upon stimulation.

Quantitative PCR

Quantitative PCR was performed as described previously[16]. Data from the reactions were analysed using StepOne Software, v 2.3(Applied Biosystems).

Immunohistochemistry

Immunohistochemical analysis was performed as previously described[8]. Airway was assessed in formalin fixed paraffin embedded sections using hematoxylin & eosin, col 15α1, and tenascin-C staining (n=4-5). Imaging was conducted using a Hamamatsu NanoZoomer (40X) and stain intensity quantified using Fiji Image J.

Histone acetyltransferase and deacetylase activity

Histone acetyltransferase results are expressed as amount of catalytic byproduct (CoA-SH) per μg protein. Histone deacetylase activity results are expressed as amount of deacetylated substrate per μg protein.

ChIP assay

Chromatin Immunoprecipitation (ChIP) assay was carried out as previously described[17] using EZ-ChIP kit (Millipore, Bayswater, VIC, Australia) according to the manufacturer's protocol. Data is presented as fold enrichment compared with the IgG negative control.

Results

COL15A1* and *TNC* are upregulated in COPD *in vitro

Initially we used a focused ECM microarray to analyse pooled cDNA (n=7 non-COPD; n=7 COPD) stimulated with TGF-β1. TGF-β1 induced greater *COL5A1, COL15A1, ITGA1,* and *TNC* expression (relative to baseline) in ASM cells from COPD smokers compared to cells from non-COPD smokers (fig 1A). We next used real-time PCR to validate these findings. TGF-β1 induced heightened levels of *COL15A1* expression

(fig. 1C) in COPD than in non-COPD smokers (~~63.21~~54.86±69.74~~15.19~~, n=7, range = 115.7 versus ~~18.51~~13.98±~~4.548~~19.78, n=7, range = 34.68, p=0.0070~~155~~)). Similarly, TGF-β1 induced *TNC* expression (fig. 1D) was also significantly (p=0.0041~~482~~, n=7) elevated in COPD (9.491~~13.65~~±4.698~~3.651~~, n=7, range =26.96) compared to non-COPD smokers (~~5.508~~5.499±1.699~~0.6006~~, n=7, range = 4.835). However, there was no statistically significant difference between groups (n=7 per group) for *COL5A1* (fig. 1B) and *ITGA1* (fig. 1E). The data indicates a selective upregulation of *COL15A1* and *TNC* expression in COPD by TGF-β1 rather than a global TGF-β1-induced upregulation of ECM genes.

Collagen type XVα1 and tenascin C proteins deposed in ASM layer in small airways of COPD patients *in vivo*

To validate our *in vitro* mRNA findings, we performed immunohistochemical staining of bronchioles from non-COPD and COPD smokers. Collagen type XVα1 (fig. 2A) and tenascin c (fig. 2B) – encoded for by genes *COL15A1* and *TNC*, respectively – are found within the smooth muscle layer *in vivo*. Of particular interest, is the specific and exclusive localization of collagen type XVα1 to the airway smooth muscle layer (figure 2A). Q~~Q~~uantification of staining intensity ~~found significantly~~ showed trends towards higher levels of collagen type XVα1 (fig. 2C) and tenascin-C (fig. 2D) deposited in COPD airways *in vivo*~~, and a trend towards higher levels of *TNC*~~.

Global HAT and HDAC activity unaltered in COPD ASM cells

To investigate if activity of epigenetic enzymes differed with disease, we carried out HAT and HDAC activity assays. We found no significant (p=0.7302~~4156~~) difference in HAT activity (fig. 3A) in the nuclear fraction of non-COPD (45.64~~41.92~~ ±21.0~~4.92~~,

n=5, range=25.58) and COPD susceptible (~~50.17~~49.18 ± 38.35~~8.81~~, n=4, range=41.73) smokers. Similarly, HDAC activity (fig. 3B) did not differ (p=0.~~4211~~6857) between groups (non-COPD: ~~21.0~~22.57 ± 5.230~~1.7~~, n=4, range=6.867. COPD: ~~25.1~~23.56 ± ~~4.5~~16.64, n=4, range=21.29).

HDAC inhibition alters *TNC* expression in COPD suggests acetylation plays a role in gene expression

Although the overall HAT and HDAC activity did not differ between groups, we theorized that the differences may exist at a gene specific level. To investigate any acetylation differences at the *COL15A1* and *TNC* promoter region, we treated cells with the pan-HAT inhibitor, curcumin (10µM) or the pan-HDAC inhibitor, TSA (100nM) prior to stimulation with TGF-β (10ng/ml) for 48 hours. HAT inhibition using curcumin resulted in no significant difference in TGF-β1-induced *COL15A1* (fig. 4A) or *TNC* (fig. 4B) expression in cells from either patient group. In contrast, TGF-β1-induced *TNC* expression differed significantly between cells from non-COPD (43.05±41.48, n=7, range=154.6) and COPD (156.9±189.3, n=7, range=246.5) susceptible smokers (p=0.0104) in the presence of TSA (fig 4D). To investigate the role of methylation at the *COL15A1* and *TNC* promoter, cells were exposed to the DNMT1 inhibitor 5-azacytidine (10µM) during cell division, resulting in significantly abrogated TGF-β1-induced *COL15A1* expression (fig. 4E) in ASM cells from non-COPD (18.79±53.61, p=0.0015, n=7, range=76.15) and COPD (23.11±74.52, p=0.0017, n=7, range=76.12) susceptible smokers, with no difference between groups.

Histone 4 acetylated at *COL15A1* and *TNC* promoter in COPD

We used ChIP-qPCR to investigate acetylation of histones H3 and H4 at the *COL15A1* and *TNC* promoter regions. Results show that histone H3 was not acetylated at the *COL15A1* (fig. 5A) or *TNC* (fig. 5C) promoters at baseline or in response to stimulation with TGF-β1. In contrast, histone H4 acetylation was significantly upregulated at the *COL15A1* (fig. 5B; ~~;~~ n=4; p~~=0.0011~~<0.05) and *TNC* (fig. 5D; n=4; p=<0.05~~0.0065~~) promoters in COPD cells in response to TGF-β1 stimulation (*COL15A1* – non-COPD: 22.95±25.02, range=26.99; COPD: 60.26±89.93, range=117.7. *TNC* – non-COPD: 19.81±56.03, range=70.81; COPD: 159.0±177.1, range=195.9).

Acetyl-histone recognition underpins *COL15A1* and *TNC* expression in COPD

To investigate the direct effect of the acetylated histone marks defined in figures 3B and 3D, cells were treated with the BET mimic JQ1(+) at a range of concentrations (1nM, 10nM, 100nM, and 1μM). JQ1(+) at 1nM and 10nM had no effect on TGF-β1-induced *COL15A1* or *TNC* expression; whilst expression of both *COL15A1* (fig. 6A; 100nM p<0.05~~=0.0009~~, non-COPD: 1.401±2.83, range=3.03 COPD: 6.594±2.647, range=4.859; 1μM p<0.05~~=0.0003~~, non-COPD: 0.322±0.311, range=0.508 COPD: 0.2198±0.176, range=0.317; n=6~~5~~ non-COPD, n=5~~5~~ COPD) and *TNC* (fig. 6B; 100nM p<0.05~~<0.0001~~, non-COPD: 0.533±0.707, range=1.559 COPD: 1.119±1.631, range=1.899; 1μM p<0.05~~<0.0001~~, non-COPD: 0.213±0.30, range=0.757 COPD: 0.513±0.340, range=0.468; n=6~~5~~ non-COPD, n=5 COPD) was significantly abrogated in cells from volunteers with COPD at 100nM and 1μM JQ1(+). These data demonstrate a role for histone H4 acetylation in aberrant ECM gene expression in ASM cells from COPD patients.

BET proteins maintain pathological Histone H4 Acetyl mark

To elucidate the extent which BET proteins play a role in histone H4 acetylation, we carried out ChIP-qPCR on hASM cells treated with JQ1(+) at the effective concentrations (100nM and 1µM) shown in figures 4A and 4C. The results show that Brd inhibition with 1µM JQ1(+) significantly ablated ~~the detection of~~ H4 acetylation at the *COL15A1* (~~100nM p=0.0017; 1µM~~ p~~=0.008~~<0.05; n=4 ~~non-COPD, n=4 COPD~~per group; non-COPD: 9.07±11.64, range=12.53 COPD: 1.310±1.043, range: 1.293) and *TNC* (~~100nM p=0.0018; 1µM~~ p~~=0.002~~<0.05; n=4 ~~non-COPD, n=4 COPD~~per group; non-COPD: 1.548±4.647, range=5.73 COPD: 6.797±12.13, range=13.24) promoter regions.

Discussion

In this study we investigated the epigenetic modulation of ECM gene expression in human ASM cells. We found that ASM cells expressed more *COL15A1* and *TNC* mRNA in COPD upon stimulation with TGF-β1 *in vitro*. The ECM proteins encoded by these genes, collagen XVα1 and tenascin-C, were shown to be deposited within the ASM layer in human COPD airways *in vivo*, with ~~collagen XVα1 being significantly~~trends towards higher deposition in COPD. HDAC inhibition significantly attenuated *TNC* expression in COPD only, whilst DNMT1 inhibition with 5-azacytidine and HAT inhibition with Curcumin lead to no difference in response between COPD and non-COPD cells. We found increased H4 acetylation at the *COL15A1* and *TNC* promoters in response to TGF-β1 stimulation in COPD only. We competitively inhibited acetyl-lysine recognition by BET proteins with concentrations of JQ1(+) to show that *COL15A1* and *TNC* mRNA expression depends on BET protein recognition of acetylated histones. Finally, we carried out ChIP-qPCR on cells pre-treated with

JQ1(+). Interestingly, 1µM JQ1(+) pre-treatment lead to complete abrogation of TGF-β1 induced histone H4 acetylation at the *COL15A1* and *TNC* promoter region. Thereby demonstrating that BET proteins play a role in maintaining TGF-β1 induced histone H4 acetylation in COPD.

To induce ECM expression, cells from each cohort were stimulated with TGF-β1; a known pro-fibrotic cytokine shown to be elevated in COPD[18]. We used cells from non-COPD and COPD smokers to delineate epigenetic aberrations relative to COPD rather than smoking status. Further, we controlled for age and gender to limit confounding factors. We found that COPD hASM cells expressed significantly higher levels of *COL15A1* and *TNC* mRNA *in vitro* and immunohistochemical staining demonstrated that these proteins are deposited within the ASM layer of small airways of COPD patients *in vivo*. This increased ECM is consistent with other studies of COPD smokers[8, 9, 19], and contributes to small airway fibrosis which has been shown to positively correlate with COPD severity[5].

ECM proteins have inherent bioactive properties in addition to providing structural support. Tenascin-C is a glycoprotein which induces myofibroblast formation, cell migration and ameliorates production of other ECM proteins *in vivo* and *in vitro*[20-22]. This emphasizes the critical role of tenascin-C in the onset and maintenance of fibrosis. Collagen XVα1 is one of the lesser understood collagens but is expressed in basement membranes and in smooth and striated muscle of numerous organ systems[23]. Although increased levels of collagen XVα1 have been noted in other fibrotic diseases[24], little is understood of its role in COPD. Its C-terminal fragment, restin, inhibits the migration of endothelial cells *in vitro* [25]).

HAT and HDAC activity were similar between non-COPD and COPD susceptible smokers. These findings agree with previous data regarding HAT activity but different to macrophages where decreased HDAC activity in COPD is reported [12]. This suggests that the epigenetic aberrations revealed by this study are a consequence of targeted gene- and cell-specific mechanisms rather than epigenome-wide changes. Cells were treated with epigenetic inhibitors curcumin, TSA, and 5-azacytidine to inhibit HATs, HDACs, and DNMT1, respectively. Our results shown no effect of HAT inhibition on *COLXVA1* and *TNC* gene expression, whilst HDAC inhibition significantly attenuated *TNC* expression in ASM cells from COPD smokers, changes in *COL15A1* were not statistically significant but they did follow a similar trend.

DNMT1 inhibition with 5-azacytidine has been shown to inhibit the maintenance of cytosine methylation[26]. 5-azacytidine reduced *COLXVA1* expression in both groups. It is well established that DNA methylation represses gene transcription[27]; given that our findings show that *COLXVA1* expression is decreased upon DNMT1 inhibition, we surmise that our result is attributed to the increased expression of an upstream inhibitor of *COLXVA1* rather than an indication that DNA methylation plays a role in the increased expression in COPD. However, this requires further experimentation to confirm this. In light of these findings, we determined that the likely mechanism underpinning increased *COLXVA1* and *TNC* expression was local changes in histone acetylation status. The use of non-selective inhibitors of these epigenetic enzymes should be confirmed in future studies using more selective agents or specific knockdown/over-expression of the target enzymes. Coupling these data with RNA-

sequencing will provide a greater overall view of the function of these epigenetic enzymes in the function of COPD ASM cells.

ChIP-qPCR demonstrated that the promoter regions for *COL15A1* and *TNC* were enriched for acetylated histone H4 in COPD. Histone acetylation leads to increased gene expression, with two mechanisms contributing to increased mRNA transcription. The first being that the addition of an acetyl moiety alters the charge of the histone, making it more negative and therein repelling the negatively charged DNA. This conformational change from tightly bound heterochromatin to loosely bound euchromatin allows transcriptional activators, co-factors, and enzymes such as RNA Polymerase II (RNAP2) access to the transcriptional start site. With Brd2 being shown to facilitate the recruitment of RNAP2[28]. Second, the acetylated lysine residue acts as a tag for a BET protein, such as Brd4. Brd4 then subsequently recruits transcriptional elongation factor b (P-TEFb), which relieves transcriptional pause of RNAP2 to initiate mRNA transcription and elongation[29]. In light of our findings we posit that histone H4 acetylation is the mechanism underpinning increased expressed of *COL15A1* and *TNC* in COPD ASM cells.

To investigate whether acetylation played a direct role in the transcription of *COL15A1* and *TNC* mRNA, we used a small molecule competitive mimic or functional inhibitor of BET proteins' acetyl-recognition site, JQ1(+). Pre-treatment of cells with 100nM and 1μM of JQ1(+) significantly attenuated increased *COL15A1* and *TNC* expression induced by TGF-β1 in COPD, whilst the negative enantiomer JQ1(-) did not. Thereby demonstrating the direct role acetylated histones play in increased *COL15A1* and *TNC* expression.

BET proteins contain two tandem acetyl-binding domains, bromodomain 1 (BD1) and (BD2). BD1 and BD2 hold high sequence homology amongst the BET protein family, thereby resulting in a paucity of inhibitors specific to each Brd. Often, BET inhibitors act via dual binding of BD1 and BD2, but selective inhibitors of BD2, such as RVX-208 have been developed[30]. The mechanism of action of JQ1(+) is well established[31] and it does not discriminate between BET proteins or BDs. The classical understanding of BET protein-mediated transcription involves their constitutive capacity as an epigenetic reader, however, Brd2 and Brd4 can also modulate acetylation and subsequent mRNA transcription in a specific and targeted manner. Brd4 knockout is embryonically lethal, however *in vitro* experiments have linked haploinsufficiency ($Brd4^{-/+}$) of Brd4 to reduced levels of H3K14 and H4K12 acetylation[32]. Brd2 and Brd4 preferentially binds to acetylated H3 and H4[29], with a higher affinity for the latter[33], and continues to remain bound to those acetylated histones associated with transcriptional start sites of genes essential for establishing basic cellular functions during mitosis[14, 29, 34]. It has been shown that at least half of the Brd4 "bookmarks" during mitosis are being carried over from interphase[35]. The capacity of BET proteins to remain associated with transcriptional start sites throughout the cell cycle is unlike most other bromodomain containing proteins and transcription factors that are evicted from the chromatin during M-phase[36, 37] along with the displacement of most acetyl marks[36, 38]. The subsequent transcription of these "marked" genes has been shown to commence early in G1[34] and indicate BET protein capacity to confer effective epigenetic memory of acetylated histones to daughter cells.

With this in mind, we carried ChIP-qPCR against acetyl-H4 on hASM pre-treated with JQ1(+). JQ1(+) (~~100nM & ~~1μM) significantly abrogated TGF-β1-induced H4 acetylation of the *COL15A1* and *TNC* promoter region whilst the negative enantiomer, JQ1(-), did not exert such an effect. Thereby demonstrating that BET proteins play a role in maintaining an acetyl mark underpinning the overexpression of the aforementioned ECM genes. A study on Brd2 chromatin occupancy in a non-small lung cancer cell line H23[33] showed that JQ1(+) treatment depleted Brd2 chromatin association globally with no effect on global histone H4 acetylation. Interestingly, it was further shown that a small subpopulation of genomic regions that did not associate with Brd2 lost histone H4 acetylation in response to treatment with JQ1(+). The study did not investigate Brd4 occupation at regions of lost histone H4 acetylation in response to JQ1(+). Brd2 and Brd4 occupancy appears to be cell type specific as a separate study using murine Th17 cells found JQ1(+) treatment to displace global occupancy of Brd4, but not Brd2[28]. Our findings showing a loss of the acetyl mark post-JQ1(+) inhibition suggests that overexpression is likely linked to a BET protein, potentially Brd4, acting as a specific transcriptional modulator, rather than a classical epigenetic reader of acetylated lysine residues. Although its role as the latter would contribute to expression of the inherited mark during interphase[34]. Future studies should utilise experiments examining the effect of selective Brd2, Brd3, and Brd4 knockdown to confirm the exact function of these related proteins in the modulation of histone acetylation and subsequent ECM gene expression. These experiments may be combined with the use of the novel selective BRD4 specific inhibitors, ZL0420 and ZL0454[39]. We assert that our findings are bolstered by investigating genes directly, rather than overall global patterns, thereby allowing for detection of aberrancies that may otherwise be overlooked using bioinformatic methods such as peak calling and principal component

analysis. We concede that our findings are based on antibody-based methods which may lead to bias in acetylated histone H4 detection post-JQ1(+) treatment which may contribute to epitope occlusion resulting from changes to nearby PTMs. Future studies may wish to bolster their findings by correlating ChIP-based methods with tandem mass spectrometry (LC-MS/MS) to confirm a decrease in acetylated histones. Recent research shows that Brd4 possesses inherent histone acetyltransferase activity[40] and our findings may be explained by this phenomenon. Although we did not observe any differences in HAT activity in COPD ASM cells, future studies should examine the activity associated with immunoprecipitated Brd4 and other HAT enzymes to confirm this. In addition, ATAC-seq linked with ChIP-seq analysis of these cells may provide greater insight into the epigenetic control of COPD ASMs.

Investigations into increased CXCL8 expression in asthmatic ASM showed increased histone H3 acetylation along with Brd3 and Brd4 binding at the CXCL8 promoter[41]; interestingly BET protein inhibition lead to significant abrogation of CXCL8 expression. Studies using primary human ASM from asthmatic patients showed pre-treatment with JQ1(+) prior to stimulation with TGF-β1 reduced Brd4 binding to the *IL6* and *CXCL8* promoters, which correlated with a decrease in IL6 and CXCL8 protein expression[42]. It has further been shown that the acetyl-lysine binding pocket of Brd2 associates with Stat3 to recruit Th17 factors and enhance recruitment of RNAP2 to inflammatory genes, at which point co-bound Brd4 activates RNAP2 to commence transcriptional elongation[28]. Thereby demonstrating the capacity of Brd2 and Brd4 to bind to the same gene with distinct roles in facilitating and sustaining gene expression. It is evident that BET proteins play an essential role in chromatin organisation, transcriptional elongation, and epigenetic memory yet there is a current

paucity of studies delineating the distinct roles of BET proteins in transcriptional activation during interphase and/or mitosis. We have shown, for the first time, that mesenchymal cells from COPD patients demonstrate an aberrant epigenetic response at the promoter region for ECM genes.

Central to all epigenetic investigations is the conundrum of which came first: the epigenetic insult predisposing a patient to disease or an epigenetic aberration as a consequence of modifiable risk factors, such as smoking. By controlling for smoking, we have demonstrated that ASM cells from patients with COPD have an inherent epigenetic predisposition to histone acetylation underpinning increased ECM gene expression in small airways. We posit that increased *COL15A1* and *TNC* expression is modulated by histone H4 acetylation; and that BET proteins act to maintain the acetylation mark in cell progeny. These novel findings shed light on a mechanism behind small airway fibrosis and highlight a new potential therapeutic avenue for the primary pathological insult in COPD.

Disclosures

The authors have nothing to disclose.

Reference

1. Organisation WH. Global Burden of Disease; 2015.
2. Hogg JC, Macklem PT, Thurlbeck WM. Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med* 1968: 278(25): 1355-1360.
3. Barnes PJ. Cellular and molecular mechanisms of asthma and COPD. *Clinical science* 2017: 131(13): 1541-1558.
4. Hirota N, Martin JG. Mechanisms of airway remodeling. *Chest* 2013: 144(3): 1026-1032.
5. Jones RL, Noble PB, Elliot JG, Mitchell HW, McFawn PK, Hogg JC, James AL. Airflow obstruction is associated with increased smooth muscle extracellular matrix. *Eur Respir J* 2016: 47(6): 1855-1857.
6. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Pare PD. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 2004: 350(26): 2645-2653.
7. McDonough JE, Yuan R, Suzuki M, Seyednejad N, Elliott WM, Sanchez PG, Wright AC, Gefter WB, Litzky L, Coxson HO, Pare PD, Sin DD, Pierce RA, Woods JC, McWilliams AM, Mayo JR, Lam SC, Cooper JD, Hogg JC. Small-airway obstruction and emphysema in chronic obstructive pulmonary disease. *N Engl J Med* 2011: 365(17): 1567-1575.
8. Chen L, Ge Q, Tjin G, Alkhouri H, Deng L, Brandsma C-A, Adcock I, Timens W, Postma D, Burgess JK. Effects of cigarette smoke extract on human airway smooth muscle cells in COPD. *European Respiratory Journal* 2014: 44(3): 634-646.

9. Krimmer DI, Burgess JK, Wooi TK, Black JL, Oliver BG. Matrix proteins from smoke-exposed fibroblasts are pro-proliferative. *Am J Respir Cell Mol Biol* 2012: 46(1): 34-39.
10. Qiu W, Baccarelli A, Carey VJ, Boutaoui N, Bacherman H, Klanderman B, Rennard S, Agusti A, Anderson W, Lomas DA. Variable DNA methylation is associated with chronic obstructive pulmonary disease and lung function. *American journal of respiratory and critical care medicine* 2012: 185(4): 373-381.
11. Vucic EA, Chari R, Thu KL, Wilson IM, Cotton AM, Kennett JY, Zhang M, Lonergan KM, Steiling K, Brown CJ. DNA methylation is globally disrupted and associated with expression changes in chronic obstructive pulmonary disease small airways. *American journal of respiratory cell and molecular biology* 2014: 50(5): 912-922.
12. Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, Barnes PJ. Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N Engl J Med* 2005: 352(19): 1967-1976.
13. Riggs AD, Porter TNJCSHMA. Overview of epigenetic mechanisms. 1996: 32: 29-45.
14. Kanno T, Kanno Y, Siegel RM, Jang MK, Lenardo MJ, Ozato KJMc. Selective recognition of acetylated histones by bromodomain proteins visualized in living cells. 2004: 13(1): 33-43.
15. Chen M-W, Hua K-T, Kao H-J, Chi C-C, Wei L-H, Johansson G, Shiah S-G, Chen P-S, Jeng Y-M, Cheng T-YJCr. H3K9 histone methyltransferase G9a promotes lung cancer invasion and metastasis by silencing the cell adhesion molecule Ep-CAM. 2010: 70(20): 7830-7840.
16. Ge Q, Chen L, Jaffar J, Argraves WS, Twal WO, Hansbro P, Black JL, Burgess JK, Oliver B. Fibulin1C peptide induces cell attachment and extracellular matrix deposition in lung fibroblasts. *Scientific reports* 2015: 5: 9496.
17. Mumby S, Gambaryan N, Meng C, Perros F, Humbert M, Wort SJ, Adcock IM. Bromodomain and extra‐terminal protein mimic JQ1 decreases inflammation in human vascular endothelial cells: Implications for pulmonary arterial hypertension. *Respirology* 2017: 22(1): 157-164.
18. Takizawa H, TANAKA M, Takami K, OHTOSHI T, ITO K, SATOH M, OKADA Y, YAMASAWA F, NAKAHARA K, UMEDA A. Increased expression of transforming growth factor-β 1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *American journal of respiratory and critical care medicine* 2001: 163(6): 1476-1483.
19. Annoni R, Lanças T, Tanigawa RY, de Medeiros Matsushita M, de Morais Fernezlian S, Bruno A, da Silva LFF, Roughley PJ, Battaglia S, Dolhnikoff MJERJ. Extracellular matrix composition in COPD. 2012: 40(6): 1362-1373.
20. Bhattacharyya S, Wang W, Morales-Nebreda L, Feng G, Wu M, Zhou X, Lafyatis R, Lee J, Hinchcliff M, Feghali-Bostwick CJNc. Tenascin-C drives persistence of organ fibrosis. 2016: 7: 11703.
21. Carey WA, Taylor GD, Dean WB, Bristow JDJAJoP-LC, Physiology M. Tenascin-C deficiency attenuates TGF-β-mediated fibrosis following murine lung injury. 2010: 299(6): L785-L793.
22. Trebaul A, Chan E, Midwood K. Regulation of fibroblast migration by tenascin-C. Portland Press Limited, 2007.
23. Tomono Y, Naito I, Ando K, Yonezawa T, Sado Y, Hirakawa S, Arata J, Okigaki T, Ninomiya Y. Epitope-defined monoclonal antibodies against multiplexin collagens demonstrate that type XV and XVIII collagens are expressed in specialized basement membranes. *Cell structure and function* 2002: 27(1): 9-20.
24. Hägg P, Hägg P, Peltonen S, Autio-Harmainen H, Pihlajaniemi T. Location of type XV collagen in human tissues and its accumulation in the interstitial matrix of the fibrotic kidney. *The American journal of pathology* 1997: 150(6): 2075.
25. Ramchandran R, Dhanabal M, Volk R, Waterman MJ, Segal M, Lu H, Knebelmann B, Sukhatme VPJB, communications br. Antiangiogenic activity of restin, NC10 domain of human collagen XV: comparison to endostatin. 1999: 255(3): 735-739.
26. Christman JKJO. 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. 2002: 21(35): 5483.
27. Deaton AM, Bird AJG, development. CpG islands and the regulation of transcription. 2011: 25(10): 1010-1022.
28. Cheung KL, Zhang F, Jaganathan A, Sharma R, Zhang Q, Konuma T, Shen T, Lee J-Y, Ren C, Chen C-H. Distinct roles of Brd2 and Brd4 in potentiating the transcriptional program for Th17 cell differentiation. *Molecular cell* 2017: 65(6): 1068-1080. e1065.
29. Wu S-Y, Chiang C-M. The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. *Journal of Biological Chemistry* 2007: 282(18): 13141-13145.
30. Gilan O, Rioja I, Knezevic K, Bell MJ, Yeung MM, Harker NR, Lam EYN, Chung C-w, Bamborough P, Petretich M, Urh M, Atkinson SJ, Bassil AK, Roberts EJ, Vassiliadis D, Burr ML, Preston AGS, Wellaway C, Werner T, Gray JR, Michon A-M, Gobbetti T, Kumar V, Soden PE, Haynes A, Vappiani J, Tough DF, Taylor S, Dawson S-J, Bantscheff M, Lindon M, Drewes G, Demont EH, Daniels DL, Grandi P, Prinjha RK, Dawson MA. Selective targeting of BD1 and BD2 of the BET proteins in cancer and immuno-inflammation. *Science* 2020: eaaz8455.
31. Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM, Keates T, Hickman TT, Felletar I, Philpott M, Munro S, McKeown MR, Wang Y, Christie AL, West N, Cameron MJ, Schwartz B, Heightman TD, La Thangue N, French CA, Wiest O, Kung AL, Knapp S, Bradner JE. Selective inhibition of BET bromodomains. *Nature* 2010: 468(7327): 1067-1073.
32. Nishiyama A, Dey A, Miyazaki J-i, Ozato K. Brd4 is required for recovery from antimicrotubule drug-induced mitotic arrest: preservation of acetylated chromatin. *Molecular biology of the cell* 2006: 17(2): 814-823.
33. Handoko L, Kaczkowski B, Hon C-C, Lizio M, Wakamori M, Matsuda T, Ito T, Jeyamohan P, Sato Y, Sakamoto K. JQ1 affects BRD2-dependent and independent transcription regulation without disrupting H4-hyperacetylated chromatin states. *Epigenetics* 2018: 13(4): 410-431.
34. Dey A, Nishiyama A, Karpova T, McNally J, Ozato K. Brd4 marks select genes on mitotic chromatin and directs postmitotic transcription. *Molecular biology of the cell* 2009: 20(23): 4899-4909.
35. Behera V, Stonestrom AJ, Hamagami N, Hsiung CC, Keller CA, Giardine B, Sidoli S, Yuan Z-F, Bhanu NV, Werner MT. Interrogating Histone Acetylation and BRD4 as Mitotic Bookmarks of Transcription. *Cell reports* 2019: 27(2): 400-415. e405.
36. Kruhlak MJ, Hendzel MJ, Fischle W, Bertos NR, Hameed S, Yang X-J, Verdin E, Bazett-Jones DP. Regulation of global acetylation in mitosis through loss of histone acetyltransferases and deacetylases from chromatin. *Journal of Biological Chemistry* 2001: 276(41): 38307-38319.
37. Martínez-Balbás MA, Dey A, Rabindran SK, Ozato K, Wu C. Displacement of sequence-specific transcription factors from mitotic chromatin. *Cell* 1995: 83(1): 29-38.

38. Long JJ, Leresche A, Kriwacki RW, Gottesfeld JM. Repression of TFIIH transcriptional activity and TFIIH-associated cdk7 kinase activity at mitosis. *Molecular and cellular biology* 1998: 18(3): 1467-1476.
39. Tian B, Liu Z, Litvinov J, Maroto R, Jamaluddin M, Rytting E, Patrikeev I, Ochoa L, Vargas G, Motamedi M, Ameredes BT, Zhou J, Brasier AR. Efficacy of Novel Highly Specific Bromodomain-Containing Protein 4 Inhibitors in Innate Inflammation-Driven Airway Remodeling. *Am J Respir Cell Mol Biol* 2019: 60(1): 68-83.
40. Devaiah BN, Case-Borden C, Gegonne A, Hsu CH, Chen Q, Meerzaman D, Dey A, Ozato K, Singer DSJNs, biology m. BRD4 is a histone acetyltransferase that evicts nucleosomes from chromatin. 2016: 23(6): 540.
41. Clifford RL, Patel JK, John AE, Tatler AL, Mazengarb L, Brightling CE, Knox AJ. CXCL8 histone H3 acetylation is dysfunctional in airway smooth muscle in asthma: regulation by BET. *Am J Physiol Lung Cell Mol Physiol* 2015: 308(9): L962-972.
42. Perry MM, Durham AL, Austin PJ, Adcock IM, Chung KFJJoBC. BET bromodomains regulate transforming growth factor-β-induced proliferation and cytokine release in asthmatic airway smooth muscle. 2015: 290(14): 9111-9121.