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RESEARCH ARTICLE

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Alveolar epithelial TET2 is not involved in the development of bleomycin-induced pulmonary fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease of unknown etiology with minimal treatment options. Repetitive alveolar epithelial injury has been suggested as one of the causative mechanisms of this disease. Type 2 alveolar epithelial cells (AEC2) play a crucial role during fibrosis by functioning as stem cells able to repair epithelial damage. The DNA demethylase Tet methylcytosine dioxygenase 2 (TET2) regulates the stemness of multiple types of stem cells, but whether it also affects the stemness of AEC2 during fibrosis remains elusive. To study the role of TET2 in AEC2 during fibrosis, we first determined TET2 protein levels in the lungs of IPF patients and compared TET2 expression in AEC2 of IPF patients and controls using publicly available data sets. Subsequently, pulmonary fibrosis was induced by the intranasal administration of bleomycin to wild-type and AEC2-specific TET2 knockout mice to determine the role of TET2 in vivo. Fibrosis was assessed by hydroxyproline analysis and fibrotic gene expression. Additionally, macrophage recruitment and activation, and epithelial injury were analyzed. TET2 protein levels and gene expression were downregulated in IPF lungs and AEC2, respectively. Bleomycin inoculation induced a robust fibrotic response as indicated by increased hydroxyproline levels and increased expression of pro-fibrotic genes. Additionally, increased macrophage recruitment and both M1 and M2 activation were observed. None of these parameters were, however, affected by AEC2-specific TET2 deficiency. TET2 expression is reduced in IPF, but the absence of TET2 in AEC2 cells does not affect the development of bleomycin-induced pulmonary fibrosis.

KEYWORDS

AEC2, DNA methylation, pulmonary fibrosis, stemness, TET2

Abbreviations: AEC2, Type 2 alveolar epithelial cells; BALF, bronchoalveolar lavage fluid; CCL2, C-C motif chemokine ligand 2; IgM, immunoglobulin M; IPF, idiopathic pulmonary fibrosis; TET2, Tet methylcytosine dioxygenase 2.

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1 | INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal lung disease with limited therapeutic options.[1,2] IPF is an age-related interstitial lung disease diagnosed at a median age of 65 years and characterized primarily by the excessive deposition of extracellular matrix (ECM) proteins by activated lung fibroblasts and myofibroblasts, resulting in reduced gas exchange and impaired pulmonary function.[3]

The pathogenesis of IPF has been intensively studied during the past decades, and current hypotheses propose that this condition arises from a failure of lung alveolar epithelial regeneration and an abnormal wound healing response in genetically susceptible and aged individuals due to repetitive epithelial injury.[4] Many polymorphisms related to epithelial cell injury and dysfunction and abnormal wound healing were identified that contribute to susceptibility to IPF.[5] During pulmonary fibrosis, specific cells within the mammalian respiratory system regenerate after injury through the activation of stem/progenitor populations or through proliferation-induced cellular expansion in order to repair epithelial damage.[6] Re-epithelialization and repair of damaged alveolar basement membranes by a specific subset of type II alveolar cells (AEC2) are thought to be protective processes that minimize the fibrogenic program in the lung,[7] but ineffective repair of a damaged alveolar epithelium has been postulated to cause pulmonary fibrosis.[8] Different cell progenitors in the lung during injury have been discovered using newly developed state of art technologies like single-cell sequencing and lineage tracing.[9-12] Among these are pulmonary progenitors/stem cells, ie, AEC2, which give rise to AEC1 and new AEC2 during lung damage,[13,14] and their intratracheal transplantation can, indeed, halt and reverse the fibrotic process in an experimental model of bleomycin-induced lung fibrosis in rats.[15]

DNA methylation, a mechanism of epigenetic regulation, is altered in lung tissue of IPF patients.[16-19] TET2 plays a key role in active DNA demethylation by catalyzing the conversion of the modified genomic base 5-methylcytosine into 5-hydroxymethylcytosine,[20,21] and has been reported to regulate the stemness of different progenitors. TET2 maintains the stemness of trophoblast stem cells and its deletion leads to a significantly slower rate of proliferation and a higher rate of EMT.[22,23] On the contrary, TET2 limits aberrant self-renewal in hematopoietic stem cells (HSCs), thereby limiting HSC frequency and suppressing leukemogenesis.[24,25] Overall, the role of TET2 in controlling the function of stem cells is divergent, either promoting or inhibiting stemness, depending on the cellular context.[26]

Considering the importance of AEC2 during pulmonary fibrosis and the crucial role of TET2 in regulating cell fate and stemness, we hypothesized that epithelial TET2 maintains stem cell-like properties of AEC2 thereby limiting the development of pulmonary fibrosis. To test this hypothesis, we generated AEC2 conditional TET2 knockout mice and subjected these to experimentally induced pulmonary fibrosis.

2 | METHODS

2.1 | Human tissues

Human lung tissues were obtained from five IPF patients and five control subjects (patients undergoing lung surgery for removal of a primary lung tumor), which were used for protein analysis by Western blot. IPF was diagnosed according to the American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Latin American Thoracic Association (ATS/ERS/JRS/ALAT) criteria.[27] Control tissues were derived from lung samples obtained from a segmented remote from the solitary tumor lesion; the normalcy of the control lung sections was verified histologically, as previously described.[28,29] The study protocol was approved by the local Institutional Ethics Committee (CPP Ile de France 1 0811760; Comité d'Ethique du CEERB Paris Nord, Biobank registration DC 2009-940). Written informed consent was obtained from all patients.

2.2 | Western blot

Total lung homogenates were separated on 10% polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked using TBS containing 0.01% Tween-20 (TBST) and 5% non-fat dry milk and then incubated with primary antibodies against TET2 (1:500; clone 21F11; Active Motif, Carlsbad, CA), fibronectin (1:2500; Abcam), α-tubulin (1:2000; Santa Cruz, CA, USA), collagen type I (1:2000; Southern Biotech, Birmingham, AL), SP-C (1:500; Millipore, Billerica, MA) and β-actin (1:1000; clone 13E5, Cell Signaling Technology, Danvers, MA) diluted in TBST containing 1% non-fat dry milk overnight at 4°C. Membranes were washed in TBST before incubation with anti-mouse IgG, HRP-linked antibody, anti-goat IgG, HRP-linked antibody or anti-rabbit IgG, HRP-linked antibody (all 1:1000, Cell Signaling Technology) for 1 hour at room temperature. Chemiluminescence was induced using LumiLightPLUS ECL substrate (Roche, Almere, The Netherlands) and visualized on an ImageQuant LAS 4000 Biomolecular Imager (GE Healthcare, Buckinghamshire, UK).

2.3 | In silico analysis of TET2 expression with publicly available data sets

Adult mouse lung single-cell sequencing data (SRA accession: SRS2874276) was visualized using the PanglaoDB database (https://panglaodb.se/index.html). Cell types in the lung and Tet2 expression in each cell type were analyzed. To analyze TET2 gene expression in human type II alveolar epithelial cells (hAEC2), the publicly available data set GEO GSE94555 which determined gene expression in hAEC2 isolated from the peripheral lung of IPF patients (n = 3)

and controls (n = 3)[30] was visualized in then Lung Gene Expression Analysis (LGEA) Web portal.[31]

2.4 | Animal model of pulmonary fibrosis

Homozygous *Tet2*$^{fl/fl}$ mice[32] were crossed with mice expressing Cre recombinase under the control of the surfactant protein C promoter (SpCcre mice)[33] to generate AEC2-specific TET2 deficient (*Tet2*$^{fl/fl}$*SpC*^{Cre}) mice; littermate *Tet2*$^{fl/fl}$ mice were used as controls in all experiments and all mice were backcrossed to C57Bl/6 at least eight times. Our laboratory previously showed that Cre expression driven by the *SpC* promoter is restricted to AEC2.[33] Pulmonary fibrosis was induced by a single intranasal dose of bleomycin (Sigma, St-Louis, MO) at 2 U/kg body weight as described before.[34] Mice were euthanized 14 or 21 days after bleomycin installation to assess fibrosis. First, the left tracheal bronchus was ligated and a one-sided bronchoalveolar lavage (BAL) was performed by flushing the lung with 2 × 0.5 mL of sterile phosphate-buffered saline. Subsequently, the right lung was homogenized in sterile saline (1:5) and used for mRNA isolation and hydroxyproline analysis. The left lung was taken for histology and stored in 10% formalin. All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center Amsterdam.

2.5 | BALF analysis

BALF was centrifuged at 1500 rpm for 10 minutes at 4°C to remove cells and debris. The supernatant was collected in a separate tube and stored at −20°C until further analysis. The pellet was resuspended in cold PBS and cells were counted using a hemocytometer (Beckman Coulter, Fullerton, CA). Cytocentrifuge slides of 40 000 cells/slide were prepared using a CytoSpin 4 (Thermo Fisher Scientific, Waltham, MA) at 250 g for 5 minutes. The slides were air-dried for 1 hour and stained with Giemsa. The percentage of macrophages was determined by counting 100 cells for each mouse.

2.6 | Quantitative real-time PCR

Total RNA was isolated from lung homogenates using NucleoSpin columns (Bioke, Leiden, Netherlands) according to the manufacturer's recommendations. All RNA samples were quantified by spectrophotometry and stored at −80°C until further analysis. cDNA was prepared using an M-MLV Reverse Transcriptase kit (Promega, Leiden, Netherlands) according to manufacturer's instruction. Gene expression analysis was performed using a Roche LightCycler 480 thermocycler with SensiFAST Real-time PCR kit (Bioline, London, UK) using the gene-specific primers listed in Table 1. The results were normalized to hypoxanthine-guanine phosphoribosyltransferase (*hprt*) expression levels.

2.7 | Immunohistochemistry (IHC) staining

Lung tissues were fixed in formalin and embedded in paraffin. Lung tissues were then cut into 4 μm sections and stained with primary mouse anti-TET2 monoclonal antibody (dilution 1:500, ab94580; Abcam, Cambridge, MA),

TABLE 1 Primers used for RT-qPCR

Primer name	Forward sequence	Reverse sequence
Tet2	AGCTGATGGAAAATGCAAGC	AAGGTGCCTCTGGAGTGTTG
Acta2	GCTACGAACTGCCTGACGG	GCTGTTATAGGTGGTTTCGTGGA
Fn1	AGAGGAGGCACAAGGTTCGG	GACAACCGCTCCCACTCCTC
Spp1	GCCGAGGTGATAGCTTGGCTTATG	CTCTCCTGGCTCTCTTTGGAATGC
Col1a1	GAGAGGTGAACAAGGTCCCG	AAACCTCTCTCGCCTCTTGC
Ccl2	AGGCTGGAGAGCTACAAGAGG	ACCCATTCCTTCTTGGGGTCA
Fizz1	CGTGGAGAATAAGGTCAAGGAAC	CACAAGCACACCCAGTAGCAG
Arg1	CTGGGAATCTGCATGGGCAA	GTCTACGTCTCGCAAGCCAA
Il6	CTTCCTACCCCAATTTCCAATGCT	TCTTGGTCCTTAGCCACTCCTT
Tnfα	CGAGTGACAAGCCTGTAGCC	CCTTGAAGAGAACCTGGGAGT
Epcam	GTCCGAAGAACCGACAAGGA	TGATGGTCGTAGGGGCTTTC
Scgb1a1	CAGACACCAAAGCCTCCAAC	ATCCTGGGCAGATGTCCGAA
Sftpc	AACGCCTTCTCATCGTGGTT	GGGCTAGGCGTTTCTGAGTT
Aqp5	CATGAACCCAGCCCGATCTT	CTCAGCGAGGAGGGGAAAAG
Hprt	AGTCAAGGGCATATCCAACA	CAAACTTTGCTTTCCGGGT

Ki-67 (D3B5) Rabbit monoclonal antibody (dilution 1:500, 12202T; Cell Signaling, Danvers, MA), anti-F4/80 antibody (1:5000, 123107, BioLegend, London, United Kingdom) or anti-proSurfactant protein C (proSP-C) antibody (1:1500, AB3786; Millipore, Billerica, MA); After incubation with HRP-linked goat anti-rabbit (IgG) secondary antibody (dilution 1:200, P0488; DAKO, Amstelveen, the Netherlands), positive staining was visualized using 3,3-diaminobenzidine (DAB, ImmunoLogic, Duiven, Netherlands); Hematoxylin or methyl green was applied as a counterstain.

2.8 | Assays

Hydroxyproline was measured by a commercially available assay (Sigma, St-Louis, MO) according to the manufacturer's instructions and as described before.[34] Mouse C-C Motif Chemokine Ligand 2 (CCL2) and immunoglobulin M (IgM) levels in bronchoalveolar lavage fluid (BALF), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in lung homogenates were measured by specific enzyme-linked immunosorbent assays (ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Total protein levels in the BALF were measured using a BCA protein assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the standard procedures.

2.9 | Histological analysis

Histological examination of the lungs was performed essentially as described before.[34,35] Lung section slides were prepared and stained with hematoxylin and eosin (H&E) or Masson's trichrome according to routine procedures. The severity of fibrosis was evaluated according to the Ashcroft scoring system based on the H&E straining.[36] Two independent observers were blinded to the treatment group and an average of 10 fields of each lung section was selected and scored. The results are reported as the average score of the individual field scores.

2.10 | Statistics

Statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software, San Diego, CA). Data are expressed as mean ± SEM. Comparisons between two conditions were analyzed using two-sided Mann-Whitney test, comparisons between multiple conditions were analyzed using Kruskal-Wallis test. *P* values of less than .05 were considered significant.

3 | RESULTS

3.1 | TET2 expression is downregulated in the lungs of IPF patients and in the lungs of bleomycin inoculated mice

To evaluate the potential role of TET2 during fibrosis, we first compared the expression of TET2 in normal lungs and IPF lungs by western blot. As shown in Figure 1A, TET2 protein levels in IPF lungs were significantly decreased compared with healthy controls. Subsequently, TET2 mRNA expression levels in the lungs of bleomycin-treated mice were determined. The expression of TET2 gradually decreased over time reaching the lowest level of expression at day 14 after bleomycin inoculation (Figure 1B). Recent single-cell sequencing data showed that *Tet2* is ubiquitously expressed in multiple cell types of adult murine lungs including AEC2 cells (SRA accession: SRS2874276) and that TET2 expression is decreased in human AEC2 cells (hAEC2) of IPF patients (GEO GSE94555 (Figure S1A,B).[30] In order to substantiate this data, we assessed TET2 expression in AEC2 cells in the lung of untreated mice. As shown in Figure 1C, proSP-C positive cells were also positive for TET2. Moreover, TET2 protein levels decreased at days 7, 14, and 21 days post bleomycin instillation (Figure 1D). Overall, these data show that TET2 expression decreases in AEC2 cells during the development of pulmonary fibrosis.

3.2 | AEC2-TET2 does not limit fibrosis progression in bleomycin-induced pulmonary fibrosis

Considering the role of AEC2 in epithelial repair and the role of TET2 in stemness, we hypothesized that TET2 may limit the development of fibrosis during the onset of the disease. In order to prove or refute this hypothesis, AEC2-TET2 deficient mice and littermate controls were intranasally inoculated with bleomycin and sacrificed after 14 or 21 days. Bleomycin administration caused a transient body weight loss in both wild-type and TET2 conditional knockout mice that were not different between groups (Figure 2A). More importantly, fibrosis development progressed similarly in wild-type and TET conditional knockout mice as reflected by similar increases in lung weight (Figure 2B) and total collagen levels, as determined by hydroxyproline levels (Figure 2C). To substantiate these findings, we next assessed the expression of the pro-fibrotic genes encoding smooth muscle-alpha-actin 2 (*Acta2*), fibronectin (*Fn1*), collagen type I (*Col1a1*), and secreted phosphoprotein 1 (*spp1*). As shown in Figure 2D, bleomycin administration induced similar expression levels of these pro-fibrotic genes in

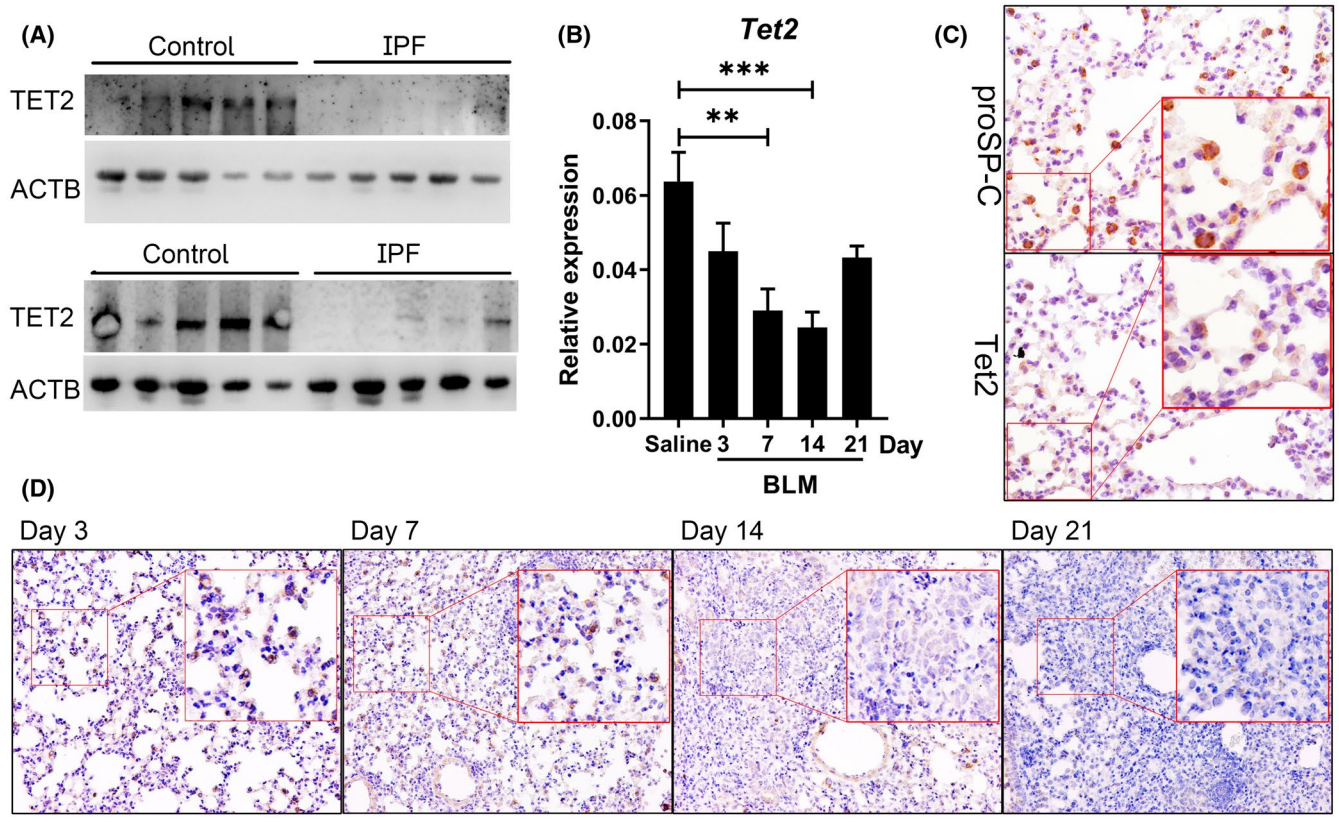


FIGURE 1 TET2 expression is downregulated in the lungs of IPF patients and in the lungs of mice upon intranasal inoculation of bleomycin compared to control. A, Representative picture of western blot analysis of TET2 expression in human lung homogenates of control and IPF lung tissue. Analysis was performed on n = 10 per group. ACTB: β-actin. B, Relative gene expression of *Tet2* determined by RT-qPCR in wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Expression levels are relative to that of *Hprt* (*hypoxanthine-guanine phosphoribosyltransferase*). Data are presented as mean ± SEM. n = 8 mice per group (BLM) or 4 mice per group (saline) at each time point. $^{**}P < .01$, $^{***}P < .001$. C, Representative pictures of proSP-C (upper panel) and TET2 (lower panel) immunohistochemical staining of lung sections collected from naive wild-type mice, magnification: 40×. The insets (right) are a 2× enlargement of the selected zone (square) in each representative picture. D, Representative pictures of TET2 immunohistochemical staining of lung sections from mice0with bleomycin for 3, 7, 14, and 21 days, magnification: 20×. The insets (right) are a 2× enlargement of the selected zone (square) in each representative picture. Treatment compared to corresponding saline control by unpaired *t* test (*) $P < .05$, (**) $P < .01$, (***) $P < .001$. Data between $Tet2^{fl/fl}SpC^{Cre}$ and control $Tet2^{fl/fl}$ mice were not significantly different at each time point

the lungs of wild-type and TET2 conditional knockout mice. To confirm these results at the protein level, fibronectin (FN) and Collagen type I (Col1) levels were determined by Western blot. In line with the gene expression levels, FN and Col1 levels were increased by bleomycin and comparable between the two groups (Figure 2E). Furthermore, total collagen levels, as determined by Masson's trichrome staining were comparable between wild-type and TET2 conditional knockouts at 14 and 21 days post bleomycin inoculation (Figure 3A). Subsequent histological analysis of the lungs showed that bleomycin induced extensive patchy areas of fibrosis culminating in severe pulmonary fibrosis at day 14 and day 21 (Figure 3B); the extent of fibrosis was, however, similar in wild-type and TET2 conditional knockout mice as evident from Ashcroft scores (Figure 3C). Taken together, these results suggest that TET2 in AEC2 does not limit fibrosis progression after bleomycin-induced pulmonary fibrosis.

3.3 | AEC2-TET2 does not affect macrophage migration or differentiation during bleomycin-induced experimental pulmonary fibrosis

Macrophages play important roles during fibrosis by either producing pro-fibrotic mediators or promoting wound healing in response to soluble factors secreted by injured epithelial cells.[37] Consequently, we quantified macrophage recruitment and differentiation during bleomycin-induced fibrosis in wild-type and TET2 conditional knockout mice. CCL2 protein levels, an important cytokine for monocyte/macrophage recruitment, were elevated at 14 and 21 days after bleomycin administration in BALF of wild-type and TET2 conditional knockout mice, which was paralleled by a rise in Ccl2 mRNA levels in lung homogenates (Figure 4A,B). Again, however, no differences were observed between wild-type

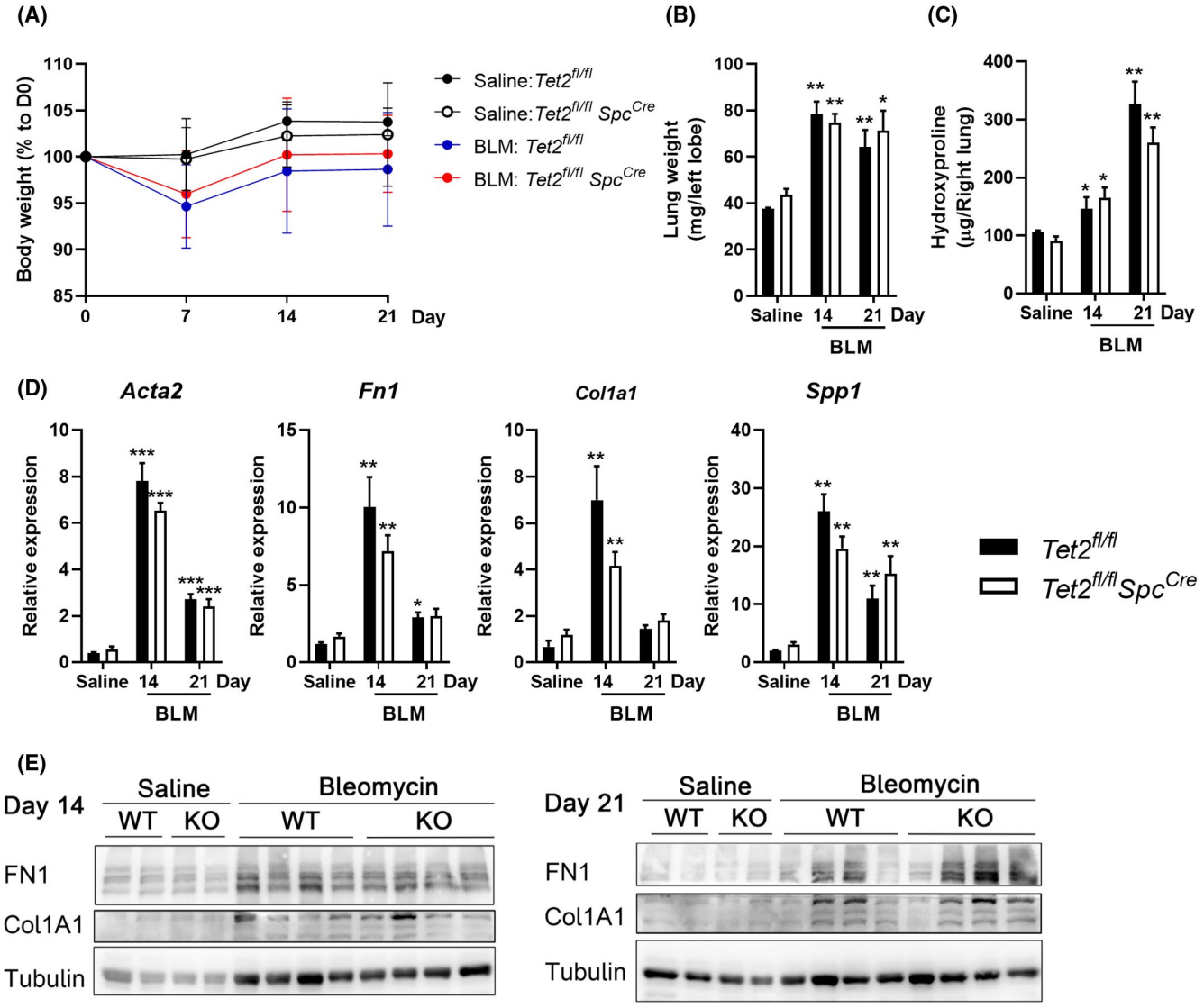


FIGURE 2 AEC2-TET2 does not affect the production of fibrotic mediators during bleomycin-induced pulmonary fibrosis. A, Total body weight of wild-type and TET2 conditional knockouts upon saline or 14 and 21 days bleomycin (BLM) administration in time. B, Lung weight of left lung lobes of wild-type and TET2 conditional knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. C, Collagen expression as measured by hydroxyproline levels in the right lung of wild-type and TET2 conditional knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. D, Relative gene expression of *Acta2* (*alpha-smooth muscle actin*), *Fn1* (*fibronectin 1*), *Col1a1* (*collagen type I alpha 1*), and *Spp1* (*secreted phosphoprotein 1*) determined by RT-qPCR in wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Expression levels are relative to that of *Hprt* (*hypoxanthine-guanine phosphoribosyltransferase*). E, Representative images of western blots for detecting protein levels of Fibronectin (FN) and Collagen type I (Col1) in lung tissues of wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Treatment compared to corresponding saline control by unpaired *t* test (*) $P < .05$, (**) $P < .01$, (***) $P < .001$. Data between $Tet2^{fl/fl}SpC^{Cre}$ and control $Tet2^{fl/fl}$ mice were not significantly different at each time point

and TET2 conditional knockout mice. Total cell numbers in BALF were increased at 14 and 21 days after bleomycin administration (Figure 4C) with the majority of these cells being macrophages (Figure 4D). However, neither the total cell numbers nor the macrophage count in BALF wasaltered by TET2 deficiency in AEC2 (Figure 4D). Similarly, macrophage numbers were also increased in fibrotic lung tissues upon bleomycin administration, which was not affected by AEC2-specific TET2 knockout (Figure S2A). Additionally, other cell types such as lymphocytes and neutrophils in the BALF were also not affected by TET2 deficiency in AEC2 (Figure S2B). Previous studies suggested that M1 and M2 macrophage activation coexist during pulmonary fibrosis,[38] therefore, we determined macrophage differentiation by assessing *Fizz1* and *Arg1* (M2 markers) and *Il6* and *Tnfα* (M1 markers) gene expression in the lungs of wild-type and TET2 conditional knockout mice. *Fizz1*, *arg1,* and *il6* expression were increased at 14 and 21 days after bleomycin

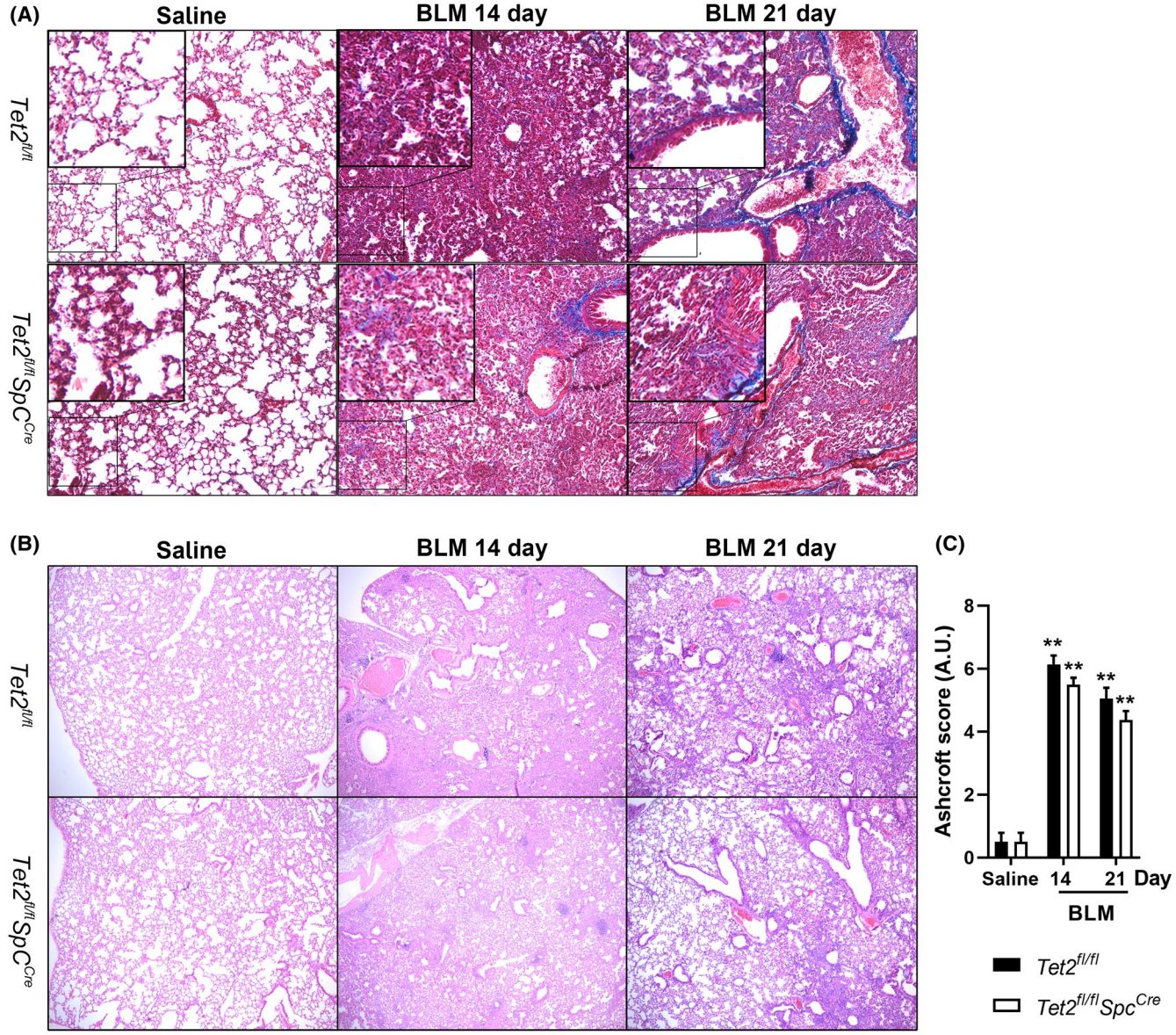


FIGURE 3 AEC2-TET2 does not affect the development of bleomycin-induced pulmonary fibrosis. A, Representative Masson's Trichrome stained lung sections of wild-type and TET2 conditional knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Magnification: 20×. The insets (up left corner) are a 2× enlargement of the selected zone (square) in each representative picture. B, Representative H&E-stained lung sections of wild-type and TET2 conditional knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Magnification: 10×. C, Quantification of pulmonary fibrosis using the Ashcroft score (AU: arbitrary units) in wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Data are presented as means ± SEM. Treatment compared to corresponding saline control by unpaired *t* test (**) $P < .01$. Data between $Tet2^{fl/fl}SpC^{Cre}$ and control $Tet2^{fl/fl}$ mice were not significantly different at each time point

administration, whereas *tnfα* expression was similar to saline-treated mice (Figure 4E,F). Most importantly, however, gene expression of these macrophage differentiation markers was not different between wild-type and TET2 conditional knockout mice in bleomycin-induced pulmonary fibrosis. In line, IL-6 and TNF-α protein levels were also similar in the lung homogenates of wild-type and AEC-specific TET2 knockout mice (Figure S3). Overall, these results suggest that AEC2-TET2 does not modify macrophage recruitment or activation during bleomycin-induced pulmonary fibrosis.

3.4 | AEC2-TET2 does not limit epithelial injury during bleomycin-induced fibrosis

Repetitive epithelial injury and abnormal lung alveolar epithelial regeneration have been recognized as causative mechanisms in pulmonary fibrosis.[4] To determine the abundance of epithelial cells in lung tissue during fibrosis, we determined the relative gene expression level of the pan epithelial cell marker *epcam*, bronchial epithelial club cell marker *scgb1a1*, AEC1 marker *aqp5,* and AEC2 marker *spftc*

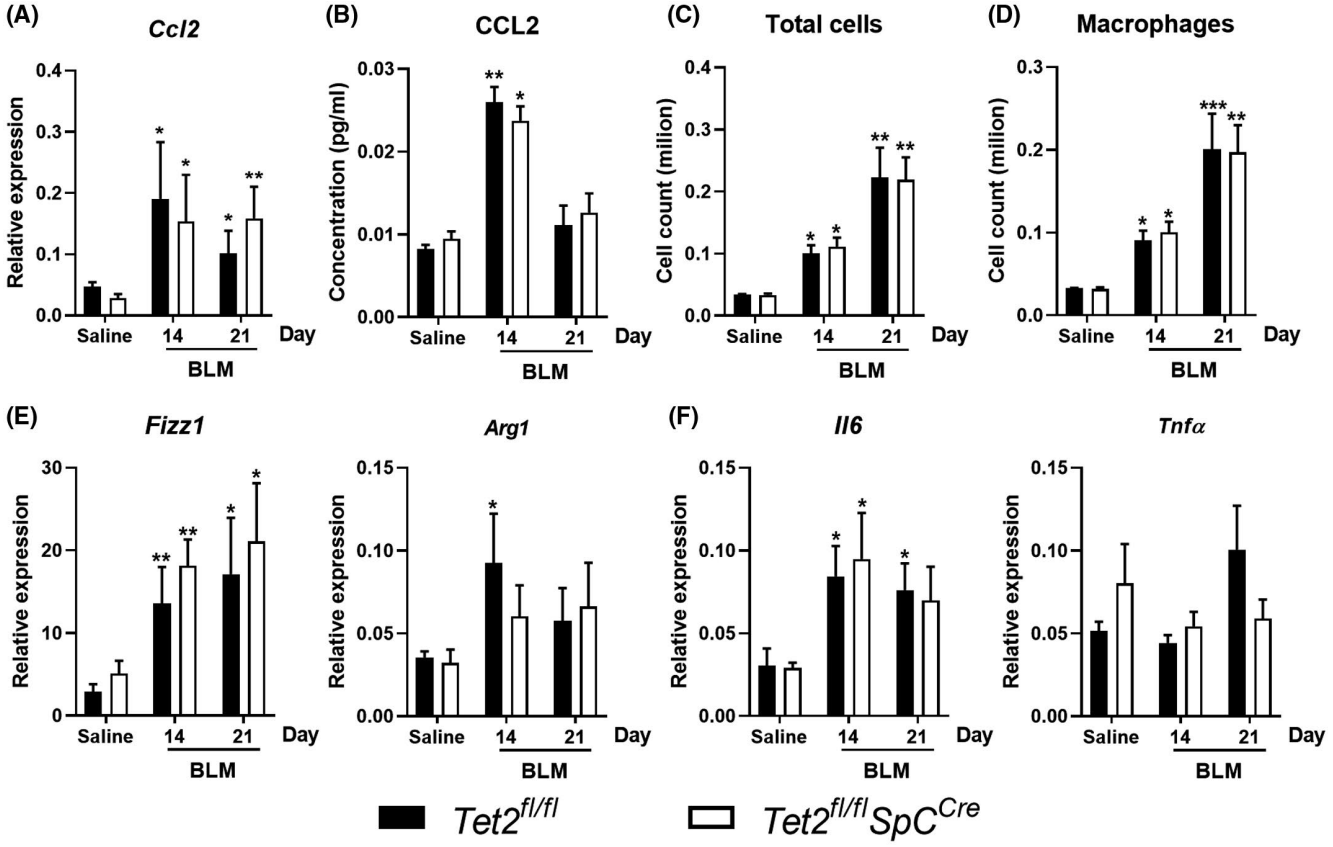


FIGURE 4 AEC2-TET2 does not affect macrophage migration or differentiation during bleomycin-induced pulmonary fibrosis. A, Relative gene expression of *Ccl2* (*C-C motif chemokine ligand 2*) determined by RT-qPCR in wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Expression levels are relative to *Hprt* (*hypoxanthine-guanine phosphoribosyltransferase*). B, CCL2 protein levels in BALF measured by ELISA in wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. C, Total cell numbers in BALF of wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. D, Macrophages numbers in BALF of wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. E-F, Relative gene expression of M2 macrophage activation genes *Fizz1* (*resistin-like molecule alpha1*) and *Arg1* (*arginase 1*) determined by RT-qPCR in wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Expression levels are relative to *hprt* (*hypoxanthine-guanine phosphoribosyltransferase*). F, Relative gene expression of M1 macrophage activation genes *Il6* (*interleukin-6*) and *Tnfα* (*tumor necrosis factor-alpha*) determined by RT-qPCR in wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Expression levels are relative to *hprt* (*hypoxanthine-guanine phosphoribosyltransferase*). Data are presented as means ± SEM. n = 8 mice per group (BLM) or 4 mice per group (saline) at each time point. Treatment compared to corresponding saline control by unpaired *t* test (*) $P < .05$, (**) $P < .01$, (***) $P < .001$. Data between $Tet2^{fl/fl}SpC^{Cre}$ and control $Tet2^{fl/fl}$ mice were not significantly different

by qPCR. Gene expression of epithelial markers was, however, unaltered by AEC2-TET2 expression upon bleomycin administration (Figure 5A). In line, western blot analysis of total lung homogenates showed that SP-C levels were not affected by bleomycin treatment or by TET2 deficiency in AEC2 (Figure S4). In addition, expression of the general cell proliferation marker Ki67 was almost absent in the lungs of saline-treated mice, but increased during bleomycin-induced pulmonary fibrosis to a similar extent in both wild-type and conditional *Tet2* deficient mice (Figure S5). Those results suggest that the regeneration of AEC1 and AEC2 cells upon injury is not affected by the loss of TET2. Epithelial injury, as measured by total protein and IgM levels in BALF, was increased after bleomycin treatment, but to a similar extent in wild-type and TET2 conditional knockout mice (Figure 5B,C). Taken together, epithelial integrity was decreased upon bleomycin administration, but not affected by TET2 expression in AEC2 cells.

4 | DISCUSSION

TET2 regulates several stem cell properties including stemness, proliferation, and differentiation,[39] and may, therefore, play a protective role during the development of pulmonary fibrosis. However, in this study, we show that the selective deletion of *Tet2* in AEC2 does not affect epithelial cell function and the development of bleomycin-induced pulmonary fibrosis.

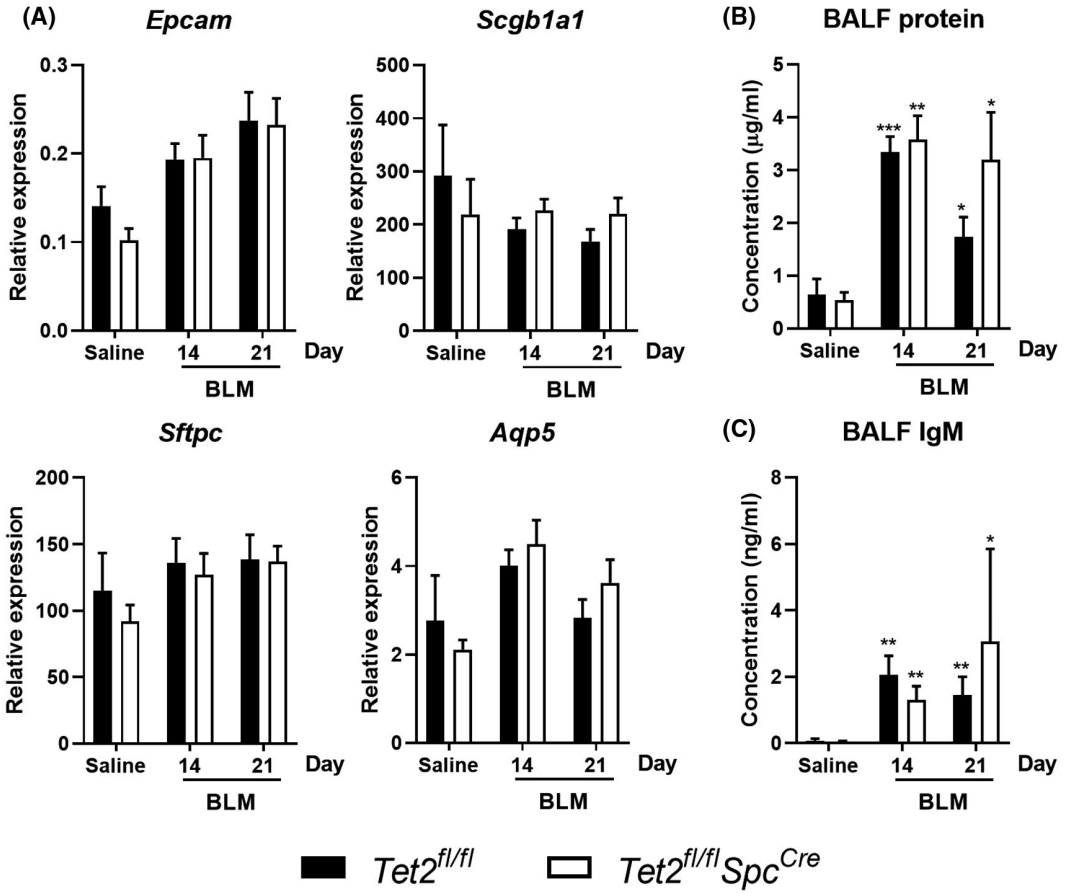


FIGURE 5 AEC2-TET2 does not limit epithelial injury during bleomycin-induced pulmonary fibrosis. A, Relative gene expression of epithelial cell marker *Epcam*, S*cgb1a1*, *Sftpc,* and *Aqp5* determined by RT-qPCR in wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Expression levels are relative to *Hprt* (*hypoxanthine-guanine phosphoribosyltransferase*). B, Total protein levels in BALF of wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. C, IgM levels in BALF of wild-type and conditional TET2 knockout mice upon saline or 14 and 21 days bleomycin (BLM) administration. Data are presented as means ± SEM. n = 8 mice per group (BLM) or 4 mice per group (saline) at each time point. Treatment compared to corresponding saline control by unpaired *t* test (*) $P < .05$, (**) $P < .01$, (***) $P < .001$. Data between $Tet2^{fl/fl}SpC^{Cre}$ and control $Tet2^{fl/fl}$ mice were not significantly different

AEC2 plays a crucial role during pulmonary fibrosis.[8] AEC2 minimizes and protect against pulmonary fibrosis by acting as stem cells to repair the damaged alveolar epithelium.[7] Indeed, AEC2 function as lung stem cells and their dysfunction during fibrosis accelerates disease development.[6,8,14] TET2 was originally recognized as a DNA methylation eraser that removes DNA methylation through a stepwise procedure working together with base excision repair mediated by thymine DNA glycosylase.[40] Alterations in DNA methylation during fibrosis have been extensively reported in different organs.[41] It is well known that DNA methylation increases during the differentiation of stem cells, but methylation patterns differ between cell types.[23,42] During the differentiation of stem cells, dysfunction of TET2 leads to abnormal cell proliferation.[25,43] Whether TET2 also protects the stemness of AEC2 by limiting DNA methylation during pulmonary fibrosis is unknown. In line with previous reports,[44] we here show extensive epithelial damage upon bleomycin instillation in the lungs of wild-type mice as reflected by increased protein and IgM levels in BALF. We hypothesized that the ablation of TET2 in AEC2 cells would aggravate pulmonary fibrosis development because of the potential loss of stemness in these cells. However, we did not observe such an effect, suggesting that TET2 does not affect the stemness of AEC2 cells during bleomycin-induced pulmonary fibrosis development. Notably, the dosing window at which bleomycin induces fibrosis in wild-type mice is narrow, with low doses failing to elicit fibrosis and high doses causing lethality[45] (and our own observations). Our results do not exclude that TET2 in AEC2 does play a role in lung fibrosis induced by bleomycin administered at doses different from those used here.

An interesting finding is that TET2 levels in lung homogenates are dramatically decreased in IPF patients. This decrease in TET2 levels could well be explained by

the loss of AEC2 cells during the development of IPF.[46] However, the scRNAseq data of IPF lungs and healthy controls showed that TET2 levels are decreased in AEC2 cells in IPF, suggesting that the reduction of TET2 levels in IPF lung homogenates is not solely due to the loss of AEC2 cells.

Aside its important role in regulating stem cell properties, TET2 also regulates cellular functions in terminally differentiated cells. TET2 proteins regulate the expression of tight junction proteins in intestinal epithelial cells thereby regulation epithelial barrier function.[47] In addition, we recently found that TET2 maintains the bronchial epithelial barrier function during acute pulmonary infection by *Pseudomonas aeruginosa*.[48] Loss of epithelial integrity may lead to the development of pulmonary fibrosis and protection of the epithelial integrity may, therefore, be protective.[49] Although epithelial damage was evident after bleomycin instillation, this was not significantly affected by the deletion of TET2 in AEC2, indicating the epithelial interaction during pulmonary fibrosis is not affected by epithelial TET2.

Upon epithelial injury, soluble factors are released by the epithelial cells in order to attract monocytes and macrophages which play critical roles during the initiation, maintenance, and resolution phases of tissue repair. Disturbances in these tightly regulated processes can affect macrophage function leading to the development of pathological fibrosis.[37] Indeed, monocyte-derived macrophages drive pulmonary fibrosis and inhibition or deletion of these macrophages limits pulmonary fibrosis.[50,51] The monocyte chemoattractant chemokine CCL2 which is predominantly produced by AEC2 has been reported to contribute to fibrosis by recruiting monocyte and fibrocytes[52,53] and in order to determine the role of epithelial TET2 in the expression of CCL2 we determined *ccl2* gene expression and CCL2 protein levels in the lung. While we observed an increase in CCL2 production and subsequent macrophage recruitment, these were not altered by TET2 deficiency in AEC2, suggesting that the epithelial-macrophage crosstalk and macrophage function during fibrosis are not regulated by epithelial TET2.

An interesting finding is the loss of TET2 expression in IPF lung and one could argue that the deletion of TET2 in AEC2 might not be the appropriate approach. TET2 expression in the bleomycin-induced pulmonary fibrosis model shows, however, that TET2 expression is still detectable in the early phase of the model and could, therefore, play a protective role in preserving stemness during this phase. We show that the complete deletion of TET2 in AEC2 from the start of the model did not attenuate fibrosis, showing that AEC2-TET2 does not protect against fibrosis development. This implies that the demethylation of target genes by TET2 during the early development of fibrosis is not beneficial and this specific epigenetic mechanism has potentially limited therapeutic potential in early pulmonary fibrosis. Whether the re-expression of TET2 in AEC2 during established pulmonary fibrosis, thereby re-activating the expression of target genes, would limit bleomycin-induced pulmonary fibrosis remains, however, elusive. Moreover, (de)methylation of target genes affecting stemness of AEC2 cells by alternative (de)methylation proteins may also still play a role in the development of pulmonary fibrosis and future studies are needed to address the importance of epigenetic regulation in AEC2 cells during pulmonary fibrosis development. In addition, although the bleomycin model is still considered the best available model to investigate pulmonary fibrosis and has been of value to the field, it also has limitations in mimicking IPF.[54,55] It would, therefore, be of interest to confirm the results of the current study using different lung fibrosis models such as silicosis or adeno-TGF-β-induced fibrosis.

Overall, we show that TET2 in AEC2 cells does not play a protective role in the development of pulmonary fibrosis in the well-established model of bleomycin-induced pulmonary fibrosis.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

W. Qin, J. Duitman, and T. van der Poll conceived and designed research; W. Qin and J. Duitman performed experiments, analyzed data, prepared figures, and drafted manuscript; W. Qin, J. Duitman, B. Crestani, C.A. Spek, B.P. Scicluna and T. van der Poll interpreted the results, edited and revised the manuscript, and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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