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Graphical abstract

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Molecular Clock REV-ERBα regulates Cigarette Smoke-Induced Pulmonary Inflammation And Epithelial-Mesenchymal-Transition

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Short running title: Rev-erbα regulates CS-induced EMT
Abstract

Cigarette smoke (CS) is the main etiological factor in the pathogenesis of emphysema/Chronic Obstructive Pulmonary Disease (COPD), which is associated with abnormal epithelial-mesenchymal-transition (EMT). Previously, we have shown an association between circadian rhythms and CS-induced lung inflammation, and nuclear-heme-receptor α (REV-ERBα) acting as an anti-inflammatory target in both pulmonary epithelial cells and fibroblasts. We hypothesized that molecular clock REV-ERBα plays an important role in CS-induced circadian dysfunction and EMT alteration. C57BL/6J wild type (WT) and REV-ERBα heterozygous (Het) and knockout (KO) mice were exposed to CS for 30 days (sub-chronic) and 4 months (chronic), and WT mice were exposed to CS for 10 days with or without REV-ERBα agonist (SR9009) administration. Sub-chronic/chronic CS exposure caused circadian disruption and dysregulated EMT in the lungs of WT and REV-ERBα KO mice, both circadian and EMT dysregulation were exaggerated in REV-ERBα KO condition. REV-ERBα agonist, SR9009 treatment reduced acute CS-induced inflammatory response and abnormal EMT in the lungs. Further, REV-ERBα agonist (GSK4112) inhibited TGFβ/CS-induced fibroblast differentiation in human fetal lung fibroblast 1 (HFL-1). Thus, CS-induced circadian gene alterations and EMT activation are mediated through a Rev-erba-dependent mechanism, which suggests activation of REV-ERBα as a novel therapeutic approach for smoking-induced chronic inflammatory lung diseases.

Key Word: Cigarette Smoke, EMT, REV-ERBα, molecular clock, COPD
Clinical Relevance

Previous studies have shown that REV-ERBα plays a role in regulating pulmonary inflammation. However, there is a lack of study investigating the role of REV-ERBα in mediating CS-induced epithelial to mesenchymal transition (EMT) in mouse lungs. Our research delves into the role of REV-ERBα in CS-induced EMT. CS-induced activation of mesenchymal markers was correlated with REV-ERBα deletion, but epithelial markers remain unaffected. Treatment with REV-ERBα agonists (SR9009 or GSK4112) exhibited protective role against CS or TGF-β induced dysregulation of EMT markers. Our study emphasized the role of the circadian clock molecule, REV-ERBα in the pathogenesis of COPD, and highlighted the potential of REV-ERBα agonist as a novel chrono-therapeutic drug against smoking-induced chronic lung diseases via EMT.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of death worldwide, and affects more than 10% of the global population (1). There are limited therapeutic strategies currently available, and the comprehensive treatment mechanisms remain unclear (2). COPD is a chronic lung disease resulting from exposure to environmental pollutants/toxicants, noxious gases, and cigarette smoke (CS). CS is a significant risk factor that causes COPD. Our previous reports showed that short-term CS exposure (acute and sub-chronic) causes inflammation/injury in mouse lungs, and chronic CS exposure results in emphysema/COPD, which could be exacerbated by influenza A virus infection (3-5).
Epithelial to mesenchymal transition (EMT) has been studied previously as a primary mechanism of COPD (6). Down-regulation of epithelial phenotypes (E-cadherin, ZO-1, and Occludin), increased mesenchymal phenotypes (Vimentin, α-Smooth Muscle Actin, and Fibronectin) and extracellular matrix remodeling, as well as altered barrier function, are all associated with CS-induced COPD-phenotypes (7, 8). Additionally, bronchial epithelium exposed to CS showed decreased barrier function with a reduced abundance of adherence junction proteins, and increased mesenchymal markers (9). Intriguingly, transcriptions of most EMT genes are regulated by E-box, which also regulates circadian clock genes (10-12). Previous research has shown that circadian clock molecules play an essential role in lung injury and chronic inflammatory lung diseases (11, 13, 14). However, there is no information available on the role of circadian clock in modulating EMT phenotypes in COPD and its exacerbations.

Circadian oscillation is a fundamental biological process occurring within various organs, such as the heart, lung, liver, and brain (12, 15). The circadian clocks in different organs are driven by a similar core molecular feedback loop. The CLOCK:BMAL1 (Brain and Muscle ARNT-Like 1; Gene symbol: Arntl) heterodimer binds to E-box and promotes the transcription/translation of either core clock molecules or downstream targets. The core clock molecules include: Retinoid Acid Receptor-related Orphan Receptor (RORα, RORβ, and RORγ), Nuclear Receptor subfamily (REV-ERBα (Gene symbol: Nr1d1) and REV-ERBβ (Gene symbol: Nr1d2)), Period 1/2/3 (PER1, PER2, and PER3) and Cryptochrome 1/2 (CRY1 and CRY2), which form two different feedback-loops that affect the activity of CLOCK:BMAL1 complexes (12). Heterodimers
containing PERs and CRYs inhibit transcription activated by CLOCK: BMAL1 complexes. In a different loop, RORs compete with REV-ERBα/β for binding with ROR response elements (RORE), with RORs contributing to the activation of BMAL1 transcription, whereas REV-ERBα/β inhibits it (12). Previous studies have shown that circadian clock molecules play an important role in the pathogenesis of chronic lung diseases, such as pulmonary fibrosis, COPD, and even lung cancer (13, 16-18).

Molecular clock REV-ERBα, a transcriptional inhibitor involved in circadian rhythms, can shorten the circadian period (19). Our previous studies identified a lower abundance of REV-ERBα protein in smokers and COPD patients in comparison to non-smokers (14). Moreover, loss of REV-ERBα worsens the inflammatory response induced by CS, while REV-ERBα agonist helped to reduce the inflammation (13). However, there is no research focused on how REV-ERBα modulates abnormal EMT induced by CS. In this study, we hypothesized that the loss of REV-ERBα promotes CS-induced EMT in mouse lungs, and that treatment with REV-ERBα agonist attenuates both lung inflammation and EMT.
Results

Activation of EMT in the lungs of smokers compared to healthy control

It has been shown that CS induces EMT in lung epithelial cells in culture, and the lungs of smokers compared to health control. As shown in Figure 1, we observed a significant increase in the abundance of type-1 collagen (COL1A1) in smokers compared to non-smokers as well as a non-significant increasing trend of TGFβ and vimentin between smokers vs. non-smokers. There was no change in the protein level of plasminogen activator inhibitor-1 (PAI-1) between smokers and non-smokers. Overall, our results confirm the upregulation in mesenchymal transition markers in the lungs of smokers compared to non-smokers. In our previous study, we have shown decreased protein levels of REV-ERBα in smokers compared to non-smokers (14). Here, we proposed to determine how REV-ERBα affects EMT activation.

REV-ERBα deficiency promotes clock dysregulation in the lungs following sub-chronic CS exposure

To determine how sub-chronic CS exposure affects circadian clock gene targets in mouse lungs, we have employed a customized NanoString nCounter panel for gene transcription analysis. Sub-chronic CS exposure causes circadian clock dysregulation in the lungs (Figure 2 and Figure S1). The core clock-controlled genes (CCGs), such as Arntl, Per2, Per3, Cry2, and Rora showed altered expression following sub-chronic CS exposure. Other circadian-related genes, such as Dbp (D site of albumin promoter binding protein), Bhlhe41 (Basic helix-loop-helix family, member e41), Bhlhe40 (Basic helix-loop-helix family, member e40), Csnk1d (Casein Kinase 1 Delta), Csnk2a1 (Casein
Kinase 2 Alpha 1), Fbxl3 (F-Box And Leucine Rich Repeat Protein 3), Mapk14 (Mitogen-Activated Protein Kinase 14), Mat2a (Methionine Adenosyl transferase 2A), Nr2f6 (Nuclear Receptor Subfamily 2 Group F Member 6), and Nfil3 (Nuclear Factor, Interleukin 3 Regulated), were all dysregulated after sub-chronic CS exposure. In REV-ERBα KO mice, sub-chronic CS exposure further up-regulated circadian gene transcription of selected genes, such as Per2, Per3, Dbp, Bhlhe41 and Hlf (Hepatic leukemia factor) (Figure 2 and Figure S1). Notably, Dbp, Bhlhe41, and Per2 genes showed a significant increase in REV-ERBα KO mice exposed to CS compared with their respective air group control. However, we only observed a remarkable dysregulation in CCGs at the gene-level. There was an increase in the protein abundance of BMAL1 after sub-chronic CS exposure (Figure S2).

**REV-ERBα deficiency accelerates EMT activation following sub-chronic CS exposure**

To further investigate the correlation between REV-ERBα and CS-induced EMT, we examined EMT-associated gene expression and protein abundances/localizations in the lungs of sub-chronic CS exposed WT and REV-ERBα KO mice (Figure 3 and Figure 4). At the gene transcription level, only Col1a1 (Collagen type I alpha I) showed significant upregulation in CS exposed REV-ERBα KO mice compared to CS exposed WT mice (Figure 3). Most of the EMT markers, especially the mesenchymal markers, including Vim (Vimentin), Tgfb1 (Transforming Growth Factor Beta 1), Serpine1 (Serpin Family E Member 1), Col3a1 (Collagen type III alpha I), Mmp2 (matrix metalloproteinase-2), and Timp1 (TIMP metallopeptidase inhibitor 1) were increased in
CS exposed mice compared to air group control (Figure 3, Figure 4C, and Figure S3). Surprisingly, we showed a decreased gene expression of Snail (Snai1) after CS exposure in both WT and KO mice (Figure S3). The epithelial marker, such as Cdh1, was decreased in CS exposed (WT or KO) mice (Figure 4C).

EMT-related protein abundances and localizations were measured to further determine the role of REV-ERBα in CS-induced EMT. All mesenchymal markers: Vimentin, COL1A1, TGFβ, PAI-1, and p53 were increased in CS exposed mice (Figure 4 and Figure 5). Additionally, protein expression of Vimentin, COL1A1, TGFβ, and Snail-Slug were augmented in CS exposed REV-ERBα KO mice (Figure 4A). To further understand the altered expression and localization of mesenchymal markers, vimentin, α-smooth muscle actin (αSMA), and snail-slug were stained immunohistochemically. In agreement with immunoblotting results, CS exposure significantly increased vimentin expression in the alveolar region. In REV-ERBα KO mice, further increase in the protein abundance of vimentin and snail-slug in the alveolar regions of the lungs was semi-quantitatively analyzed (Figure 4B and Figure S4). Expression of αSMA was also increased in alveolar epithelium of CS exposed mice independent of REV-ERBα (Figure 4B and Figure S4). Most of the EMT markers, such as αSMA, vimentin, and snail-slug were expressed near airway, while CS exposure augmented the expression of mesenchymal markers in alveoli (Figure S4). PAI-1 and p53 protein levels were increased after sub-chronic CS exposure in both WT and REV-ERBα KO mice (Figure 5). The epithelial markers: E-cadherin and ZO-1 were decreased in CS exposed WT and REV-ERBα KO mice (Figure 5).
MMPs are the essential components in ECM remodeling and deposition; MMP2, 9, and 12 are up-regulated after CS exposure. We evaluated the protein abundance of MMP2, MMP9, and MMP12 in mouse lung after 30 days exposure (Figure S5). We only found increased protein level of MMP12 in CS-exposed REV-ERBα KO mice, whereas MMP2 and MMP9 showed no alteration in protein abundance (Figure S5). However, we observed an increase in MMP2 gene expression in both CS exposed WT and REV-ERBα KO mice (Figure 3). There was no difference in gene expression of MMP9 among all the treatment groups (Figure S3).

REV-ERBα deficient mice exposed to sub-chronic CS show altered ECM and lung remodeling

We showed that CS-induced mesenchymal transition is associated with circadian clock gene REV-ERBα in the lungs. We investigated this association by utilizing histological analysis of lung sections for alveolar destruction/airspace enlargement and ECM-remodeling (Figure 6 and Figure S6). We measured lung mean linear intercept (Lm) analysis using H&E stained images and Gomori’s Trichrome staining to quantify collagen deposition in the lungs from different experimental groups. Type1-collagen and fibronectin were also immunohistological stained to determine the ECM-remodeling. We found that the mean linear intercept (Lm) of airspace was increased, but not significant in WT mice exposed to CS compared to air exposed controls. However, REV-ERBα KO mice exposed to sub-chronic CS exhibited significantly increased Lm compared to their respective air group control (Figure 6A and Figure S6). As expected, various immune cells were found in the airspace of both CS exposed WT and REV-ERBα KO mice
compared to respective air exposed controls (Figure 6A and Figure S6). Additionally, a significant increase in collagen deposition around airway was found in CS exposed REV-ERBα KO mice, while CS exposed WT mice showed similar collagen deposition compared to the air exposed control (Figure 6B). We also found a higher type-1 collagen deposition in CS exposed REV-ERBα KO mice compared to air control (Figure 6C), complementing the trichrome staining results. However, we did not observe any significant difference in fibronectin localization and expression (Figure 6D).

We exposed REV-ERBα Het and WT C57 mice to CS for 4 month, and we found similar alterations of lung morphometry and inflammation (Figure 7). We found increased lung compliance and decreased lung resistance and elastance in CS exposed REV-ERBα Het mice compared to WT mice or air group (Figure 7A). In concurrence with the increased compliance, we have observed airspace enlargement in CS exposed REV-ERBα Het mice compared to WT mice (Figure 7B). We observed that the baseline Lm results vary between 1 month and 4 month mouse groups due to mouse batch differences, such as their age and quantitation involved in scoring. Further, we found that chronic CS exposure increased inflammatory cell influx in BALF with increased macrophage, neutrophils, T-lymphocytes and total cells (Figure 7C). However, REV-ERBα Het mice did not show significant difference in altered inflammatory cell counts compared to WT mice (Figure 7C). To support our finding for lung morphometry and inflammatory responses, we found a similar change histologically (Figure 7B and Figure S7). Increased airspace levels were only observed in REV-ERBα Het CS exposed mice (10x and 20x magnification pictures, denoted by green arrows); lung
injury and infiltration of inflammatory cells in airspace were observed in both WT and REV-ERBα Het CS exposed mice (10X and 20x magnification pictures, denoted by red arrows) (Figure S7).

**REV-ERBα agonist treatment attenuates lung inflammatory response caused by acute CS exposure**

We have shown that REV-ERBα deficiency promotes abnormal mesenchymal transition and our previous study has shown that REV-ERBα is associated with increased lung inflammation induced by sub-chronic CS exposure (13). Here, we demonstrate the therapeutic potential of REV-ERBα agonist (SR9009) in acute CS-induced inflammation. After 10 days CS exposure, total cells from BAL fluid increased in both vehicle and Rev-erb agonist (SR9009) treatment groups (Figure S8A). The percentages of inflammatory cells were determined by flow cytometry, and the percentages of macrophages, CD4 T-cells, and CD8 T-cells were similar in acute CS-exposed mice treated with vehicle or SR9009 (Figure S8A). Intriguingly, acute CS-induced increase in neutrophils was inhibited by REV-ERBα agonist (SR9009) treatment (Figure S8A).

We measured the BAL fluid cytokines by Luminex to identify the inflammatory responses induced by acute CS exposure with or without SR9009 treatment. We found that only CS-induced increase in IL-5 (p= 0.06) and GM-CSF (p=0.07) were inhibited by SR9009 treatment without significant difference. Other BAL fluid cytokines, such as MCP-1 and KC, had significantly increased in the CS alone and CS+SR9009 treatment groups (Figure 8B and Table 1).
REV-ERBα agonist treatment reduced alterations in EMT markers caused by acute CS exposure

We have shown that sub-chronic CS exposure resulted in mesenchymal transition in a REV-ERBα-dependent manner. Here, we demonstrate the protective role of SR9009 against dysregulated EMT following acute CS exposure (Figure 9 and Figure 10). As expected, Vimentin, COL1A1, TGF-β, and Snail-Slug were up-regulated by acute CS exposure, and SR9009 treatment significantly reduced COL1A1 and Snail-Slug protein abundance. Additionally, SR9009 treatment also inhibited the CS-induced increase in Vimentin and TGFβ, which was not significantly different between air- and CS-exposed SR9009 treatment groups. (Figure 9A). We also measured the gene expression levels of Tgfb1, Col1a1, Vim, Snai1, and Snai2 (Figure 9B). We observed increased Tgfb1 and Col1a1 after CS exposure, and SR9009 administration was able to inhibit the upregulation of Tgfb1 but not Col1a1 (Figure 9B). There was no difference in Vim expression among groups (Figure 9B). Interestingly, we found decreased gene expression of Snai1 and Snai2 after 10 days CS exposure (Figure 9B). Other mesenchymal markers: PAI-1 and p53 were increased after acute CS exposure in both CS alone and CS+SR9009 treatment groups (Figure 10). We also measured the protein abundance of epithelial markers. We found that acute CS exposure decreased E-cadherin protein levels, while CS+SR9009 treatment further decreased E-cadherin levels. Interestingly, there was no change in the protein levels of ZO-1 between air and CS exposed mice, while SR9009 treatment augments ZO-1 in air+SR9009 treatment group compared to both air alone and CS+SR9009 treatment groups (Figure 10).
We showed increased protein abundance of MMP12 in mouse lungs after 30 days CS exposure, and increased gene transcript level of MMP2. We measured the protein expression levels of MMP2, 9 and 12; and gene levels of Mmp2, Serpine1, and Fn1 in 10 days exposed mouse lungs as well (Figure S8). Surprisingly, we observed a decreased protein expression level of MMP12 after 10 days CS exposure (Figure S8), whereas increased MMP12 was found after 30 days CS exposure (Figure S5). Similarly, gene expression of MMP2 was upregulated after CS exposure, and SR9009 administration failed to attenuate MMP2 gene expression. There was no difference in the protein expression of MMP9 and MMP2, as well as the gene transcripts of Fn1 and Serpine1 (Figure S8).

Finally, we measured the protein abundance of a few key circadian clock molecules to understand the possible protective role of SR9009 treatment in acute CS exposed mice. Immunoblot analysis of circadian clock targets confirmed an increase in the protein abundance of REV-ERBα in both acute air- and CS-exposed mice treated with SR9009 (Figure S9). We found increased protein abundance of RORα in CS-exposed mice treated with SR9009 compared to vehicle/CS-exposed controls. BMAL1 protein levels remain unaffected among different exposure and treatment groups, and CLOCK protein expression was decreased in CS+SR9009 treatment group compared to the air+SR9009 group (Figure S9). Overall, results revealed REV-ERBα agonist treatment mediates attenuation of lung inflammation and EMT phenotype via augmenting REV-ERBα expression in the lungs.
REV-ERBα agonist treatment prevents fibroblast differentiation in vitro in HFL1 cells

In order to determine if REV-ERBα plays an essential role in CS-induced EMT. We investigated the role of REV-ERBα in fibroblast differentiation. Human Lung Fibroblast (HFL1) cells were treated with TGFβ with or without GSK4112 (REV-ERBα agonist) for 3 days (Figure 11A). TGFβ-induced increase in gene expression of ACTA2 (α-smooth muscle actin) was significantly inhibited by GSK4112 treatment in HFL1 cells, whereas mRNA levels of COL1A1 and FN1 (Fibronectin) were reduced by GSK4112, but not significantly. We also treated HFL-1 cells with CS extract (CSE) with or without GSK4112, and measured the same fibroblast differentiation markers (Figure 11B). We found a high concentration of CSE (0.25%) showed reduced expression of ACTA2 and FN, while low 0.1% CSE increased gene expression of ACTA2 and COL1A1, without change in the expression of FN1 (Figure S10). Similarly, we showed a significant reduction in gene expression of ACTA2, COL1A1 and FN1 after treatment of 0.1%CSE + GSK4112 (Figure 11B). These findings indirectly support the role REV-ERBα during inflammation induced mesenchymal differentiation in the lungs (Figure 11).
Discussion

CS exposure induces lung inflammation, followed by the development of chronic lung diseases, such as emphysema/COPD and lung remodeling. Previously, we have shown that the CS-induced inflammatory response in mice which was associated with circadian molecular clock dysfunction (13, 16). REV-ERBα expression was reduced in smokers and COPD patients compared to healthy controls as well as in LPS treated peripheral blood mononuclear cells (PBMCs) (14). In this study, we found that CS-induced EMT was associated with REV-ERBα in both acute and sub-chronic mouse models. Based on our findings, the role of REV-ERBα in mesenchymal activation following CS exposure in mice was warranted. As expected, REV-ERBα agonists (SR9009 and GSK4112) both showed potential therapeutic effects in fibroblast phenotype dysregulation, and SR9009 treatment showed attenuation against CS-induced acute lung inflammatory responses. NanoString data analysis confirmed that the circadian clock gene targets were affected by sub-chronic CS exposure, which may be responsible for the abnormal lung development (EMT) and reduced repair at the early stage of CS-induced lung injury.

Circadian rhythms exist in almost all the organs, such as lungs, liver, heart, and spleen. Previous studies have reported that several physiological processes, such as body temperature, hormone release, and sleep-wake cycle are regulated by circadian rhythms. Additional evidence suggests that loss of specific circadian clock molecules, such as BMAL1, REV-ERBα, and CRY1, could promote an inflammatory response and metabolic dysfunction in target organs (20-22). Our results are in agreement with a
previous study describing the association between lung inflammation and decreased levels of BMAL1 (23). Previous studies demonstrated the anti-inflammatory effects of certain clock molecules including: REV-ERBα, CRY1, CRY2, RORα, and BMAL1 (24-27). In this study, we showed that the expression of several key circadian clock targets were significantly downregulated following sub-chronic CS exposure. From our previous study, we also observed decreased CRY2, BMAL1, and REV-ERBα in smoker and COPD patients compared to healthy control (14). The heterodimer BMAL1:CLOCK activates the transcription of CRYs, PERs, and RORα; down-regulation of BMAL1, CRYs and RORα showed the tendency of circadian feedback loop inhibition (28).

However, we also observed an increase in the expression of PERs (Per1/2/3) and DBP, following sub-chronic CS exposure. It should be noted that the expression of genes within the mammalian PER families (Per1/2/3) not only regulated by BMAL1:CLOCK activated E-boxes, but also additionally regulated by DBP bond D-boxes (28). Overall, our results suggest that acute and sub-chronic CS exposure induced alterations in circadian clock gene expression which may have an impact in disrupting the positive-negative feedback loops, thus culminating in the circadian clock dysfunction in the lungs.

Our data on transcriptomic analysis by NanoString revealed sub-chronic CS exposure-induced circadian clock dysfunction in the lungs. REV-ERBα deficient mice showed alterations in the expression of circadian clock-controlled genes such as Per1, Per2, Per3, Dbp, and Bhelhe41, whereas the expressions of Arntl, Cry1, and Cry2 were not affected. As previously reported, REV-ERBα KO showed increased transcription of Dbp (24), and DBP is capable of activating the expression of PERs (28). In this study, we
found that REV-ERBα deletion resulted in CS-induced expression of Dbp and Per2. Interestingly, Per2 also served as a tumor suppressor, targeting cell proliferation and cell-cycle progression (18). The role of Per2 and Dbp need further investigation, especially their interaction with REV-ERBα. Based on our gene transcription data, selective clock antagonists/agonists specific for CRYs, RORα, and PERs and activators of protein kinases showed protective effects. However, their therapeutic role against CS-induced circadian clock dysfunction requires further studies.

We have shown that EMT activation was observed in smokers relative to non-smokers, and that sub-chronic CS exposure in mice had induced EMT in a REV-ERBα-dependent manner. It is obvious that that EMT induced by TGFβ down-regulates epithelial markers (E-cadherin, and ZO-1) along with activation of mesenchymal markers (Vimentin, Collagen, Fibronectin, and αSMA) (29). CLOCK:BMAL1 heterodimer, the major transcription activator of REV-ERBα, has been shown to be associated with EMT markers E-cadherin and N-cadherin (30). Similarly, silencing BMAL1 attenuated the TGFβ-induced EMT in mouse lungs as well as in lung epithelium (31). Further, TGFβ induction in mouse lungs resulted in increased BMAL1 along with decreased REV-ERBα and RORα levels (31). The indirect connection between EMT markers and circadian molecules cannot be ruled out. In this study, sub-chronic CS exposure reduced transcriptional levels of BMAL1 and CLOCK, while increasing BMAL1 at the protein levels. The dysregulated BMAL1 and CLOCK might be one of the reasons for EMT induced by CS, and the detailed mechanisms need further investigation. Interestingly, REV-ERBα has been associated with the pathogenesis of pulmonary
fibrosis with possible an interaction with the transcription factor TBPL1 (17). We have shown that dysregulated EMT markers which are associated with REV-ERBα KO are mostly mesenchymal markers (Snail-Slug, Vimentin, COL1A1, and TGFβ), whereas epithelial markers showed the reduced expression between REV-ERBα KO and WT group. Our results, in part, highlight the important role of REV-ERBα in fibroblasts, these cells having a crucial role in exacerbating fibrotic responses in the lungs (17). The robust circadian gene oscillations in fibroblasts present the importance of circadian rhythmicity in fibroblasts and also has been shown to regulate the wound healing response in vitro and in vivo (32, 33). In addition to the importance of fibroblast in stress responses, diminished REV-ERBα levels in lung epithelium could also enhance the inflammation induced by LPS (21). We have also observed that vimentin snail-slug, and collagen accumulation were increased by CS exposure in the alveolar and bronchial epithelium, and REV-ERBα KO mice showed further augmentation of mesenchymal markers. Our results emphasize the importance of REV-ERBα regulating EMT markers in lung epithelium during CS-induced lung inflammation. We also measured the protein abundance and gene levels of MMPs (MMP2, 9, and 12) after acute or sub-chronic CS exposure, however, we observed little or no changes in MMPs associated with REV-ERBα. Currently, there is no report on the dysregulated EMT associated with REV-ERBα specifically in lung epithelium. Our data demonstrate the accumulation of mesenchymal markers in lung epithelium after sub-chronic CS exposure. Understanding the role of the specific circadian clock targets using genetic (knockout and overexpression) and pharmacological (selective agonist and antagonist) models
associated with altered EMT will shed light into the cellular processes that play crucial role at early stage of chronic lung disease.

Previously, we have shown that sub-chronic CS exposure induced lung inflammation in a REV-ERBα-dependent manner (14), which is in agreement with our data presented in another study (13). REV-ERBα KO mice exposed to sub-chronic CS (30 days) increased neutrophil percentage (13), and our data demonstrates that REV-ERBα agonist (SR9009) treatment reduced the neutrophil percentages after 10 days acute CS exposure. Previously, we showed both LPS and CS extract induced inflammatory response in human small airway epithelial cells (SAEC) and mouse lung fibroblasts (MLF) were attenuated by REV-ERBα agonist (GSK4112) (13). Here, we showed that GSK4112 treatment could ease TGFβ-induced fibroblast differentiation by inhibiting the activation of mesenchymal markers in HFL1 cells.

Interestingly, some of the EMT genes showed rhythmic oscillation during a 24h period, includes: Snai2, Col1a1, Col3a1, and Col5a2, as confirmed by CIRCA database (http://circadb.hogeneschlab.org). A recent study has explained that collagen secretion and homeostasis are regulated by the circadian clock (34). Others, such as Serpine1 (PAI-1), Tgfb1, and Tjp3, also showed rhythmic expression at the transcript level. Since PAI-1 expression peaks at 6 a.m., and it has been shown that BMAL1:CLOCK heterodimer induced activation of the Serpine1 promoter through E-box (35), this may lead to an increased risk of cardiovascular disorders during early morning hours (36). Similarly, rhythmic expression of Tgfb1 was also regulated by the BMAL1:CLOCK heterodimer through E-boxes and CLOCK deficiency attenuated the oscillation (37).
TGFβ1 is a common activator of EMT, and TGFβ pathway activation could also induce the abnormal ECM deposition, such as collagen and αSMA. Targeting the novel gene transcriptional rhythms which regulate canonical signaling pathways related to EMT may be critical to devise potential therapeutic strategies against chronic lung disease where ECM remodeling plays an important role.

In this study, we investigated the therapeutic potential of REV-ERBα agonist in abnormal EMT activated by CS exposure, as well as the anti-inflammatory effects. We and others have reported that REV-ERBα agonists were capable of attenuating inflammatory responses (13, 21, 24). Recently, REV-ERBα agonists have been shown to modulate fibrotic responses in mice and ex-vivo human lung tissue (17). Additionally, SR9009 treatment in tendon fibroblasts showed a decrease in collagen fiber formation per cell, and CRY1/2 agonist: KL001, enhance the transcript levels of genes (Sec61a2, Mia3, Pde4d and Vps33b) that regulate collagen trafficking and fiber assembly, and the number of collagen fiber formation per cell (34). Furthermore, SR9009 exhibited anti-tumor effects on small-cell lung cancer through REV-ERBα-mediated autophagy (38). Based on our data, the dysregulation in EMT and TGFβ-induced fibroblast differentiation was modulated by REV-ERBα agonists, SR9009 and GSK4112.

Apart from SR9009 and GSK4112, there are other REV-ERBα ligands such as SR9011, GSK2945, SR12418, GSK0999, GSK5072, and GSK2667 that are available and currently being tested (39). SR9011 and SR9009 are designed based on the chemical structure of GSK4112 for in vivo evaluation since GSK4112 has poor solubility in PBS.
as well as poor pharmaceutical properties (39, 40). We have proved that SR9009 is
able to modulate CS-induced inflammation and EMT. Similarly, GSK2945 was
designed based on the similarities observed in the structure of GSK4112, while
GSK2945 showed both REV-ERBα activation and/or inhibition that varies due to the
cell-type/organ specificity (39, 41, 42). Another agonist SR12418, which is a modified
based on the chemical structure of SR9009 that showed higher efficiency in boosting
the level of REV-ERBα and inhibiting the expression of IL-17A and NLRP3 in THP-1
cells (43). Besides, some other REV-ERBα agonists, such as GSK0999, GSK5072, and
GSK2667, were synthesized with GSK2945 in the same study based on the chemical
structure of GSK4112 and SR9009, all of them showed inhibition of IL-6 upregulation
induced by LPS on THP-1 cells (41). There are multiple REV-ERBα agonists
synthesized currently, and most of them showed anti-inflammation effects which makes
them as a promising anti-inflammatory candidate (39). However, applications of REV-
ERBα agonists into other diseases are lacking and need verification of their efficacy.
Our study showed that REV-ERBα agonist is capable of attenuating CS-induced lung
inflammation and abnormal EMT.

Although several new REV-ERBα agonists have been currently synthesized and
evaluated to overcome the limitations of GSK4112 (poor solubility and short half-life),
there are still challenges that may restrict the clinical translational value of REV-ERBα
agonists. The in vivo half-life (oral gavage administration) of GSK2945 is approximately
2h, while SR9009 is 0.5h, and GSK0999, GSK5072, and GSK2667 are only 0.25h (41).
It takes total 24hrs to clear the GSK2945 in serum, while only 4hrs for SR9009,
GSK0999, GSK5062, and GSK2667 (41). Even though, there are new drugs synthesized based on molecular clock target, the half-life is still too short to satisfy the clinical needs. Frequent dosing is required because of the short half-life and fast clearance, and that is why we daily injected SR9009 intraperitoneally before CS exposure for 10 days. Additionally, SR9009 also presents with side-effects on cellular proliferation and metabolic progress (44). Administration of SR9009 showed similar dose-dependent cytotoxicity, cellular proliferation, and mitochondrial respiration between wild-type and REV-ERBα/β double KO mouse embryonic stem cells (44). Short-duration time in biological system and off-target effects diminish the translational values of REV-ERBα agonists. However, multiple studies including our previous reports have identified that REV-ERBα is essential and critical in pulmonary injury, and REV-ERBα agonists possess the potential to combat lung inflammation and injury induced by environmental toxicants.

Further, RORα inhibitor: SR1001, presented a therapeutic effect in hypoxia-induced pulmonary hypertension (45), and the expression of RORα was up-regulated after CS exposure as well as in COPD/emphysema patients compared to healthy control (46). The casein kinases (CKs) are required in the phosphorylation of PERs, which are also important in circadian rhythm regulation (47). The inhibitor of CK1δ/ε: PF670462, was found with anti-fibrotic ability either in TGFβ induced cell-differentiation, or bleomycin administered mice (48). Furthermore, CKs were identified to interact with other targets in the Wnt signaling pathway, which is one of the key pathways that regulate EMT (49). Altogether, investigation of various circadian agonists in different lung disease models is
necessary to understand the role of circadian clock targets and their therapeutic potentials for alleviating chronic inflammatory lung diseases. The combination of REV-ERBα agonist SR9009 with other steroidal vs. non-steroidal drugs could possibly be a new therapeutic strategy for chronic lung disease that needs to be tested.

In conclusion, REV-ERBα deficiency causes circadian clock dysfunction in the lungs, as well as abnormal mesenchymal transition induced by sub-chronic CS exposure. We found that REV-ERBα deletion in mice exposed to sub-chronic CS showed increased airspace enlargement and ECM remodeling compared to CS-exposed WT mice. Furthermore, inflammation and aberrant activation of EMT caused by acute CS exposure were partially abrogated by REV-ERBα agonist treatment. Taken together, our results show a promising role of circadian clock molecular target, REV-ERBα in management of chronic lung diseases associated with EMT dysregulation. The REV-ERBα agonist, SR9009 treatment showed potential effects against lung inflammation or inhibiting mesenchymal transition. Targeting multiple circadian clock molecules using selective agonists/antagonists (e.g., SR9009, and KL001) could be a novel approach for treating chronic lung disease associated with mesenchymal transition and abnormal ECM remodeling, such as COPD and lung fibrosis.
Methods

The description of experimental details, please refer to supplementary information. All cellular and molecular analysis details including, inflammatory cell and cytokine measurement, western blotting, qRT-PCR, Nanostring quantification, Hematoxylin and Eosin (H&E) staining, Gomori Trichrome (collagen) staining, and Immunohistochemistry (IHC) staining, are provided as supplementary information (see support information file).

Human lung tissues

Lung tissue specimens from 14 subjects, including 7 non-smokers and 7 smokers, were procured from National Disease Research Interchange (NDRI) and generously provided by Dr. Vuokko L. Kinnula (Table S1). Lungs were homogenized in RIPA buffer, and protein concentration was determined by the Pierce BCA Assay Kit (Cat#: 23227, ThermoFisher Scientific).

Cell culture and treatment

Human fetal lung fibroblast (HFL-1) was purchased from ATCC, and cultured in DMEM:F12K medium (Cat#:11320033, ThermoFisher Scientific) with 10%FBS (Cat#:10082147, ThermoFisher Scientific), 1% Penicillin-Streptomycin-Glutamine (Cat#: 10378016, ThermoFisher Scientific), and 1% Non-Essential Amino Acids (Cat#: 11140076, ThermoFisher Scientific). Before treatment, cells were serum-deprived for 12hrs and then treated with 5 ng/mL TGF-β with or without 10 μM GSK4112 (Cat#: 3663, TOCRIS) for 3 days; or 0.1% CSE with or without 10 μM GSK4112 or 0.25% CSE.
for 2 days. CSE stock was always prepared freshly right before the treatment as described previously (13). In brief, cigarette smoke (3R4F, University of Kentucky, KY) was bubbling into 10mL FBS-free DMEM: F12K medium (Phenol-red free) as 10% CSE stock. The 0.25% and 0.1% CSE working solutions were diluted from the stock CSE. Quality control of the CSE was based on the absorbance at 320nm (1.00 ± 0.05).

**Animals and cigarette smoke (CS) exposure**

Adult C57BL/6 (WT) and *REV-ERBα* global KO (*REV-ERBα* KO) mice (male and female mice, 4-5-month-old) were housed in a 12:12 light/dark cycle in the inhalation core facility at the University of Rochester. *REV-ERBα* KO mice were generously provided by Ronald Evans, PhD, from Salk Institute (La Jolla, CA) (19). WT mice were injected intra-peritoneally (i.p. veh/SR9009, 100 mg/kg body weight) with SR9009 dissolved in 15% cremophor (vehicle) 1h before CS exposure, SR9009 or vehicle were injected at ZT5 (11 am), every day for 10 days. Mice were exposed to CS for 10 days or 30 days; 2 h/day with ~300 total particular matter (TPM, mg/m³), and exposed to CS for 4 months (chronic exposure) with ~300 TPM 2hr everyday using the Baumgartner Jaeger mainstream smoking machine. Air group mice were i.p. injected with SR9009 or vehicle at the same time for 10 days without CS exposure. Bodyweight was recorded daily during the SR9009 treatment period. Mice were sacrificed 24 hrs after the last exposure; lungs were either snap-frozen for gene/protein analysis, or fixed (10% formalin) for histological observation; bronchoalveolar lavage fluid (BALF) was collected for inflammatory measurement. Lung mechanical properties were analyzed 24h after
the last exposure via Flexivent FX1 system (Scireq) as per the manufacturer’s instructions. Each measurement was performed three times per animal.

**Inflammatory cells and cytokine analysis**

Mice were euthanized with ketamine/xylazine, and lungs were lavaged with total 1.8 mL saline (0.6 mL/each time, for a total of 3 times). The BAL fluids were spun down to collect inflammatory cells, and supernatants were transferred for cytokine analysis. The cells were suspended and labeled with specific antibodies to identify the differentiated inflammatory cells. Specific cells were identified by Guava® easyCyte™ flow cytometer (Millipore Sigma) and analyzed by Guava® InCyte (50). Inflammatory cytokines were measured by Bio-plex Pro mouse cytokine 23-plex immunoassay kit (Cat#: M60009RD, Bio-Rad), and plates were read through the Luminex Flexmap 3D (Luminex Corp).

**Protein isolation and Western blot**

Proteins were isolated from the snap-frozen lungs (~30 mg) in RIPA buffer with protease inhibitor (Cat#: 78440, ThermoFisher Scientific) with mechanical homogenization. An equal amount of protein (20 µg/lane) was used and then separated through 10% SDS-polyacrylamide electrophoresis (SDS-PAGE). The gels with protein samples were transferred onto a nitrocellulose membrane (Cat# 1620112, Bio-Rad). The membranes were blocked with 5% non-fat milk at room temperature for 1 h, followed by primary antibodies incubation (overnight at 4°C). After primary antibody probing, membranes were washed with TBS-T, 10 min each for 4 times, and probed
with Goat-anti-Rabbit Secondary antibody (1:5000, #1706515, BioRad) for 1 h at room temperature. Membranes were developed with Pierce™ ECL Western Blotting Substrate (Cat#: 32106, Thermo Scientific™) and imaged by Bio-Rad ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The normalization was done based on β-actin (1:2500, ab20272, Abcam) or GAPDH (1:1000, ab9482, Abcam), and fold-change was normalized to the WT air group, or air group treated with vehicle. Full unedited blots/images are provided as additional supporting file (see attached file for unedited full blot images).

**RNA isolation**

First, Snap frozen lung lobes were homogenized in QIAzol reagent (Cat#:79306, Qiagen) mechanically. Lung homogenates were mixed with chloroform and vortexed for 10s, followed by centrifugation at 12,000 RPM, for 15 min in 4°C. After centrifugation, aqueous phase was transferred to new RNase-free tubes and then mixed with isopropanol. The mixtures were kept at -20°C for 2 h and then spun down at 15,000 RPM, for 15 min in 4°C, and discarded the supernatant. The RNA pellets were washed with 75% EtOH, and spun down again at 15,000 RPM, for 15 min in 4°C. Discarded the EtOH, and re-suspended RNA with RNase-free water. All the RNA samples were cleaned up by RNeasy Plus Mini Kit (Cat#: 74136, Qiagen) based on the manufacture's protocol. RNA samples were stored at -80°C until analysis.

**Nanostring quantification**
RNA samples isolated from lung lobes were quantified through Nano-drop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA), and 100 ng RNA samples were prepared for nanostring analysis. The customized panels from nanostring, including circadian genes and epithelial-mesenchymal-transition (EMT) genes, were used in this study. All the gene expressions were presented as normalized counts and measured by nCounter SPRINT Profiler (NanoString Technologies, Inc.), and analyzed by nSolver 4.0 software.

**Quantitative-RT-PCR**

RNA samples isolated from lung lobes were quantified by Nano-drop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription was done by RT² First Strand Kit (Cat# 330401, Qiagen), and qRT-PCR was based on SYBR green expression master-mix (Cat# 330509, Qiagen). Quantitative PCR was done by the Bio-Rad CFX96 qPCR instrument, and the expression level of mRNA was determined by 2^{ΔΔCt} methods with normalization using GAPDH as housekeeping control.

**Hematoxylin and Eosin (H&E) staining**

The staining protocol has been described in our previous study (51). Briefly, lung sections (5 µm) were deparaffinized, rehydrated with xylene, and gradient percentage of ethanol (100%, 95%, and 70%). Then, sections were soaked in hematoxylin for 1 min and 7% ammonia-water for 10s. The sections were stained with eosin for 1 min and
rinsed in 95% ethanol for 1 min. After staining, slides were dehydrated in 95% and 100% ethanol and xylene. All slides were mounted for further analysis.

**Gomori Trichrome (collagen) staining**

The staining protocol has been described in our previous study (51). Lung sections were stained using the Gomori's Trichrome staining kit that was commercially available (Cat#: 87020, Thermo Fisher Scientific) based on the manufacturer's protocol. The slides were dehydrated and mounted for observation and Ashcroft scoring (52). The Ashcroft scoring was done based on the previous study in a blind manner (5, 51).

**Immunohistochemistry (IHC) staining**

The staining protocol has been described in our previous study (51). Lung sections (5 µm) were deparaffinized and rehydrated, and then unmasked the antigens with antigen retrieval solution (10x) (Cat#: S1699, Dako, Denmark). Sections were blocked with 10% normal goat serum and then incubated with primary antibodies at 4°C overnight. Then, slides were soaked in 0.3% hydrogen peroxide for 15 min, then washed with TBS. Secondary antibody (1:1000, ab7090, Abcam) was applied to the section at room-temperature for 1h. Slides were developed with DAB Quanto Chromogen and Substrate (Cat#: TA-125-QHDX, Thermo fisher scientific) and then counter stained with hematoxylin. Slides then dehydrated and mounted for further analysis.

**Lung morphometry**
The mean linear intercept (Lm) of airspace was measured through H&E stained lung section (20x) by MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Pictures were taken randomly from 2-4 sections per slide in a blinded manner, >8 pictures were used for Lm calculation, and all the pictures were used for analysis followed manual threshold as described previously (53).

**Statistical analysis**

The statistical differences among samples were analyzed through either one-way ANOVA or student's t-test in GraphPad Prism software (Version 8.0, La Jolla, CA). Results were presented as the mean ± SEM, and p < 0.05 was considered as a significant difference.

**Study approval**

This study was performed according to the standards from the United States Animal Welfare Act, National Institutes of Health (NIH). All the animal experiments followed the protocol approved by The Animal Research Committee of the University of Rochester (UCAR).

**Declarations**

**Human participants, human data and human tissue:** The lung tissues were obtained from NDRI and/or NIH- Lung Tissue Research Consortium (LTRC), and/or provide by Dr. Vuokko L. Kinnula
Human ethical approval: Not applicable

Consent for publication: Not applicable.

Competing interests: The authors have declared that no competing interest.

Availability of data and material: All data and materials are described in the manuscript.

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Competing interests: None

Author Contributions:

QW, IKS, and IR conceived and designed the experiments. QW, JHL, TM, and IKS conducted the experiments. QW and TM analyzed the data. QW, JHL, TM, IKS and IR wrote and revised the manuscript.

References


Figure legends

Figure 1. **Mesenchymal markers increased in smokers compared to non-smokers**

Lungs from smokers and non-smokers were homogenized and probed with EMT markers, and protein abundance of different markers were analyzed by Western blotting. Representative blot images of target proteins (Vimentin, COL1A1, TGFβ, and PAI-1) were shown in (A). Densitometry is used for fold change of specific protein targets (B), β-actin were used as endogenous controls for normalization. All the bands of different targets were probed on the same membrane. Vimentin and PAI-1 were probed in the same membrane, and COL1A1 and TGF-β were probed in the same membrane. Data are shown as mean ± SEM (n=7, * P < 0.05, paired Student’s t-test).
Figure 2. **Sub-chronic CS exposure affects circadian focused mRNA expression analyzed by NanoString**

Total RNA was isolated from the lungs of mice exposed to air and CS for 30 days. Our customized NanoString panel (circadian gene focused) was used to screen the potential targets via nCounter® SPRINT Profiler. Normalization of absolute RNA count and data analysis were done by nSolver software. The overview of all the dysregulated targets is shown as a heatmap (A), and selected gene transcription changes are shown separately (B). Data are shown as mean ± SEM. (n=6. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between groups; *# $P < 0.05$, ## $P < 0.01$ compared with CS exposed WT group; & & $P < 0.01$ compared with WT air control, One-way ANOVA with Šidák correction).
Figure 3. **Sub-chronic CS exposure affects EMT focused mRNA expression analyzed by NanoString**

Total RNA was isolated from the lungs of mice exposed to air and CS for 30 days. Our customized NanoString panel (EMT gene focused) was used to screen the potential targets via nCounter® SPRINT Profiler. Normalization of absolute RNA count and data analysis were performed by nSolver software. The overview of all the dysregulated targets was shown as a heatmap (A), and selected gene transcription changes were shown separately (B). Data are shown as mean ± SEM. (n=6. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between groups; # $P < 0.05$ compared with CS exposed WT group; One-way ANOVA with Šidák correction).
Figure 4. Sub-chronic CS exposure induced mesenchymal transition in REV-ERBα-dependent manner

Protein abundance of mesenchymal markers in mouse lung affected by sub-chronic CS exposure (1 month/30 days) were analyzed by Western blotting. (A). Representative blot images (TGF-β, Vimentin, COL1A1, and Snail-Slug) are shown, and densitometry analyses are done individually. Different groups were run on the same membrane, but were noncontiguous. COL1A1 and Vimentin were probed in the same membrane, and β-actin was used as an endogenous control (n=5/group); (B). The localizations of dysregulated Vimentin, α-smooth
muscle actin (α-SMA), and Snail-Slug were observed via immuno-histochemical staining. Regions of interest were pointed by red-arrow. Relative IHC score based on positive staining intensity was performed in a blind-manner (n=3-4/group); specific protein accumulation in alveoli was denoted by red arrows (40x, scale bar = 50 μm) (C). RNA isolated from lung tissues were used to determine the gene expression (Serpine1, Vim, Cdh1, and Fn1) by qRT-PCR. GAPDH was used as an endogenous control, and gene fold change was calculated by 2^{-ΔΔCt} method (n=4-5/group). Data are shown as mean ± SEM. (* P < 0.05, ** P < 0.01, *** P < 0.001 between groups; # P < 0.05 compared with CS exposed WT group; & P < 0.05 compared with WT air control; One-way ANOVA with Šidák correction).
Figure 5. **Protein abundance of EMT markers were affected by sub-chronic CS exposure**

Lungs from mice exposed to CS for 30 days were homogenized and protein abundances were measured by Western blotting. Representative blot images are shown and relative protein fold change of E-cadherin, ZO-1, p53, and PAI-1 are analyzed based on densitometry with β-actin as the endogenous control. Different groups were run on the same membrane, but were noncontiguous. Data are shown as mean ± SEM. (n=5, * P < 0.05 between groups; † P < 0.05 compared with WT air control; One-way ANOVA with Šidák correction).
Figure 6. **Airspace enlargement and abnormal ECM deposition were induced by sub-chronic CS exposure in REV-ERBα KO mice**

Sub-chronic 30 days cigarette smoke exposure induced airspace enlargement observed by (A). H&E (40x) (green arrows indicated airspace enlargement and red arrows indicated inflammatory responses, scale bar = 50 µm), and abnormal ECM deposition observed via (B). Gomoris’ Trichrome (20x) staining (scale bar = 100 µm), (C). Immuno-histochemical staining COL1A1 (20x, scale bar = 100 µm) and (D). Fibronectin (20x, scale bar = 100 µm). Relative IHC score based on positive staining intensity was performed in a blind-manner and denoted by red arrows. Data are shown as mean ± SEM. (n=3-4/group, * P < 0.05; One-way ANOVA with Šidák correction).
Figure 7  **Altered airspace enlargement, lung mechanics and inflammation were induced by chronic CS exposure in REV-ERBα Het mice**

Lung mechanics, inflammatory cell influx in BALF and airspace enlargement induced by chronic CS exposure (4 months) were determined by Flexivent, flow-cytometry, and lung morphometry. 
(A) Lung mechanics (Lung compliance resistance, and elastance) were measured after chronic CS exposure. (B). Lung histological analysis were conducted using H&E stained sections, and mean linear intercept (Lm) analysis was performed using Metamorph software (20x, scale bar = 100 μm) from H&E stained images (green arrows indicated airspace enlargement and red arrows indicated inflammatory responses). (C). Total cell was counted by Bio-Rad cell counter using Trypan blue staining. Differential inflammatory cell counts (Macrophage, Neutrophil, T-lymphocytes) were determined (cells/mL) by flow cytometry. Data are shown as mean ± SEM. (n=5-10.  * P < 0.05, ** P < 0.01, between groups; # P < 0.05  ## P < 0.01 compared with CS exposed WT group; One-way ANOVA with Šidák correction).
Figure 8. **Acute CS exposure-induced inflammatory response was reduced by REV-ERBα agonist**

Pro-inflammatory cell influx and cytokines in BALF induced by acute CS exposure (10 days) were determined by flow-cytometry and Luminex. (A) Total number of inflammatory cell was counted by Bio-Rad cell counter using Trypan blue staining. Differential inflammatory cell counts (Macrophage, Neutrophil, CD4/CD8 T-lymphocytes) were determined (cells/mL) by flow cytometry. (B) Relative cytokines were determined by Luminex, total 2 technical repeats were done to calculate the final concentration. Data are shown as mean ± SEM. (n=10. * P < 0.05, ** P < 0.01, *** P <0.001, between groups; ## P < 0.01 compared with CS exposed WT group; One-way ANOVA with Šidák correction).
Figure 9. **Acute CS exposure-induced mesenchymal transitions were inhibited by REV-ERBα agonist**

Lungs from mice exposed to acute CS exposure with or without REV-ERBα agonist (SR9009) administration were homogenized and protein abundance were analyzed by Western blotting. (A). Representative blot (TGF-β, Vimentin, COL1A1, and Snail-Slug) images are shown, and densitometry analyses are done individually. Different groups were run on the same membrane, but were noncontiguous, and β-actin was used as an endogenous control. (B). RNA isolated from lung tissues were used to determine the gene expression (Tgfb1, Col1a1, Vim, Snai1 and Snai2) by qRT-PCR. GAPDH was used as an endogenous control, and gene fold change was calculated by $2^{-\Delta\Delta Ct}$ method (n=11-12/group). Data are shown as mean ± SEM. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, between groups; ## $P < 0.01$, ### $P < 0.001$ compared with CS exposed group; One-way ANOVA with Šidák correction).
Figure 10. **Protein abundance of EMT markers were affected by acute CS exposure**

Lungs from mice exposed to CS for 10 days with SR9009 administration were homogenized and protein abundance were measured by Western blotting. Representative blot images are shown and relative protein fold changes of E-cadherin, ZO-1, p53, and PAI-1 are analyzed based on densitometry with β-actin as the endogenous control. E-cadherin and ZO-1 were probed in the same membrane. Different groups were run on the same membrane, but were noncontiguous. Data are shown as mean ± SEM. (n=11-12. * P < 0.05, *** P < 0.001 between groups; &&& P < 0.001 compared with WT air control; One-way ANOVA with Šidák correction).
Figure 11. TGFβ/CS-activated fibroblast differentiation were inhibited by REV-ERBα agonist

Human lung fibroblast (HFL-1) was treated with (A) TGFβ with or without REV-ERBα agonist (GSK4112) for 3 days or (B). 0.1% CSE with or without GSK4112 for 2 days. RNA isolated from cells were used to determine the gene expressions (ACTA2, COL1A1, and FN1) by qRT-PCR. GAPDH was used as an endogenous control for normalization. Data are shown as mean ± SEM. (n=3 for A, and n=4-9 for B; * P < 0.05, ** P < 0.01, *** P < 0.001, between groups; One-way ANOVA with Šidák correction for A, and unpaired student’s t-test for B).
Table. 1. Acute CS exposure increased pro-inflammatory cytokines in BALF

<table>
<thead>
<tr>
<th>Analytes (pg/mL)</th>
<th>Air</th>
<th>CS</th>
<th>Air+SR9009</th>
<th>CS+SR9009</th>
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<tr>
<td>IL-1α</td>
<td>8 ± 0.3</td>
<td>13 ± 1.3</td>
<td>12 ± 0.65</td>
<td>12 ± 1.5</td>
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<tr>
<td>IL-2</td>
<td>22 ± 0.67</td>
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<tr>
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<td>0.9 ± 0.11</td>
<td>40 ± 10 ^</td>
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<tr>
<td>IL-10</td>
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<tr>
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<td>IL-12p70</td>
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<td>IFN-γ</td>
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<td>13 ± 1.7 *</td>
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<td>MCP-1</td>
<td>49 ± 8.6</td>
<td>2318 ± 614 *</td>
<td>41 ± 2.1</td>
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<tr>
<td>RANTES</td>
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<td>KC</td>
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<tr>
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<tr>
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<td>23 ± 1.7 ***</td>
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<td>45 ± 12 **</td>
<td>11 ± 1.3</td>
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</table>

Data are shown as mean ± SEM (n=2-10/group), total 2 technical repeats were done to calculate the final concentration. (* P < 0.05, ** P < 0.01, *** P < 0.001 compared with Air group; ### P < 0.001 compared with CS group; && & P < 0.001 with Air +SR9009 group; One-way ANOVA with Dunn–Šidák correction).