Epigenetic silencing of IRF1 dysregulates type III interferon responses to respiratory virus infection in epithelial to mesenchymal transition

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Chronic oxidative injury produced by airway disease triggers a transforming growth factor-β (TGF-β)-mediated epigenetic reprogramming known as the epithelial–mesenchymal transition (EMT). We observe that EMT silences protective mucosal interferon (IFN)-I and III production associated with enhanced rhinovirus (RV) and respiratory syncytial virus (RSV) replication. Mesenchymal transitioned cells are defective in inducible interferon regulatory factor 1 (IRF1) expression by occluding RelA and IRF3 access to the promoter. IRF1 is necessary for the expression of type III IFNs (IFNLs 1 and 2/3). Induced by the EMT, zinc finger E-box binding homeobox 1 (ZEB1) binds and silences IRF1. Ectopic ZEB1 is sufficient for IRF1 silencing, whereas ZEB1 knockdown partially restores IRF1-IFNL upregulation. ZEB1 silences IRF1 through the catalytic activity of the enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), forming repressive H3K27me3 marks. We observe that IRF1 expression is mediated by ZEB1 de-repression, and our study demonstrates how airway remodelling/fibrosis is associated with a defective mucosal antiviral response through ZEB1-initiated epigenetic silencing.

The epithelial barrier also plays a major role in the episodic decompensations commonly triggered by viral respiratory tract infections, particularly respiratory syncytial virus (RSV) in infants and rhinovirus (RV) in children and adults. RSV and RV replicate in the airway epithelium, triggering an innate inflammatory response. Pathogen-associated molecular patterns (PAMPs) produced by RV and RSV replication are recognized by the membrane-anchored pattern recognition receptor (PRR) TLR3 and the cytoplasmic PRRs, RIG-I and MDA5 (refs 8, 9), to trigger cascades of interferon regulatory factor (IRF) responses. The IRF cascade is initially triggered by constitutively expressed IRF3, the activation of which induces expression of IRF1 and -7 in epithelial cells. IRF1/7 amplification expression of the RIG-I pattern recognition receptor and of protective type I IFNs (IFN-α/β)11. Type I IFNs trigger the intracellular Jak-STAT pathway controlling ~300 IFN-stimulated genes (ISGs) that inhibit viral replication, activate innate lymphocytes and stimulate leukocyte recruitment. Viral PAMPs also activate epithelial IFN-III (IFNLs) expression by mechanisms that are as yet incompletely understood9,15. In contrast to IFN-I, IFN-III binds to an epithelial-restricted class II cytokine receptor converging on Jak-STAT activation and ISG expression.

Consequently, IFN-III plays a role in innate immunity to mucosal pathogens, including RV (refs 14, 15).

Severe asthmatics are susceptible to sino-pulmonary infections16. Undoubtedly, the aetiology for this impaired mucosal innate immunity is multifactorial; reduced production of IFN-III (ref. 17) has been related to exacerbation severity18. One study reported that RV replicates more efficiently in asthmatic epithelium associated with defective IFN-III production19. The relationship between the airway remodelling and enhanced viral replication in epithelial cells subjected to mesenchymal reprogramming and implicates a role for epigenetic silencing of the IRF1 pathway via the EZH2 histone methyltransferase activity.

Results

Mesenchymal transition silences the epithelial IFN-III response. We examined TLR3 and RIG-I antiviral signalling in a standardized model of mesenchymal reprogramming using primary human small airway epithelial cells (hSAECs)20. TGF-β treatment of hSAECs triggers the characteristic genomic21,22 and proteomic23 signatures of the mesenchymal state. The epithelial–mesenchymal transition (EMT) is accompanied by coordinate repositioning of activating and repressing histone epigenetic marks—H3K4Ac and H3K27me3, respectively—on the enhancers of ~3,000 genes in the TGF-β network. To illustrate, control and TGF-β-treated hSAECs were analysed by confocal microscopy. Untreated controls showed peripheral cytoplasmic distribution of polymeric actin, while TGF-β-treated hSAECs (EMT-hSAECs) showed a marked enhancement of organized mesenchymal stress fibres throughout the cytoplasm. Loss of the epithelial marker E-cadherin (CDH1) and acquisition of mesenchymal marker vimentin (VIM) further illustrates that the TGF-β-treated hSAECs have undergone EMT (Fig. 1a).

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**Figure 1 | Deficient type I/III IFN responses in TGF-β-induced EMT.**

a. Confocal immunofluorescence imaging of hSAECs (Con) and EMT-hSAECs (EMT, induced by 10 ng ml⁻¹ TGF-β treatment for 15 days). The distribution of F-actin, E-cadherin (E-Cad) and vimentin (VIM) was shown by staining with Alexa 568-conjugated phalloidin or secondary antibody (red); nuclei were counterstained with DAPI (blue). Shown is representative staining from five images.

b–g. qRT-PCR analysis of type I interferons IFNA1 (b) and IFNB1 (c), type III interferons IFNL1 (d) and IFNL2/3 (e) and interferon-stimulated genes (ISGs) MX1 (f) and RIG-I (g) in hSAECs (Con) and EMT-hSAECs (EMT), stimulated by poly(I:C) (50 µg ml⁻¹) for 0, 4 and 6 h. The results were quantified relative to the signal of DNA polymerase beta (POLB) and shown as the fold change of mRNA abundance normalized to unstimulated samples (Con).

h–i. qRT-PCR analysis of the ISGs MX1 (h) and RIG-I (i) in hSAECs (Con) and EMT-hSAECs (EMT) infected by RV16 for 0, 4, 6 and 24 h.

j–k. qRT-PCR analysis of relative virus load in hSAECs (Con) and EMT-hSAECs (EMT), infected by RSV (j) or RV16 (k) for 0, 12 and 24 h. Results are shown as fold changes of RV16 viral RNA 5′ untranslated region (UTR) and RSV N viral RNA, respectively. Data are shown as means ± s.d. from n = 3 biological replicates.

l. Virus plaque assays of hSAECs (Con) and EMT-hSAECs (EMT) infected by RSV or RV16 for 24 h (multiplicity of infection (MOI) = 1). Virus plaques were detected in triplicate in HEp-2 (RSV) or HIHeLa cells (RV16), using serial twofold dilutions of cell culture supernatants or freeze-thawed cellular lysates, respectively. Plaques were stained at 5 days (RSV) or 9 days (RV16) p.i. Shown are representative triplicates at the same dilution. Complete views of the virus plaque data are presented in Supplementary Fig. 1.
Experiments comparing IFN secretion patterns in the epithelial versus mesenchymal state were first conducted after activating Toll-like receptor (TLR3) signalling using extracellular poly(I:C). IFNA1 mRNA expression showed a fourfold induction, peaking after 6 h in control hSAECs. EMT-hSAECs had a slightly more rapid response, peaking after 4 h of stimulation (Fig. 1b). By contrast, expression of IFNB1 was dramatically affected. In the control cells we observed an 11-fold induction of IFNB1 mRNA by 4 h of poly(I:C) stimulation, whereas less than threefold induction was produced in the EMT-hSAECs (P < 0.05, Fig. 1c). An even more marked inhibition of IFN-III production was observed in EMT-hSAECs. The 2,100-fold increased expression of IFNL1 (IL-29) in control hSAECs was less than 40-fold in EMT-hSAECs, and the 450-fold expression of IFNL2/3 (IL-28A/B) was similarly attenuated (Fig. 1d,e).

To determine whether these changes functionally reduced downstream ISG expression, we measured the expression of MX dynamin-like GTPase 1 (MX1) and retinoic acid inducible gene-1 (RIG-I). MX1 was highly induced, by 79-fold in hSAECs but by less than twofold in EMT-hSAECs after 4 h of poly(I:C) stimulation (Fig. 1f). Similar results were observed for the 400-fold induction of RIG-I in hSAECs versus <10-fold in EMT-hSAECs (Fig. 1g). These data indicate that IFN-I and III expression and the downstream ISG response were attenuated by the mesenchymal transition.

We next explored the differential responses to RNA viruses associated with exacerbations of reactive airway disease, including RSV and RV. Both viruses trigger innate inflammation via RIG-I (refs 8, 9). Similar attenuation of MX1 (Fig. 1h) and RIG-I (Fig. 1i) expression were observed after RV infection in EMT-hSAECs. We noted enhanced release of RV virions at 12 and 24 h post infection (p.i.) in EMT-hSAECs (Fig. 1j). A more striking enhancement of RV replication was observed in EMT-hSAECs, with a greater than 3-log increase in plaque-forming units (p.f.u.) in RV virions at 12 and 24 h (Fig. 1k). Images of the plaque assays are shown in Fig. 1l and Supplementary Fig. 1. These data demonstrate that the defects in the IFN-I/III ISG pathway in the EMT-hSAECs were functionally important.

Mesenchymal transition silences IRF1 expression by promoter occlusion. We further explored the mechanism for defective IFN-I/III production in EMT-hSAECs. We focused on the IRF1 promoter because the inducible IRF1 and -7 proteins play a major role in type I IFN expression by amplifying RIG-I expression. IRF1 mRNA was induced 13-fold after 4 h of poly(I:C) stimulation in hSAECs, but its basal and poly(I:C)-induced activities were completely blocked in EMT-hSAECs (P < 0.05, all conditions, Fig. 2a). By contrast, IRF7 expression was slightly enhanced in EMT-hSAECs (Fig. 2b), while IRF3 expression was not affected (Supplementary Fig. 2). This differential IRF1 expression was evident in western blot (Fig. 2c). To confirm defective IRF1 induction, we measured IRF1 DNA-binding activity by a quantitative microaffinity binding assay28 using a high-affinity ISG duplex from the IFNB1 promoter29. We observed strong induction of IRF1 DNA binding in hSAECs, which was significantly decreased in EMT-hSAECs (Fig. 2d). The effect of mesenchymal transition on defective IRF1 expression was analysed by immunofluorescence staining. In the absence of stimulation, no IRF1 staining was detected; however, poly(I:C) induced strong IRF1 nuclear accumulation in hSAECs (Fig. 2e, top). By contrast, IRF1 staining in EMT-hSAECs was significantly lower, irrespective of poly(I:C) stimulation (Fig. 2e, bottom; also note the dramatic mesenchymal phenotype with phallolidin staining).

In epithelial cells, IRF1 is induced through combinatorial actions of NF-κB and IRF3. To understand how the mesenchymal state interferes with IRF1 expression, we performed a two-step chromatin–immunoprecipitation (XChIP) assay30. Using XChIP, we quantitated NF-κB/RelA and IRF3 binding to the IRF1 promoter in hSAECs stimulated with poly(I:C), or infected with RSV, versus that in EMT-hSAECs. Basal binding of IRF3 and RelA was decreased in EMT-hSAECs (Fig. 2f). This difference was even more apparent in response to poly(I:C) and RSV infection, where IRF3 and RelA binding was largely induced in hSAECs, but dramatically blocked in EMT-hSAECs (Fig. 2f). Because IRF3 and RelA expression is unaffected by the EMT31, these data indicate to us that the chromatin environment of IRF1 in the mesenchymal state is not permissive for viral-inducible transcription factor binding.

We next determined whether the decrease in IRF1 expression/translocation was sufficient to reduce binding to the endogenous IFNB1 promoter, and measured IRF1 binding to IFNB1 by XChIP32. Poly(I:C) induced an 11-fold increase in IRF1 binding to IFNB1 relative to control treatment in hSAECs. This was reduced to less than fivefold in EMT-hSAECs (Fig. 2g, left panel). IRF1 mediates IFNL expression downstream of reactive oxygen species (ROS) generation by the peroxisosome32. Analysis of the IFNL1 promoter also showed a highly inducible, 38-fold increase in IRF1 binding in poly(I:C)-stimulated hSAECs that was reduced to ~13-fold in EMT-hSAECs (Fig. 2g, right panel). Binding of IRF7 to endogenous IFNB1 and IFNL1 showed a similar 2.8-fold induction in XChIP (Fig. 2h). Interestingly, despite the increase in IRF7 expression with the mesenchymal transition (Fig. 2c), IRF7 binding to the endogenous IFNB1 and IFNL1 promoters was greatly reduced in EMT-hSAECs. We excluded the unlikely possibility that enhanced IRF7 expression is somehow responsible for silencing IFNB1/IFNL1 expression by coactivator competition in trans by conducting an IRF7 knockdown experiment in EMT-hSAECs (Supplementary Fig. 3). IRF7 depletion slightly inhibited poly(I:C)-induced MX1 induction (Fig. 2i) and had no effect on RIG-I induction (Fig. 2j). Collectively, these data indicate that the mesenchymal transition is associated with decreased IRF1 expression due to promoter inaccessibility.

Modulation of IRF1 expression reverses type I/III IFN expression and restores antiviral immunity. Viral pattern-inducible IFN expression is controlled by transcription factor complexes of the NF-κB, IRF and AP-1 families33. To determine whether IRF1 is necessary for IFN-I/III expression in the mesenchymal background, we performed a time course of poly(I:C) stimulation of EMT-hSAECs complemented with ectopic IRF1 (Supplementary Fig. 4). IRF1 transfectants induced significantly more IFNB1 and IFNL1 mRNAs than in empty vector controls (Figs. 2k,l). In the same transfectants infected with RV, IFNB1 and IFNL1 mRNAs were significantly induced (Supplementary Fig. 5) and RV titres were significantly reduced at 8 and 24 h.p.i. in the IRF1-transfectants (Fig. 2m). These data indicate that IRF1 expression is limiting for maximal IFN-I and III responses and the restriction of viral replication.

To determine whether IRF1 expression is required for IFN-I/III production in normal hSAECs (non-EMT background), we silenced IRF1 expression using genome editing. Stable transfectants were isolated and effects on poly(I:C)-inducible IRF1 expression determined by western blot. Poly(I:C) induced high IRF1 expression in control transfectants, but IRF1 expression was completely abolished in those subjected to CRISPR/Cas 9 genome editing (Fig. 3a). We noted that the rapid induction of IFNB1 2 h after poly(I:C) activation was completely lost in the IRF1-deficient hSAECs, although 4 h later IFNB1 expression was induced via a compensatory mechanism (Fig. 3b). The induction of IFNL1 was more dramatically affected in the IRF1-deficient hSAECs at all time points (Fig. 3c). RV infection in IRF1-deficient hSAECs showed that CRISPR/Cas 9-mediated IRF1 knockout decreased the induction of IFNB1 (Fig. 3d) and IFNL1 (Fig. 3e) and increased RV virus replication (Fig. 3f). Collectively, these studies indicate that...
Figure 2 | IRF1 silencing dysregulates the IFN response in TGF-β-induced EMT. a, qRT-PCR analysis of IRF1 (a) and IRF7 (b) in hSAECs (Con) and EMT-hSAECs (EMT), stimulated with 50 µg ml⁻¹ poly(I:C) for 0, 4 and 6 h. c, Western blot analysis of IRF1 and IRF7 in cellular nuclear fractions of hSAECs (Con) and EMT-hSAECs (EMT) stimulated with 50 µg ml⁻¹ poly(I:C) for 0, 1 and 3 h. LaminB2 was used as a loading control. Shown are representative blots from three experiments. d, Microaffinity capture of poly(I:C)-inducible ISG/DNA-binding proteins. Nuclear extracts from hSAECs (Con) and EMT-hSAECs (EMT) in the absence or presence of poly(I:C) stimulation (50 µg ml⁻¹ for 3 h) were affinity-purified by biotinylated ISG duplex DNA or non-biotinylated competitor, captured by streptavidin beads and probed with the indicated antibodies (Abs) on western blots. Shown are representative blots from two experiments. e, Confocal immunofluorescence imaging for IRF1 in hSAECs (Con) or EMT-hSAECs (EMT) stimulated with 50 µg ml⁻¹ poly(I:C) for 3 h. The secondary Ab was Alexa Fluor 488 (green). Shown is representative staining from five images. f, XChIP assay for IRF3 and RelA binding to the IFN1 promoter in hSAECs (Con) or EMT-hSAECs (EMT) stimulated with 50 µg ml⁻¹ poly(I:C) for 3 h or infected with RSV at a MOI of 0.5 for 15 h. Data were quantified relative to the input signal and shown as fold change normalized to unstimulated samples (Con). g, XChIP assay of IRF1 binding to the IFNL1 promoter in hSAECs (Con) or EMT-hSAECs (EMT) stimulated with 50 µg ml⁻¹ poly(I:C) for 3 h. h, XChIP assay of IRF7 binding to IFNL1 promoters in hSAECs (Con) or EMT-hSAECs (EMT) stimulated with 50 µg ml⁻¹ poly(I:C) for 3 h. i, qRT-PCR analysis of MX1 (i) and RIG-I (j) in control small interfering RNA (siRNA) (EMT-siCon)- or IRF7 siRNA (EMT-siIRF7)-transfected EMT-hSAECs, stimulated with 50 µg ml⁻¹ poly(I:C) for 0, 2, 4 and 6 h. k, qRT-PCR analysis of IFNB1 (k) and IFNL1 (l) in lentiviral IRF1 stably transduced EMT-hSAECs (EMT-IRF1) stimulated with 50 µg ml⁻¹ poly(I:C) for 0, 2, 4, 6 h. The empty lentiviral-transduced EMT-hSAECs were used as controls (EMT-Con). m, qRT-PCR analysis of RV16 viral RNA 5’ UTR in EMT-Con and EMT-IRF1 cells infected with RV16 (MOI = 1) for 0, 8 and 24 h. Data are presented as mean ± s.d. from n = 3 biological replicates.
IRF1 is rate-limiting for maximal IFN-I and III expression and the restriction of RV infection.

The core EMT regulator zinc finger E-box binding homeobox 1 (ZEB1) silences IFN-I and III expression. The mesenchymal transition is a multistep cell state change driven by a double-negative ZEB1-SNAI feedback loop with miRNAs. In hSAECs, the TGF-β-induced mesenchymal transition induces robust activation of SNAI1, ZEB1/2 and, to a lesser extent, Twist1 at the mRNA levels. Because ZEB1 has been shown to regulate IFN-III gene expression and is rate-limiting for mucosal IFN

IFNL-1 and 2/3 mRNAs were also significantly decreased in ZEB1-expressing transfectants (Fig. 4h,i). Consistently, RV16 also replicated more efficiently in the ZEB1 transfectants, releasing a 2.5-fold higher titre of infectious virions 24 h p.i. (Fig. 4j). These data indicate that ZEB1 is sufficient for silencing IFN-I and III expression in response to viral PAMPs.

ZEB1 silencing restores mucosal IFN production in EMT-hSAECs. Although ZEB1 expression in the differentiated hSAECs suppressed mucosal IFN production, we next asked whether ZEB1 expression is required in the mesenchymal background. For this purpose, we evaluated the silencing effect of three doxycycline (Dox)-inducible lentiviral short hairpin RNAs (shRNAs) specific for ZEB1. All three significantly reduced ZEB1 expression; shRNA1 was slightly more effective by quantitative real-time PCR (qRT-PCR) (Supplementary Fig. 10) and western immunoblot (Fig. 4k). hSAECs were transitioned into the mesenchymal state by tonic TGF-β stimulation in Dox-free medium. ZEB1 was then silenced by Dox administration prior to a time course of poly(I:C) challenge. Remarkably, the ZEB1-depleted cells showed a much stronger induction of IFNB1 (Fig. 4l) and IFNL1 (Fig. 4m) across all time points. Collectively, these data show that ZEB1 downregulates IFN-I and III expression in both normal and EMT-hSAECs.

ZEB1 inhibition of mucosal IFNs is mediated by IRF1 repression. Because we observed that IRF1 was rate-limiting for mucosal IFN production and ZEB1 has been shown to bind to the IRF1 promoter in ChIP-seq experiments (Supplementary Fig. 8), we tested whether ZEB1 represses IRF1 as its mechanism for inhibition of IFN expression. First, we asked whether inducible IRF1 expression is restored in the EMT-hSAECs after ZEB1 silencing. EMT-hSAECs expressing ZEB1-targeting shRNA1 were TGF-β-transitioned, treated ± Dox, and then stimulated with poly(I:C). Western blot (Fig. 4e), as well as spindle-shaped morphological changes, consistent with its actions on cytoskeletal remodelling (Supplementary Fig. 9). Control or ZEB1-expressing hSAECs were then stimulated with poly(I:C) and the time course of IFN-I/III expression examined. We found that both IFNB1 and IFNL1 mRNA induction were significantly decreased in ZEB1-expressing transfectants relative to controls (Fig. 4f). In response to RV16 infection,
**Figure 4 | ZEB1 downregulates the IFN response in hSAECs and TGF-β-induced EMT-hSAECs.** a, qRT-PCR analysis of EMT core transcription factors (SNAIL1, TWIST1, ZEB1 and ZEB2) in hSAECs (Con) and EMT-hSAECs (EMT). b, Western blot analysis of ZEB1 and ZEB2, using cellular nuclear extracts from hSAECs (Con) and EMT-hSAECs (EMT) stimulated with 50 µg ml\(^{-1}\) poly(I:C) for 0 and 3 h. LaminB2 was used as the loading control. s.e., short exposure; l.e., long exposure. c, Confocal immunofluorescence imaging for ZEB1 in hSAECs (Con) or EMT-hSAECs (EMT). The secondary Ab was Alexa Fluor 488 (green). d, Confocal immunofluorescence staining of ZEB1 in mouse lung from chronic TGF-β-treated mouse lung. e, Confocal immunofluorescence imaging for ZEB1 in hSAECs (Con) or EMT-hSAECs (EMT). The secondary Ab was Alexa Fluor 488 (green). e, Con, Western blot analysis of ZEB1 and α-tubulin as loading control, using total protein extracts from hSAECs stably transduced with lentiviral ZEB1. The empty lentiviral-transduced hSAECs were used as controls. Shown are representative blots from two experiments. n.s., nonspecific bands. f–g, qRT-PCR analysis of IFNB1 (f) and IFNL1 (g) in Con and ZEB1 cells used in c, stimulated with 50 µg ml\(^{-1}\) poly(I:C) for 0, 2, 4 and 6 h. h–i, qRT-PCR analysis of IFNL1 (h), IFNL2/3 (i) and RV16 viral RNA 5′ UTR (j) in Con and ZEB1 cells used in c, infected with RV16 (MOI = 1) for 24 h. Data are presented as fold change normalized to unstimulated cells (Con). k, Western blot analysis of ZEB1 and α-tubulin as loading control, using total protein extracts from hSAECs stably transduced with three inducible lentiviral ZEB1 shRNAs (shR1, shR2 and shR3). Cells were first induced to EMT by 10 ng ml\(^{-1}\) of TGF-β for 15 days and then treated with 2 µg ml\(^{-1}\) doxycycline for 72 h. Cell samples were treated and collected in biological duplicates. Non-silencing shRNA-transduced cells under the same conditions were used as controls (Con). Shown are representative blots from two experiments. l–m, qRT-PCR analysis of IFNB1 (l) and IFNL1 (m) in ZEB1 shRNA (shR1)-depleted EMT-hSAECs (same treatment as in k) stimulated with 50 µg ml\(^{-1}\) poly(I:C) for 0, 2, 4 and 6 h. Data are presented as mean ± s.d. from n = 3 biological replicates.
Figure 5 | ZEB1 downregulates the IFN response via epigenetic silencing of IRF1.

a. qRT-PCR analysis of IRF1 in ZEB1 shRNA (shR1)-depleted EMT-hSAECs stimulated with 50 μg/ml poly(I:C) for 0, 2, 4, and 6 h (under the same experimental conditions as in Fig. 4l,m). **P < 0.0001.

b. XChIP assay of IRF1 in cells used in a and stimulated with 50 μg/ml poly(I:C) for 3 h. Chromatin was crosslinked and immunoprecipitated with anti-IRF1 Ab. Shown is probe-based Q-gPCR analysis of specific promoters for IFNB1, IFNL1, and IFNL2. Data were quantified relative to the signal of the input and are shown as fold change normalized to non-silencing shRNA control (EMT-Con). **P < 0.0001.

c. XChIP assay in hSAECs (Con) or EMT-hSAECs (EMT) stimulated with 50 μg/ml poly(I:C) for 3 h. Chromatin was crosslinked and immunoprecipitated with Abs for ZEB1, CtBP, EZH2, H3K4me3 and H3K27me3, respectively. Shown is probe-based Q-gPCR analysis of the IFR1 promoter region. **P < 0.0001.

d. XChIP assay of CtBP, EZH2, H3K4me3 and H3K27me3 in cells used in a and stimulated with 50 μg/ml poly(I:C) for 3 h. Chromatin was crosslinked and immunoprecipitated with Abs for ZEB1, CtBP, EZH2, H3K4me3 and H3K27me3, respectively. Shown is probe-based Q-gPCR analysis for the IFR1 promoter. Data were quantified relative to the signal of the input and are shown as fold change normalized to shRNA control (EMT-Con). **P < 0.0001.

e. qRT-PCR analysis of IRF1 (e), IFNL1 (f), and ZEB1 (g) in EMT-hSAECs treated with the specific EZH2 methyltransferase inhibitor GSK126 for a time series of 0, 24, 48, 72, and 96 h. Data are expressed as fold change relative to solvent-treated controls (0 h). **P < 0.0001.

h–k. qRT-PCR analysis of IRF1 (h), IFNB1 (i), IFNL1 (j), RSV N viral RNA and RV16 viral RNA 5′ UTR (k) in EMT-hSAECs pretreated with the specific EZH2 methyltransferase inhibitor GSK126 for 72 h, followed by infection with RV16 or RSV (MOI = 1) for 24 h. Data are shown as fold change normalized to unstimulated cells (Con). Data are presented as means ± s.d. from n = 3 biological replicates. **P < 0.0001.

(1:C). We observed a 48-fold induction of IRF1 mRNA in the ZEB1-silenced cells versus 18-fold in controls after 4 h of poly(I:C) stimulation (Fig. 5a).

The effect of ZEB1 silencing on IRF1 binding to the endogenous IFN-I/III promoters was then assayed in ZEB1-silenced cells by XChIP®. In the EMT-hSAECs, poly(I:C) induced weak binding of
IRF1 to the IFNB1 promoter; this induction was significantly enhanced by ZEB1 silencing (Fig. 5b, left panel). Similarly, IRF1 binding to the IFNL1 promoter was dramatically enhanced, to 28-fold in the ZEB1 silenced cells versus 8-fold in the control cells (Fig. 5b, middle panel). A similar enhancement of IRF1 binding was observed for the IFNL2 promoter (Fig. 5b, right panel).

A ZEB1-C-terminal binding protein CtBP-polycomb repressive complex forms repressive H3K27(me3) marks on the IRF1 promoter. Mesenchymal transition involves the inhibition of epithelial genes by inducing the accumulation of repressive histone marks as a mechanism for epigenetic regulation27. In this regard, ZEB1 inactivates epithelial genes during the mesenchymal transition by complexing with CtBP, which functions as a molecular bridge with the polycomb repressor complex (PRC) 2, containing the EZH2 methyltransferase responsible for forming repressive histone H3 trimethylated lysine (K) 27 [H3K27(me3)] marks37,38. XChIP assays were performed in the absence or presence of poly(I:C) stimulation for ZEB1, CtBP, EZH2 and activating H3K4 (me3) and repressive H3K27(me3) marks. In hSAECs, we observed that ZEB1 was associated with the IRF1 promoter and this association was significantly decreased by poly(I:C) stimulation (Fig. 5c, left; further evidence for specificity is presented in Supplementary Fig. 11). Conversely, the levels of ZEB1 binding to the IRF1 promoter were increased in EMT-hSAECs, consistent with the induction of ZEB1 by mesenchymal transition (Fig. 4a–c) and although poly(I:C) reduced ZEB1 binding, these levels were comparable to those of (silenced) IRF1 in control hSAECs (Fig. 5c, left).

A similar pattern of CtBP and EZH2 binding to IRF1 was found in hSAECs and EMT-hSAECs as that observed for ZEB1 binding, consistent with the known ZEB1-CtBP-EZH2 protein–protein complex39,37. Analysis of the histone modifications showed that the activation-associated H3K4(me3) mark was found on the IRF1 promoter in hSAECs and increased with poly(I:C) stimulation, consistent with IRF1 mRNA expression (Fig. 5c, c.f. Fig. 2a). By contrast, H3K4(me3) abundance was significantly lower in both unstimulated and poly(I:C)-stimulated EMT-hSAECs relative to that in hSAECs (Fig. 5c). The repressive H3K27(me3) mark was increased on the IRF1 promoter in control EMT-hSAECs and although this was decreased by poly(I:C) stimulation, the levels were comparable to those on the silenced IRF1 promoter in control hSAECs (Fig. 5c).

The effect of ZEB1 silencing on CtBP and EZH2 binding and histone modifications on the IRF1 promoter was analysed in EMT-hSAECs by XChIP (Fig. 5d). In ZEB1 shRNA-depleted EMT-hSAECs, the activating H3K4(me3) was increased on the IRF1 promoter while the repressive H3K27(me3) was decreased in association with CtBP and EZH2 binding (Fig. 5d). We interpret these findings to suggest that the ZEB1-CtBP-EZH2 complex mediates mesenchymal silencing of IRF1 by inducing the accumulation of repressive epigenetic marks. Conversely, a component of the mechanism of IRF1 induction by poly(I:C) in normal hSAECs involves promoter de-repression via decreased ZEB1-CtBP-EZH2 binding.

The association between ZEB1-CtBP-EZH2 binding and H3K27 (me3) accumulation on the IRF1 promoter prompted us to evaluate whether the EZH2 methyltransferase of the PRC2 mediates IRF1 expression. We tested the effect of GSK126, a potent, highly selective small-molecule inhibitor of EZH2 methyltransferase activity40. Previous studies have shown that GSK126 decreases global H3K27 (me3) levels and reactivates repressed PRC2 target genes40. We observed that, relative to solvent-treated controls, GSK126 enhanced IRF1 mRNA expression in EMT-hSAECs, peaking at 17-fold 96 h after addition (Fig. 5e). A similar release of inhibition of the IFNL1 mRNA response increased 27-fold at the same time point (Fig. 5f), consistent with our earlier findings that IRF1 expression is rate-limiting in IFNL production. Although GSK126 produced a transient induction of ZEB1 mRNA after 24 h, this treatment

Figure 6 | ZEB1-mediated epigenetic regulation of IRF1. Schematic model of the IRF1 promoter in epithelial cells (left) and mesenchymal cells (right). In unstimulated epithelial cells, IRF1 is in a metastable state associated with activating histone H3 K4(me3) and suppressive histone H3 K27 (me3) marks, controlled by the ZEB1-CtBP complex. Upon activation by viral patterns, ZEB1-CtBP is cleared from the promoter, associated with recruitment of activating NF-kB and IRF3. By contrast, in the mesenchymal transition, upregulated ZEB1-CtBP replaces the activating histone marks with H3K27(me3) mediated by EZH2, functionally repressing the promoter and downstream expression of IFN-III.
had no stable effect on ZEB1 expression (Fig. 5g). Interestingly, GSK126 treatment enhanced the increase in IRF1 expression by either RV16 or RSV infection in EMT-hSAECs (Fig. 5h). We also noted that GSK126 treatment slightly increased IFNB1 production (Fig. 5i) and significantly increased IFNL expression for all treatments (Fig. 5j). The viral replication of both RV16 and RSV was lower as well (Fig. 5k).

Discussion

Diseases associated with chronic airway mucosal injury and repair are also associated with defects in innate immunity. This study focuses on the effects of cellular reprogramming on the expression of IFN-I/III, two major classes of paracrine mediators of the mucosal antiviral response. A major discovery in this work is that the TGF-β-dependent mesenchymal transition dramatically affects the expression of IFN-IIIIs and, to a lesser extent, IFN-β, through silencing of IRF1 expression. Although the virus-inducible expression of IFN-I (IFN-β) has been extensively studied, the control mechanisms for inducible IFNLs are incompletely understood. Our studies demonstrate that IRF1 is the major rate-limiting transcriptional regulator of IFNL expression and confirm that IFNL expression plays a significant role in restricting RV replication, more so than RSV. Although the mechanisms for inducible control of IRF1 via NF-κB are well documented, little is known about its epigenetic regulation. Our studies indicate that the chromatin environment of IRF1 is located in a metastable state in epithelial cells, with both activating histone marks (H3K4me3) and repressive marks (H3K27me3). We demonstrate that the mesenchymal transition silences IRF1, making the promoter inaccessible to RelA and IRF3 transactivators associated with the accumulation of repressive H3K27(me3) and ZEB1-PRC complex interaction with the IRF1 promoter in both cell states (Fig. 6). In normal cells, in the basal state, IRF1 is in a semi-open chromatin environment, repressed by low levels of ZEB1-CtBP interaction. In response to poly(I:C) stimulation, ZEB1-CtBP binding is cleared from the promoter, a de-repression event followed by the binding of the activators NF-κB/RelA and IRF3 to mediate its dramatic upregulation. Conversely, in mesenchymal transitioned cells, ZEB1-CtBP is much more abundant and, although this complex is slightly decreased upon activation of the innate pathway, this is not sufficient to clear ZEB1-CtBP or repressive H3K27(me3) marks to enable gene activation. Others have shown that IRF1/type I IFN expression is developmentally controlled in embryonal stem cells, being silenced via an unknown mechanism. It will be of interest to examine the role of ZEB1-PRC2 in this developmental phenomenon.

This work provides new insights into innate immune defects associated with chronic remodelling and repair. In reactive airway disease, the TGF-β pathway is activated to promote mucosal repair and renewal and may also be reprogramming the mucosal IFN response. Our study provides a testable mechanism for how severe asthma is associated with defects in epithelial secretion of type III IFNs and enhanced susceptibility to RV replication. Similarly, mesenchymal transition is an important pathological process in idiopathic pulmonary fibrosis and cystic fibrosis, where an upregulated TGF-β pathway and ZEB1 have been observed. Our findings using the GSK126 ZEB1 inhibitor suggest that EZH2 methylemerase may be a target for epigenetic modulation that could reverse defects in mucosal innate immunity.

Methods

Cell culture and treatment. hSAECs55-56, authenticated by short tandem repeat (STR) analysis in our laboratory, were not listed in the database of commonly misidentified cell lines maintained by ICLAC and NCBI BioSample. The hSAECs were mycoplasma-free and grown as submersed monolayers in SAGM small airway epithelial cell growth medium (Lonza) with a low glucose content of 1.081 g/l (6 mM). To induce EMT, hSAECs were stimulated with TGF-β (10 ng ml⁻¹; PeproTech) for 15 days56. Poly(I:C) (Sigma-Aldrich) was solubilized in phosphate buffered saline (PBS) and used at 50 µg ml⁻¹ in cell culture. GSK126 (Selleckchem) was solubilized in DMSO as a 10 mM stock and used at 1 µM for 3 days56.

Virus preparation and infection. RSV A2 strain was grown in HEp-2 cells (ATCC CCL-23) and prepared as previously described51. Rhinovirus serotype 16 (RV16, a gift from J. E. Gern52) was grown in H1 HeLa cells at 35 °C as described in ref. 48. The viral titre of RSV or RV16 was determined by standard methylcellulose plaque assay. Purified viral pools were aliquoted, quick-frozen in dry ice-ethanol and stored at −80 °C until use.

Immunofluorescence microscopy. hSAECs were plated on coverslips pretreated with rat tail collagen (Roche). After treatment, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, blocked, and incubated with the primary rabbit polyclonal antibodies53.54. After incubation with Alexa-goat anti-rabbit antibody, cells were washed and mounted using ProLong Diamond Antifade Mountant with 4′-diamidino-2-phenylindole (DAPI) (Molecular Probes). The cells were visualized on a Zeiss fluorescence LSM510 confocal microscope using a ×63 objective lens.

Subcellular fractionation and western immunoblot analysis. Sucrose cushion-purified Nuclear proteins were used55. For western blots, the membrane was cut into semiconfluent monolayers with or without 5 mM puromycin.

Microarray analysis (biontinated DNA pulldown) of ISG DNA-binding proteins. A microarray DNA pulldown assay for ISG DNA-binding proteins was adapted56.57. The natural positive regulatory domain I (PRD1) DNA element from the interferon beta promoter was chemically synthesized with or without 5′-biotin (labeling) (IDT)57. The duplex oligonucleotide sequences are listed in Supplementary Table 1. In brief, 50 pmol of biotinylated duplex was incubated with 1 mg of sucrose-cushion-purified nuclear protein in 1 ml of binding buffer (8% vol/vol glycerol, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 150 mM KCl, 1 mM EDTA and 12 mM HEPES pH 7.9) in the presence of 10 µg of DNA for 1 h at 4 °C. After incubation, bound proteins were captured by adding 50 µl of a 50% slurry of Dynabeads M-280 streptavidin (Invitrogen) for 20 min at 4 °C with mixing. The beads were washed and washed twice with binding buffer. ISG DNA-binding proteins were eluted with 50 µl of 1 × SDS–PAGE loading buffer for western blot analysis. A fibroblast cell line was used as a negative control and a non-biotinylated DNA duplex was added in the initial binding reaction.

Quantitative real-time PCR (qRT–PCR). Total RNA was isolated using TRI reagent (Sigma-Aldrich). For gene expression analyses, 5 µg of RNA was reverse-transcribed using Super Script III in a 20 µl reaction mixture with or without gene-specific primers (Supplementary Table 2). The PCR plates were denatured for 3 min at 95 °C and then subjected to 40 cycles of 10 s at 95 °C and 30 s at 58 °C in a CFX96 real-time PCR detection system (Bio-Rad). PCR products were subjected to melting curve analysis to assure that a single amplification product was produced. Quantification of relative changes in mRNA levels was determined by the ΔΔCt method (normalized to DNA polymerase beta, POLR) and expressed as the fold change between experimental and control samples52.

Two-step chromatin immunoprecipitation (XChIP). XChIP was performed as described in ref. 30. Briefly, 2 × 10⁶ to 4 × 10⁶ hSAECs per 100 mm dish were washed twice with PBS. Protein–DNA crosslinking was performed with or without 5′-biotin (labeling) (IDT)57. The duplex oligonucleotide sequences are listed in Supplementary Table 1. In brief, 50 pmol of biotinylated duplex was incubated with 1 mg of sucrose-cushion-purified nuclear protein in 1 ml of binding buffer (8% vol/vol glycerol, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 150 mM KCl, 1 mM EDTA and 12 mM HEPES pH 7.9) in the presence of 10 µg of DNA for 1 h at 4 °C. After incubation, bound proteins were captured by adding 50 µl of a 50% slurry of Dynabeads M-280 streptavidin (Invitrogen) for 20 min at 4 °C with mixing. The beads were washed and washed twice with binding buffer. ISG DNA-binding proteins were eluted with 50 µl of 1 × SDS–PAGE loading buffer for western blot analysis. A fibroblast cell line was used as a negative control and a non-biotinylated DNA duplex was added in the initial binding reaction.

Quantitative real-time genomic PCR (Q-gPCR). Gene expression in ChIP was determined by Q-gPCR using region-specific PCR primers and probes (Supplementary Table 3). The fold change of DNA in each immunoprecipitate was determined.
was determined by normalizing the absolute amount to an input DNA reference and calculating the fold change relative to the amount in unstimulated cells.

**Lentivirus-mediated gene silencing and overexpression.** Human IRF1 and ZEB1 cDNAs (gifts from C. M. Rice4 and Kumiko UiTei (Addgene plasmid # 42100)5 respectively) were amplified and cloned into the XbaI/EcoRI sites of the lentiviral vectors pLV-tet-OX (Addgene plasmid # 8260) and pLV-tet-R (Addgene plasmid # 8260) through Gibson assembly. The empty vector was used as a negative control.

TRIPZ inducible lentiviral shRNAs of human ZEB1 were purchased from Thermo Scientific (V2THS_226625, V2THS_116659 and V2THS_116660). The mature antisense sequences of ZEB1 shRNAs are listed in Supplementary Table 4. TRIPZ inducible lentiviral non-silencing shRNA (RHS4743) was used as a negative control.

To produce infectious lentivirus, each construct was transfected into 293FT packaging cells together with the lentiviral packaging plasmids using lipofectamine 2000 (Invitrogen). The supernatant media were collected 48 h after transfection and frozen in aliquots at −80 °C. hSACs were infected in the presence of 4 µg ml−1 POLYBRENE and selected 48 h later with 2 µg ml−1 of puromycin for 3 days. Stable transfectants from a mixed population were used in the experiments. The lentiviral shRNA stable transfectants were treated with 2 µg ml−1 of doxycycline (Sigma-Aldrich) for 72 h to induce shRNA-mediated mRNA knockdown.

**CRISPR/Cas9-mediated IRF1 gene knockout.** The LentCRISPRv2 system (a gift from F. Zhang (Addgene plasmid #52961)7) was used to prepare lentivirus for lentiviral CRISPR/Cas9-mediated IRF1 gene knockout. The gRNA sequence 5’-ACAGAGATGCGCTGTTCCTG-3’ targeting exon 3 (Supplementary Fig. 7F)7,77. The lentiviral CRISPR plasmid targeting human IRF1 was constructed by single-step golden gate gRNA cloning. hSACs were infected with virus supernatant in the presence of 4 µg ml−1 POLYBRENE and selected 48 h later with 2 µg ml−1 of puromycin for 3 days. At 10 days later, the pooled cells were stimulated with 50 µg ml−1 poly(I-C) for 3 h and analysed for IRF1 abundance by western immunoblot. Lenti lentiviral-CRISPRv2-transduced hSACs were puromycin-selected and used as a negative control.

**TGF-β-induced pulmonary fibrosis in mice.** The design and procedures of animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch (protocol no. 1312058). Male C57BL/6j mice aged 16 weeks (The Jackson Laboratory, Bar Harbor, ME) were randomized into two groups (n = 5 in each group) and housed under pathogen-free conditions with food and water ad libitum. Mice in treated group were given repetitive challenges with TGF-β (1 µg per mouse, intranasally) every other day for a total of 15 TGF-β treatments. Ten days after the last TGF-β treatment, all the mice were killed and lung tissues taken and fixed for immunofluorescence assays, blindly.

**Statistical analysis.** One-way analysis of variance (ANOVA) was performed when looking for time differences, followed by Tukey’s post hoc test to determine significance. P < 0.05 was considered significant.

**Data availability.** The data that support the findings of this study are available from the corresponding authors upon reasonable request. Complete blots for all findings of this study are available. The data that support the findings of this study are available from the corresponding authors upon reasonable request. Complete blots for all findings of this study are available.

Received 1 July 2016; accepted 25 April 2017; published 5 June 2017

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