Paracrine signalling during ZEB1-mediated epithelial–mesenchymal transition augments local myofibroblast differentiation in lung fibrosis

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Abstract
The contribution of epithelial–mesenchymal transition (EMT) to human lung fibrogenesis is controversial. Here we provide evidence that ZEB1-mediated EMT in human alveolar epithelial type II (ATII) cells contributes to the development of lung fibrosis by paracrine signalling to underlying fibroblasts. Activation of EGFR–RAS–ERK signalling in ATII cells induced EMT via ZEB1. ATII cells had extremely low extracellular matrix gene expression even after induction of EMT, however conditioned media from ATII cells undergoing RAS-induced EMT augmented TGFβ-induced pro-fibrogenic responses in lung fibroblasts. This epithelial–mesenchymal crosstalk was controlled by ZEB1 via the expression of tissue plasminogen activator (tPA). In human fibrotic lung tissue, nuclear ZEB1 expression was detected in alveolar epithelium adjacent to sites of extracellular matrix (ECM) deposition, suggesting that ZEB1-mediated paracrine signalling has the potential to contribute to early fibrotic changes in the lung interstitium. Targeting this novel ZEB1 regulatory axis may be a viable strategy for the treatment of pulmonary fibrosis.

Introduction
Epithelial–mesenchymal transition (EMT), a dynamic and reversible biological process by which epithelial cells lose their cell polarity and down-regulate cadherin-mediated cell–cell adhesion to gain migratory properties, is involved in embryonic development, wound healing, fibrosis and cancer...
metastasis [1]. EMT is executed in response to pleiotropic signalling factors, including the transforming growth factor β (TGFβ) superfamily, Sonic Hedgehog (Shh), Wnt/β-catenin, fibroblast growth factor (FGF) and epidermal growth factor (EGF). These factors regulate the expression of specific transcription factors (TFs) called EMT-TFs (e.g. Snail, ZEB, Twist and others) that promote repression of epithelial features and induction of mesenchymal characteristics [2, 3]. Unlike EMT in cancer, which is detrimental, wound-healing-driven EMT induced in response to injury is beneficial, but exaggerated healing responses can lead to fibrosis or tissue scarring.

Fibrosis is a hallmark of many chronic degenerative disorders and is associated with reduced organ function and eventual organ failure. Fibrotic disease is on the increase; for example, idiopathic pulmonary fibrosis (IPF), the most common type of idiopathic interstitial pneumonia, occurs with similar frequency to that of stomach, brain and testicular cancer [4]. IPF is now generally regarded as a consequence of multiple interacting genetic and environmental risk factors, with repetitive local micro-injuries to ageing alveolar epithelium playing a central role [5]. These micro-injuries initiate the progressive accumulation of extracellular matrix (ECM) deposited by myofibroblasts. The origin of these myofibroblasts has been debated for many years, with EMT being considered as a potential source by driving the transformation of epithelial cells into ECM producing myofibroblasts [6–10]. However, lineage tracing in transgenic mice indicates that the contribution of those cells to the population of myofibroblasts is negligible [11–14].

In this study, we identify a novel regulatory axis involved in lung fibrosis whereby EMT contributes to the fibrotic process via paracrine activation of fibroblasts. We demonstrate that epidermal growth factor receptor (EGFR)-RAS-extracellular signal-regulated kinase (ERK) signalling induces the transcription factor ZEB1, which not only controls EMT but also regulates the production of locally-induced pro-fibrogenic signalling by enhancing TGFβ-induced pro-fibrogenic responses in fibroblasts. Consistent with this, increased ZEB1 nuclear expression was detected in alveolar epithelium adjacent to sites of ECM deposition in IPF lung tissue. Thus, rather than contributing directly to the mesenchymal population, our data suggest that ZEB1-dependent EMT of ATII cells contributes to fibrosis via epithelial–fibroblast crosstalk. The occurrence of ZEB1 activation at sites of local ECM deposition in IPF lung tissue is consistent with the concept that ZEB1-regulated paracrine signalling contributes to the development of a pro-fibrogenic microenvironment leading to interstitial lung fibrosis.

Results

Activation of EGFR signalling induces EMT in alveolar epithelial cells

To investigate IPF associated signalling pathways, we analysed differentially expressed genes in IPF and control lung tissue from a publicly available microarray dataset (GSE24206) [15]. Using a false discovery rate (FDR) corrected P value of 0.05, we identified 7668 genes to be differentially expressed out of a total of 54,675 probe sets. Gene network analysis using the Consensus Pathways Database [16] identified a number of pathways. Of these the EGFR–ERK pathway was the top-ranked pathway with 150 of 458 pathway candidates being significantly (Q-value < 0.05) overrepresented in the dataset (Supplementary Fig. S1a).

Based on the transcriptomic data, we hypothesised an important role of EGFR signalling in IPF. Identification of pathological mechanisms of IPF has been challenging; however, dysregulation of alveolar type 2 (ATII) epithelial cells is thought to be central [5]. We therefore treated a human ATII cell line (ATIIER:KRASV12) [17, 18] with EGF (Fig. 1b–d; Supplementary Fig. S1b) or transforming growth factor α (TGFα) (Supplementary Fig. S1b) to activate EGFR signalling. The human ATII cell line grows in continuous culture and expresses the ATII cell marker, pro-surfactant protein C (ProSP-C) (Figs. 1a, 2f). Our results showed that treatment of ATIIER:KRASV12 cells with EGF for 24 h induced EMT, reflected by a change in their morphology from typical cuboidal epithelial cells to more elongated mesenchymal cell phenotype with a reorganisation of the actin cytoskeleton as demonstrated using Phalloidin staining of filamentous actin (F-actin) (Fig. 1b). This phenotypic switch was accompanied by a significant increase in mRNA expression of ZEB1 and VIM (Vimentin), and a reduction in CDH1 (E-cadherin); mRNA levels of other EMT-TFs, such as SNAI1, SNAI2, TWIST and ZEB2 were not increased by activation of EGFR signalling (Fig. 1c). The changes in ZEB1 and E-cadherin were further confirmed by Western blot analysis (Fig. 1d; Supplementary Fig. S1b).

Similar results were obtained using primary human ATII cells treated with EGF where an increase in ZEB1 expression was associated with down-regulation of E-cadherin (Fig. 1e). Under the same conditions, however, TGFβ was not able to induce EMT in the primary human ATII cells (Fig. 1e). Together, these results demonstrate that activation of EGFR signalling is able to activate the EMT programme in ATII cells, which is supported by a morphology change, the induction of the EMT-TF ZEB1 and a mesenchymal marker Vimentin as well as a reduction in E-cadherin expression.
Activation of the RAS pathway drives EMT via ERK–ZEB1 in ATII cells

RAS signalling is one of the most important pathways downstream of EGFR activation and is involved in a variety of physiological and pathological responses, including EMT [19–21]. To investigate whether the RAS pathway is important for EMT in ATII cells, we utilised a RAS-inducible ATII cell model. KRASV12 (containing a single amino acid mutation in KRAS, glycine to valine at position 12) fused to the oestrogen receptor (ER) ligand-binding domain [22] was introduced into ATII cells to generate ATIIER:KRASV12 cells cultured in the absence or presence of 100 ng/ml EGF for 24 h. Rhodamine-phalloidin was used to stain F-actin. DAPI (blue) was used to stain nuclei. Scale bars: 40 μm. a Immunofluorescence staining of Pro-surfactant protein-C (Pro-SP-C) (green) in ATIIER:KRASV12 cells. DAPI (blue) was used to stain nuclei. Scale bars: 40 μm. b Immunofluorescence staining of F-actin (red) in ATIIER:KRASV12 cells cultured in the absence or presence of 100 ng/ml EGF for 24 h. Rhodamine-phalloidin was used to stain F-actin. DAPI (blue) was used to stain nuclei. Scale bars: 40 μm. c Fold change in mRNA levels of CDH1 (E-cadherin), VIM (Vimentin), SNAI1 (Snail1), SNAI2 (Snail2), TWIST, ZEB1 and ZEB2 in ATIIER:KRASV12 cells cultured in the absence or presence of 100 ng/ml EGF for 24 h. GAPDH-normalised mRNA levels in control cells were used to set the baseline value at unity. Data are mean ± s.d. n = 3 samples per group. **P < 0.01, ***P < 0.001. d Protein expression of E-cadherin, ZEB1 and phospho-ERK (p-ERK) in ATIIER:KRASV12 treated with 100 ng/ml EGF for 8 or 24 h. β-actin was used as a loading control. e Protein expression of E-cadherin, ZEB1, phospho-Smad2 (p-Smad2), phospho-ERK (p-ERK) in primary human ATII cells treated with 100 ng/ml EGF or 5 ng/ml TGFβ over 7 days. β-actin was used as a loading control.

![Fig. 1](image-url)
demonstrated by a reduction in E-cadherin, and an increase in ZEB1 and Vimentin expression (Fig. 2f). These observations suggest that EGFR signalling and the downstream RAS pathway are able to induce EMT in ATII cells.

Since RAS activity regulates both the RAF–ERK and phosphoinositide 3-kinase (PI3K)–protein kinase B (AKT) signalling pathways, we next investigated which one is required for EMT in the ATII cells using inhibitors for these pathways. Treatment with the ERK inhibitor U0126 in ATII\textsuperscript{ER,KRAS\textsubscript{V12}} cells was sufficient to inhibit RAS-induced ZEB1 and Vimentin expression, as well as to restore the expression of E-cadherin and the epithelial morphology; in contrast, the AKT inhibitor AKT VIII failed to do so (Fig. 3a, b; Supplementary Fig. S3a).
We next investigated which EMT-TFs are important for RAS-induced EMT in ATII cells. 

**ZEB1 RNA interference (RNAi), but not SNAI1 or SNAI2 RNAi, was able to restore E-cadherin expression and the epithelial morphology in 4-OHT-treated ATIIER:KRASV12 cells (Fig. 3c, d; Supplementary Fig. S3b), in line with the fact the ZEB1 was the only EMT-TF induced by EGFR–RAS signalling (Figs. 1, 2).**

Taken together, our results identify that RAS activation in human ATII cells drives EMT via ERK–ZEB1 pathway. **ZEB1 is highly expressed in IPF alveolar epithelium and is critical for transcriptional regulation of secreted factors that mediate crosstalk between ATII cells and fibroblasts**

Given our in vitro findings, we compared ZEB1 expression in IPF and control lung tissue. In IPF tissue, we detected strong nuclear expression of ZEB1 not only in fibroblastic foci (Fig. 4a) but also in epithelial cells of thickened alveoli septae where collagen deposition in the interstitium was evident (Fig. 4b); in contrast, little ZEB1 staining or collagen deposition was observed in alveoli of control lung tissue (Fig. 4c). The presence of nuclear ZEB1 staining in alveolar epithelial cells within IPF lung tissue suggests that these cells are undergoing EMT; furthermore, the presence of ECM suggests induction of mesenchymal responses, either directly via the repairing epithelial cells undergoing EMT or by crosstalk with underlying fibroblasts.

Comparison of the relative expression of ECM components in RAS-activated ATIIER:KRASV12 cells and fibroblasts highlights that ATII cells produce extremely low levels of ECM genes even after the induction of EMT (Supplementary Fig. S4a), suggesting that ECM production in fibrosis is more likely to be a consequence of fibroblast activation than direct deposition by epithelial cells undergoing EMT. Therefore we investigated whether ATII cells undergoing RAS-induced EMT produce paracrine factors that activate fibroblasts. For these experiments, we took advantage of the

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**Fig. 3** Activation of the RAS pathway drives EMT via ERK–ZEB1 in ATII cells. **a** Protein expression of E-cadherin, ZEB1, phospho-AKT (p-AKT) and phospho-ERK (p-ERK) in ATIIER:KRASV12 treated with 250 nM 4-OHT in the absence or presence of inhibitors AKT VIII (10 μM) or U0126 (10 μM) for 24 h. DMSO was used as a vehicle control and β-actin was used as a loading control. **b** Fold change in mRNA levels of CDH1 (E-cadherin), VIM (Vimentin) and ZEB1 in ATIIER:KRASV12 treated with 250 nM 4-OHT in the absence or presence of inhibitors AKT VIII (10 μM) or U0126 (10 μM) for 24 h. DMSO was used as a vehicle control. GAPDH-normalised mRNA levels in control cells were used to set the baseline value at unity. Data are mean ± s.d. n = 3 samples per group. **P < 0.01. ***P < 0.001. **c** Protein expression of E-cadherin, ZEB1, Snail1 and Snail2 in ATIIER:KRASV12 cells transfected with the indicated siRNA followed by treatment of 250 nM 4-OHT for 24 h. β-tubulin was used as a loading control. **d** Fold change in the mRNA level of CDH1 (E-cadherin) in ATIIER:KRASV12 cells transfected with the indicated siRNA followed by treatment of 250 nM 4-OHT for 24 h. GAPDH-normalised mRNA levels in control cells were used to set the baseline value at unity. Data are mean ± s.d. n = 3 samples per group. ***P < 0.001.
ability of 4-OHT to induce RAS pathway activation in ATII<sub>ER:KRAS<sup>V12</sup></sub> cells, as this was not dependent on exogenous growth factors that might directly affect fibroblast responses. We treated the MRC5 or primary human parenchymal lung fibroblasts with conditioned media (CM) from control or 4-OHT-treated ATII<sub>ER:KRAS<sup>V12</sup></sub> cells in the absence or presence of TGFβ, and evaluated the fibroblast responses by measuring the expression of α-smooth muscle actin (α-SMA, a myofibroblast marker) and other ECM genes, including COL1A1, COL3A1 and FN1. On its own, CM from RAS-activated ATII<sub>ER:KRAS<sup>V12</sup></sub> cells (4-OHT-treated ATII CM) had little effect on the activation of fibroblasts (Fig. 5). However, 4-OHT-treated ATII CM together with TGFβ achieved a synergistic effect in activating fibroblasts, reflected by a larger increase in α-SMA (ACTA2), COL1A1 and FN1 levels (Fig. 5a, b). Of note, 4-OHT-treated ATII CM did not augment Smad2 phosphorylation suggesting a Smad2-independent response (Fig. 5a, c). Similar results were obtained using primary human lung fibroblasts from IPF patients (IPF fibroblasts, IPFFs) and control donors (normal human lung fibroblasts, NHLFs) (Fig. 5c; Supplementary Fig. S4b).

Given the important role of ZEB1 in mediating RAS-induced EMT and the fact that ZEB1 is highly expressed in the alveolar epithelium of IPF patients, we hypothesised that ZEB1 may determine the paracrine signalling produced by ATII cells undergoing RAS-induced EMT. ZEB1 RNAi (Fig. 6a) in ATII cells completely abolished the effects of
CM from RAS-activated ATII cells on TGFβ-induced activation of fibroblasts (Fig. 6b; Supplementary Fig. S5), highlighting ZEB1 as a key regulator of EMT as well as the paracrine signalling between ATII cells and fibroblasts.

**ZEB1 regulates the expression of tissue plasminogen activator, which acts as a paracrine regulator of TGFβ-induced fibroblast activation**

By performing quantitative proteomic analysis of the CM from control or 4-OHT-treated ATIIER:KRASV12 cells, we identified ~430 secreted proteins whose levels changed during RAS-induced EMT. We then checked their expression in pulmonary epithelial cells from control and IPF lung tissue using a publicly available dataset [24], and identified a total number of 25 genes/proteins that were elevated in IPF lung epithelial cells as well as in CM from 4-OHT-treated ATIIER:KRASV12 cells (Supplementary Table S1). Of these, PLAT, which encodes tPA was most up-regulated in IPF epithelial cells (Fig. 7a; Supplementary Table S1) and...
we confirmed enhanced secretion of tPA in the CM from 4-OHT-treated ATIIER:KRASV12 cells by Western blotting (Fig. 7b). As we had identified ZEB1 as the key regulator of epithelial–mesenchymal crosstalk, we scanned the promoter of PLAT for the presence of ZEB1 binding motifs (5′-CANNTG-3′) and found a ZEB1 binding site −419 bp upstream of the transcriptional start site (TSS) (Supplementary Fig. S6a). Further experiments showed that the mRNA expression of PLAT was increased upon RAS-activation in ATII cells and this was repressed by ZEB1 RNAi (Fig. 7c).

To validate the ZEB1 binding site in the PLAT promoter, we first performed a chromatin immunoprecipitation (ChIP) assay. An anti-ZEB1 antibody was used to precipitate formaldehyde cross-linked ZEB1-DNA complexes in ATIIER:KRASV12 cells treated without or with 4-OHT. The presence of PLAT promoter DNA sequences in the immunoprecipitate was verified by PCR using primers amplifying the region between −547 and −345 upstream of the TSS, and we found RAS activation in ATII cells increased ZEB1 occupancy on the PLAT promoter (Fig. 7d; Supplementary Fig. S6b). We next generated two PLAT promoter constructs (−689 to −1 upstream of the TSS) which were cloned into a pGL3 basic luciferase reporter plasmid and transfected into ATII cells; the pGL3 basic-PLAT (−689 to −1) construct contained the ZEB1 motif whereas this was deleted in the second construct (delta −419 to −414 upstream of the TSS) (pGL3 basic-Δ ZEB1 motif). RAS activation by 4-OHT in ATIIER:KRASV12 cells resulted in a significant increase in pGL3 basic-PLAT (−689 to −1) luciferase activity. Under the same conditions, luciferase activity was not increased using pGL3 basic-Δ ZEB1 motif (Fig. 7e). These data confirm that PLAT (tPA) is a transcriptional target of ZEB1 in response to RAS activation in ATII cells.

Consistent with a previous report [25], tPA synergistically promoted TGFβ-induced α-SMA expression in human lung fibroblasts (Supplementary Fig. S6c). Like ZEB1, PLAT RNAi (Supplementary Fig. S6d) in ATII cells completely abolished the effects of CM from RAS-activated ATII cells on TGFβ-induced α-SMA expression in fibroblasts (Fig. 7f), demonstrating tPA as a key paracrine factor secreted by ATII cells undergoing RAS-induced EMT. These results provide clear evidence that a ZEB1-tPA axis is involved in the paracrine signalling between ATII cells undergoing RAS-induced EMT and fibroblasts to augment their differentiation into myofibroblasts caused by TGFβ.

Finally, in view of the requirement for exogenous TGFβ to demonstrate an effect of the 4-OHT-treated ATII CM on fibroblasts, we investigated whether ATII cells in fibrotic tissue in vivo or those undergoing injury/repair in vitro expressed endogenous TGFβ. Using a publicly available dataset [24], we found that the major TGFβ isoform expressed by alveolar epithelial cells in vivo was TGFβ2 and that this was expressed at significantly higher levels in IPF compared with control lung tissue (Supplementary Fig. S7a). In contrast with the study in kidney [14], the data also revealed that Snail2 is up-regulated in IPF vs. control lung epithelial cells, but not Snail1 or Twist (Supplementary Fig. S7b). As we have previously shown that scrape-wounding of bronchial epithelial cells stimulates release of TGFβ2 independently of EGFR activation [26], we examined whether damage of ATII cells similarly affected TGFβ2 expression. This showed that scrape-wounded ATII cells expressed more TGFβ2 and this increased in proportion to the extent of injury (Supplementary Fig. S7c). These data suggest that damaged ATII cells are a potential source of TGFβ in vivo.

**Discussion**

Fibrotic diseases are a major cause of morbidity and mortality worldwide and their prevalence is increasing with an ageing population. Abnormal wound healing responses appear to make major contributions to the scarring process, but the underlying pathological mechanisms are unclear, especially the role of EMT. In this study, we have used a variety of approaches to show that activation of EGFR–RAS–ERK signalling in ATII cells induces EMT via the transcriptional regulator ZEB1. Importantly, beyond its effects on the epithelial cell phenotype, we have identified that ZEB1 is a regulator of paracrine signalling between lung epithelial cells and fibroblasts, as ATII cells undergoing RAS-induced EMT secrete tPA to augment TGFβ-induced myofibroblast differentiation (Fig. 8). This may be an important profibrotic event as, relative to epithelial cells, the ability of fibroblasts to synthesise ECM is orders of magnitude greater.

Consistent with previous findings [27, 28], we found strong expression of ZEB1 in the epithelium in proximity to fibroblastic foci in IPF lung tissue. However, we also found ZEB1 was expressed in epithelial cells of thickened alveolar septae where ECM deposition was evident. This suggests that ZEB1 is induced as an early response to alveolar epithelial injury and that, by regulating the expression of factors involved in paracrine signalling, ZEB1 may promote TGFβ-induced fibroblast activation in IPF. While this may be a normal physiological response to injury, persistent epithelial injury and/or failure to resolve the lesion may sensitise the underlying fibroblasts to drive a pathologic profibrogenic response. In line with this, exposure of human lung cells to nickel (Ni), an environmental and occupational pollutant linked to lung fibrosis [29], caused ZEB1-dependent EMT, which was irreversible even after the termination of Ni exposure [30]. Thus, it is conceivable that...
repetitive environmental exposures to metals such as Ni could lead to deregulation of ZEB1 to cause persistent EMT and exaggerated profibrogenic crosstalk during the initiation of IPF.

Fig. 7 ZEB1 regulates the expression of tissue plasminogen activator (tPA), which acts as a paracrine regulator of TGFβ-induced fibroblast activation. a Increased expression of PLAT (tPA) in IPF epithelial cells is shown by an online LGEA web portal (https://research.cchmc.org/pbge/lunggens/mainportal.html). b Quantitative secreteome analysis identifies an increased level of tPA in the conditioned media (CM) from 4-OHT-treated ATIIER:KRASV12 cells and a representative tPA Western blot of CM from control or 4-OHT-treated ATIIER:KRASV12 cells. Data are individual values with mean and s.d. n = 3 samples per group. Values were normalised to total fmol of each sample multiplied by 10,000. c Fold change in mRNA levels of ZEB1 and PLAT (tPA) in ATIIER:KRASV12 cells with indicated treatments. β-actin-normalised mRNA levels in control cells were used to set the baseline value at unity. Data are mean ± s.d. n = 3 samples per group, ***P < 0.001. d ChIP assays of ZEB1’s ability to bind the PLAT (tPA) promoter in ATIIER:KRASV12 cells with indicated treatments. The amplified PLAT (tPA) promoter region (−547 to −345) contains a ZEB1 binding site at −419. Values represent relative binding in relation to input (2%), normalised against control (1.0). Data are mean ± s.d. n = 4 samples per group, ***P < 0.001. e PLAT promoter reporter assays in ATIIER:KRASV12 cells with indicated treatments. Values represent relative fold of firefly luciferase in relation to Renilla luciferase, normalised against control (1.0). Data are mean ± s.d. n = 3 samples per group, **P < 0.01, ***P < 0.001. f Protein expression of α-SMA and phospho-Smad2 (p-Smad2) in MRC5 lung fibroblasts with indicated treatments. β-tubulin was used as a loading control.

EMT in the ATII cells was strongly induced by EGFR activation. The EGFR is a transmembrane receptor tyrosine kinase activated by members of the EGF family, including EGF and TGFα [31]. EGFR dimerisation activates one or
Diagram summarising a critical role of ZEB1–tPA axis regulated by EGFR–RAS–ERK pathway in the development of lung fibrosis (details provided in Discussion)
mesenchymal cells, so creating a profibrogenic micro-
vironment which leads to the development of fibrosis.
Based on the relative low levels of ECM biosynthesis by
epithelial cells and the relatively small numbers of
mesenchymal cells identified in lineage tracing studies [11,
12, 14, 43], this mechanism may be more important than
direct conversion of epithelial cells into mesenchymal cells.
While the finer details of these paracrine mechanisms may
vary according to disease and tissue location, our identi-

While the diagnosis of IPF according to international consensus
guidelines [49].

Methods

Lung tissue sampling

All human lung experiments were approved by the South-
ampton and South West Hampshire and the Mid and South
Buckinghamshire Local Research Ethics Committees, and
all subjects gave written informed consent. Clinically indi-
cated IPF lung biopsy tissue samples and non-fibrotic con-
trol tissue samples (macroscopically normal lung
sampled remotely from a cancer site) were deemed surplus
to clinical diagnostic requirements. All IPF samples were
from patients subsequently receiving a multidisciplinary
diagnosis of IPF according to international consensus
guidelines [49].

Cell culture, reagents and transfections

Primary parenchymal lung fibroblast cultures were estab-
lished from IPF or control lung tissue as described pre-
viously [50]. Fibroblasts were cultured in Dulbecco’s
Modified Eagle’s Medium (DMEM) supplemented with
10% foetal bovine serum (FBS), 50 units/ml penicillin, 50
µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium
pyruvate and 1× non-essential amino acids (DMEM/FBS)
(all from Life Technologies).

Primary human ATII cells were isolated from macro-
scopically normal regions of surgically resected lung par-
enchyma as described previously [50, 51]. The alveolar
epithelial cells were resuspended in fresh DCCM-1 (Bio-
logical Industries Ltd.) supplemented with 10% new-born
calf serum (NBCS) (Life Technologies), 1% penicillin, 1%
streptomycin and 1% L-glutamine (all from Sigma Aldrich)
and plated on collagen I (PureCol 5005-b, Advanced Bio-
Matrix Inc.) coated 96 well plates at 60% density; purity of
the cultures was determined by staining for alkaline
phosphatase.

ATIIER<sup>ErKRASV12</sup> cells [17, 18] were cultured in DCCM-1
(Biological Industries Ltd.) supplemented with 10% NBCS
(Life Technologies), 1% penicillin, 1% streptomycin and 1%
L-glutamine (all from Sigma Aldrich). To induce RAS ac-
tivation in ATIIER<sup>ErKRASV12</sup> cells, 250 nM 4-OHT (Sigma-
Aldrich) was added [17, 18]. MRC5 cells were obtained from
the European Collection of Authenticated Cell Cultures
(ECACC) and were cultured in DMEM (Thermo Fisher
Scientific). Both cell culture media were supplemented with
10% FBS (Thermo Fisher Scientific), 1% penicillin/strepto-
mycin and glutamine (Thermo Fisher Scientific). All cells
were kept at 37 °C and 5% CO₂. For 3D culture, ATIIER<sup>Er-
KRASV12</sup> cells were cultured as previously described [23] in
Matrigel (BD Biosciences). TGFβ1 was from Fisher Scientific
UK Ltd. TGFβ1 was from PeproTech. EGF and recombinant
human tPA protein were from Bio-Techne. AKT VIII and
U0126 were from Sigma Aldrich. No mycoplasma con-
tamination was detected in the cell lines used.

Short interfering RNA (siRNA) oligos against ZEB1
(MU-006564-02-0002), SNAI1 (Snail1) (MU-010847-00-
0002), SNAI2 (Snail2) (MU-017386-00-0002) and PLAT
(tPA) (MU-005999-01-0002) were purchased from Dhar-
macon. Sequences are available from Dharmacon, or on
request. As a negative control, we used siGENOME RISC-
Free siRNA (Dharmacon). ATIIER<sup>ErKRASV12</sup> cells were
transfected with the indicated siRNA oligos at a final con-
centration of 35 nM using DharmaFECT 2 reagent
(Dharmacon).

Western blot analysis

Western blot analysis was performed with lysates from cells
with urea buffer (8 M urea, 1 M thiourea, 0.5% CHAPS, 50
mM DTT and 24 mM Spermine). Primary antibodies were
from Santa Cruz (β-actin, sc-47778; ZEB1, sc-25388;
ZEB2, sc-48789; E-cadherin, sc-21791; Snai1, sc-10436).
Abcam (β-tubulin, ab6046), Cell Signalling Technology (α-
SMA, 14968; phospho-AKT, 9271; phospho-ERK, 9101;
Snail1, 3879; Snail2, 9585; TWIST, 46702; Phospho-
SMad2, 3104; β-tubulin, 86298), BD Transduction Laborato-
ries (E-cadherin, 610405; Vimentin, 550513) and Millipore (proSP-C, AB3786; tPA, 05-883). Signals were
detected using an Odyssey imaging system (LI-COR), and
evaluated by ImageJ 1.42q software (National Institutes of
Health).

qRT-PCR

Total RNA was isolated using RNeasy mini kit (Qiagen)
according to manufacturer’s instructions and quantified using
a Nanodrop Spectrophotometer 2000c (Thermo Fisher Sci-
entific). Real-time quantitative RT-PCR was carried out
using gene-specific primers (QuantiTect Primer Assays,
Qiagen) for CDH1 (E-cadherin) (QT00080143), SNAI1 (Snail1) (QT00010010), SNAI2 (Snail2) (QT00044128), ZEB1 (QT00008555), ZEB2 (QT00008554), TWIST (QT00011956), VIM (QT00095795), COL1A1 (QT00037793), COL3A1 (QT00058233), FNI (QT00038024), ACTA2 (α-SMA) (QT00088102), PLAT (tPA) (QT00075761), TGFβ1 (QT0000728), TGFβ2 (QT00025718), GAPDH (QT01192646) or ACTB (β-actin) (QT01680476) with Quant-iNova SYBR Green RT-PCR kits (Qiagen). Relative transcript levels of target genes were normalised to GAPDH or ACTB (β-actin).

**Immunofluorescence microscopy**

Cells were fixed in 4% PBS-parafomaldehyde for 15 min, incubated in 0.1% Triton X-100 for 5 min on ice, then in 0.2% fish skin gelatin in PBS for 1 h and stained for 1 h with an anti-Prosurfactant Protein C (proSP-C) antibody (1:100, Millipore AB3786, rabbit polyclonal) or anti-ZEB1 (1:100, Santa Cruz sc-25388, rabbit polyclonal). Protein expression was detected using Alexa Fluor (1:400, Molecular Probes) for 20 min. DAPI (Invitrogen) was used to stain nuclei (1:1000). Rhodamine-phalloidin was used to visualise filamentous actin (F-actin) (Molecular Probes). For immunofluorescence staining of 3D cultures from ATIIER:KRASV12 cells, spheres were fixed with 4% PBS-parafomaldehyde for 40 min, permeabilised in 0.5% Triton X-100 for 10 min on ice and stained with rhodamine-phalloidin for 1 h at room temperature. Spheres were counterstained with DAPI. Samples were observed using a confocal microscope system (Leica SP8). Acquired images were analysed using Photoshop (Adobe Systems) according to the guidelines of the journal.

**Immunohistochemistry, haematoxylin and eosin (H/E) and tinctorial stains**

Control or IPF lung tissues (n = 3 donors) were fixed and embedded in paraffin wax; tissue sections (4 μm) were processed and stained as previously described [20]. Briefly, the tissue sections were de-waxed, rehydrated and incubated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. Sections were then blocked with normal goat serum and incubated at room temperature with a primary antibody against ZEB1 (1:500, Sigma), followed by a biotinylated secondary antibody (1:500, Vector Laboratories Ltd., UK); antibody binding was detected using streptavidin-conjugated horse-radish peroxidase and visualised using DAB (DAKO) before counterstaining with Mayer’s Haematoxylin. For H/E stain, Shandon Varistain 24-4 automatic slide stainer (Thermo Fisher Scientific) was used. For tinctorial stain, Trichrome stain (Abcam ab150686) was used according to the manufacturers’ instructions. Images were acquired using an Olympus Dotslide Scanner VS110.

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were carried out using SimpleChIP enzymatic chromatin IP kits (Cell Signalling Technology) as per the manufacturer’s instructions. Briefly, ATIIER:KRASV12 cells with indicated treatments were incubated for 10 min with 1% formaldehyde solution at room temperature, followed by incubation with 125 mM glycine. Antibodies used for ChIP were as follows: rabbit anti-ZEB1 (PA5-28221, Invitrogen, rabbit polyclonal, 5 μg per IP sample), normal rabbit IgG (2729, Cell Signalling Technology, 5 μg per IP sample). For the ZEB1 binding site at position −419 of the human PLAT (tPA) promoter, the primers amplifying the region between −547 and −345 were as follows: forward 5′-GGAAAGTCCCGGAGGCCACCTA-3′ and reverse 5′-TGGAAACACTTTGTGTGGTCG-3′. DNA fragments were quantified by qPCR. PCR products were analysed in a 1.5% agarose gel by ethidium bromide staining.

**Luciferase constructs and luciferase reporter assays**

The human PLAT (tPA) promoter (sequence −689 to −1 upstream of the TSS) was amplified from human genomic DNA by PCR, and was subsequently cloned into pGL3 basic vector (Promega), termed pGL3 basic-PLAT (−689 to −1). The putative ZEB1 binding site, positioned −419 to −414 on the human PLAT promoter, was removed from pGL3 basic-PLAT (−689 to −1) construct to create the pGL3 basic-ΔZEB1 motif construct.

For the luciferase reporter assays, ATIIER:KRASV12 cells were transfected using Lipofectamine 3000 (Invitrogen) with 80 ng of pRL-CMV (Promega), which constitutively expresses the Renilla luciferase reporter, plus 600 ng of pGL3 basic-PLAT (−689 to −1) or pGL3 basic-Δ ZEB1 motif per well in the presence or absence of 4-OHT. Finally, the transcriptional assay was carried out using the Dual-Luciferase reporter assay system (Promega) following the manufacturer’s protocol.

**Quantitative proteomic analysis of the secretome and the subsequent data analysis**

Serum-free CM from ATIIER:KRASV12 cells treated without or with 4-OHT (250 nM, 24 h) were analysed using an enrichment strategy based upon Strataclean resin (Agilent) in combination with the quantitative label-free approach, LC-MS², to provide in-depth proteome coverage and estimates of protein concentration in absolute amounts [52] (details provided in Supplementary Methods).
Raw data were processed and collated into a single.csv document. Values were then normalised to total fmol of each sample multiplied by 10,000. Pseudo-counts were applied to the normalised values to replace missing ones, to allow for full statistical analysis to be completed [53]. We first sorted the normalised values in each column in order of abundance, in ascending order, then the minimum value of each sample identified. This minimum was used to replace all missing values in the data set. A two-tailed, unpaired Student’s t-test was used to compare two groups for independent samples. \( P < 0.05 \) was considered statistically significant.

In order to highlight their implications in IPF, differentially expressed proteins/genes identified in the quantititative secretome analysis were searched in LGEA web portal (https://research.cchmc.org/pbge/lunggens/mainportal.html) for their levels in pulmonary epithelial cells from control and IPF lung tissue.

**Bioinformatics**

IPF transcriptomic data was downloaded from the NCBI’s Gene Expression Omnibus (GEO). We used data from GSE24206 [15], a microarray study comparing samples from 11 IPF patients undergoing lung transplantation or diagnostic biopsy to six normal lung samples taken from lung transplantation donors. Microarray series matrix files were imported into R, and differential expression analysis comparing normal to IPF samples performed using the R package limma [54]. Data were log-transformed before comparison normal to IPF samples performed using the R code. Values were then normalised to total fmol of each sample multiplied by 10,000. Pseudo-counts were applied to the normalised values to replace missing ones, to allow for full statistical analysis to be completed [53]. We first sorted the normalised values in each column in order of abundance, in ascending order, then the minimum value of each sample identified. This minimum was used to replace all missing values in the data set. A two-tailed, unpaired Student’s t-test was used to compare two groups for independent samples. \( P < 0.05 \) was considered statistically significant.

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**Statistical analysis and repeatability of experiments**

Each experiment was repeated at least twice. Unless otherwise noted, data are presented as mean and s.d., and a two-tailed, unpaired Student’s t-test was used to compare two groups for independent samples. \( P < 0.05 \) was considered statistically significant.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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