Pretreatment of aged mice with retinoic acid supports alveolar regeneration via upregulation of reciprocal PDGFA signalling

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INTRODUCTION

Several lung diseases are associated with advanced age, such as lung cancers, COPD and end-stage adult interstitial lung diseases (ILDs), including idiopathic pulmonary fibrosis (IPF). IPF is characterised by extensive fibrosis, hyperproliferation of abnormal alveolar type 2 cells (AT2) and loss of alveolar type 1 cells (AT1), causing progressive respiratory decline and mortality usually within 5 years of diagnosis.1–3 While the pathogenesis of IPF remains unclear, chronic alveolar epithelial cell injury and fibrotic activation of fibroblasts are linked to the disorder.4 However, an understanding of the epithelial–mesenchymal crosstalk during chronic epithelial injury and in the context of ageing remains limited. Reciprocal communication between epithelial and mesenchymal cells drives lung branching morphogenesis and epithelial differentiation during lung development and repair.5–7 Single-cell RNA sequencing (RNA-Seq) analyses have provided more clarity regarding the heterogeneity of normal epithelial and mesenchymal cells during development, including identifying several distinct heterogeneous pulmonary fibroblast populations.7 8 Of all the pulmonary stromal cells, mesenchymal alveolar niche cells (MANCs)1 and LGR5+ resident alveolar fibroblasts9 have received recent attention due to their capacity to support the alveolar niche and differentiation of the alveolar epithelium. When used in alveolar organoid cultures, MANCs support large organoid formation with high AT1/AT2 ratios. MANCs are Wnt-responsive AXIN2+...
cells that also express platelet-derived growth factor receptor A (PDGFRα) and transcriptionally overlap with LG85+ resident alveolar fibroblasts. In addition to the WNT2+/PDGFRα+ MANCs, there are AXIN2+/PDGFRα+, PDGFRα+ and AXIN2+ resident fibroblast populations. An important role of these PDGFRα+ fibroblast populations in normal septation, real- veolarisation and bleomycin-induced fibrosis has been demonstrated by multiple previous studies.10–19 Interstitial PDGFRα+ fibroblasts are a mixed population of three functionally distinct fibroblast stages: contractile myofibroblasts, matrix synthesising and remodelling matrix fibroblasts and lipofibroblasts such as Acta2, Perilipin and Elastin have overlapping expression in both MANCs and other PDGFRα+ fibroblasts. Myofibroblasts, which express low levels of Pdgfra, induce smooth muscle actin expression during postnatal secondary septation and during alveolar regeneration in young adult mice.14–17 Induction of the contractile function is important for the initiation and formation of secondary septa; however, overabundant myofibroblast induction in aged mice results in the failure to regenerate alveolar septa after partial pneumonectomy (PNX).22–24 Moreover, these activated myofibroblasts contribute to bleomycin-induced fibrosis.19 Developmental and regeneration studies suggest that interstitial myofibroblast transitions to a matrix synthesising and remodelling fibroblast stage to stabilise the newly formed septa.14 15 25 In some lung reaveolarisation models, retinoic acid (RA) reinitiates myofibroblast activation and septation in young adult rats and mice.16 26 27 In this study, we used preconditioning of aged mice with RA treatment before PNX to understand the downstream mechanisms that result in regenerative activation of fibroblasts, leading to alveolar repair with advanced age. We demonstrate that RA indirectly reinitiates alveolar septation by reducing myofibroblast activation and increasing the activation of matrix fibroblast function. Our in vitro experiments support the idea that PDGFRα-expressing matrix and myofibroblasts are fibroblast activation stages rather than distinct lineages as PDGFA supplementation activates the matrix function. We used gene expression studies after PNX and in vitro organoid models to further study the role of matrix fibroblast in alveolar resepta- tion and alveolar epithelial differentiation, both important func- tions for a ‘regenerative activation’ of interstitial lung fibroblasts.

One important problem with tissue fibrosis as a therapeutic target is that it is a late event in the natural history of IPF. By the time fibrotic tissue deposition is sufficient to cause symp- toms prompting the patient to seek medical attention, disease progression has advanced to a stage where parenchymal lung fibrosis cannot be repaired in human lungs. As explanted lungs are end-stage disease, we used the organoid model to identify molecular events that occur earlier in the pathogenesis of IPF and are potentially reversible, in contrast to temporally late events such as fibrotic tissue derangement, which is irreversible. In this study, we have focused on the matrix fibroblast stage that is lost with advanced age and supports proper AT2/AT1 differentia- tion. We evaluated primary alveolar epithelial cells and fibro- blasts isolated directly from patients with IPF; we studied live cells in an environment permitting them to communicate with and influence each other, and we identified PDGFA signalling as an important reciprocal messenger to allow for proper AT2/AT1 cell differentiation. These studies provide insights into future treatment options for lung regeneration and early potentially reversible interventions in IPF.

MATERIALS AND METHODS

Mouse husbandry and left lobe PNX

Wild-type mixed background mice were used for flow cytometry analyses and organoid culture. Young mice were 12–16 weeks of age, and aged mice were >40 weeks of age. Based on our previous studies on RA and alveolar regeneration,16 we treated aged mice (Aged RA) with trans-RA (Sigma R2625) at a dose of 2 μg/g body weight dissolved in dimethyl sulfoxide and peanut oil administered via intraperitoneal injection daily for 10 days prior to pneumonec- tomy with a 2-day break after the first 5 days. Mice were then subjected to PNX or sham surgery (Sham) and harvested 5 or 21 days after surgery.28 For each experimental group (Young Sham, Young PNX, Aged PNX and Aged RA pretreated), n>3 were collected for each experiment.

Tissue harvest

Prior to sacrifice, mice were lethally injected with ketamine, xylazine and acepromazine. For optimal lung histology, lungs were inflation-fixed with 4% paraformaldehyde in phosphate- buffered saline (PBS) at 25 cm H2O pressure via a tracheal cannula and closed chest to ensure comparable histology of fixed tissue. Lungs were fixed overnight at 4°C. All lungs were washed with PBS, dehydrated through a graded series of ethanol solutions and processed for paraffin embedding. Sections (5 μm) were loaded onto polylysine-coated slides for analysis. All samples were processed at the same time to ensure no differ- ence between Sham and PNX-operated mice. Use of animals was approved by Cincinnati Children’s Hospital Medical Center (CCHMC) Institutional Animal Care and Use Committee.

Morphometric point and intersection counting analysis

As in previously published studies, we used morphometric point intersection analysis to quantify alveolar simplification of fractional airspace area on histological sections of inflation-fixed adult lungs at 21 days after PNX.15 16 For each animal, three to five sections from different parts of the lung, showing all five lobes, were analysed. A 120-point grid was overlaid over five to seven random pictures of each lobe. The alveolar space was calculated as the percentage of grid intersections over the alve- olar space versus the alveolar tissue. Each symbol represents the average percentage of airspace of one animal. Values for each individual animal were averaged per experimental group.29

Patient samples

Donor patient samples were obtained from healthy lungs rejected for transplant, and the CCHMC Institutional Review Board declared that donor tissue samples were Institutional Review exempt, in accordance with protocol 2013-3356. Samples were collected from explanted lungs of patients with ILD at the time of lung transplant, and informed consent was obtained from each subject in accordance with the Partners Institutional Review Board (2013P002332), Boston, Massachusetts, USA. Patient clinical data are available in online supplemental table S6.

Second harmonic generation and immunofluorescence

IPF and donor-fixed lung tissue in optical cutting tempera- ture (OCT) blocks were sectioned at 250 μm and cleared via the passive clarity (PACT) protocol, followed by whole-mount antibody stain.30 In short, OCT is removed with PBS, and then the tissue is placed in a cold 4% polyacrylamide (1610140, Bio-Rad) hydrogel with 0.25% photoinitiator (VA-044, Wako) solution overnight. The tissue was polymerised at 37°C for 4 hours, washed with PBS to remove excess hydrogel and then
incubated at 37°C overnight in an 8% sodium dodecyl sulfate in PBS solution for permeabilisation. After washing with PBS, a 25% Quadrol (122262-1L, Sigma Aldrich) solution in PBS was added for 16 hours on a 37°C rotator, after which the samples were washed in PBS and are ready for immunostaining. For immunostaining, the PACT-cleared tissue sample was incubated with 4% donkey serum blocking solution at room temperature and then incubated with primary antibodies aSMa (Sigma A5228) and Abca3 (Seven Hills WMB-17G524) at 4°C for 3 days. Unbound antibody was removed with PBS, followed by fluorescent labelling with secondary antibodies. After washing with PBS, the tissue was mounted in refractive index matching solution (RIMS) solution for second harmonic generation (SHG) imaging on a Nikon FN I upright microscope and analysed using Nikon Elements and Imaris software.

Organoid generation
Human and mouse lung tissue were dissociated into single-cell suspensions using dispase and DNase. Cell suspensions were incubated with fragment crystallizable receptor (FcR) blocking reagent in magnetic-advanced cell sorting (MACS) MACS buffer (Miltenyi Biotec), followed by isolation of PDGFRA (CD140+) and EPCAM (CD326+) cells as described earlier. PDGFRA (CD140+) fibroblasts were co-cultured with EPCAM (CD326+) cells in a ratio of 10:1, which was determined and optimised in pilot studies based on previously published data. Mixed cells were combined with Matrigel (laminin, coll-iv, entactin) in a 1:1 ratio and cultured on a transwell insert in 24-well plates and incubated at liquid-air interface in MTEC plus media (Dulbeccos Modified Eagle Medium-Ham’s F-12, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), penicillin and streptomycin, fungizone, insulin, transferrin, cholera toxin, epithelial growth factor (EGF) and bovine pituitary extract). Organoid cultures were grown for 21 days and then fixed for immunofluorescence analysis. Murine organoids were generated by a pool of three to six mice for independent experiments, resulting in three to six technical replicates, and repeated more than three times. Human organoids were generated from two donors (>50 years): five patients with IPF and one patient with I LD (51–70 years). It is important to note that for RA-treated cells, RA was not used in the organoid cultures but was administered to the mice 2 weeks before PNX surgery and cell harvest to generate organoids.

RESULTS
To identify the role of resident PDGFRA+ interstitial fibroblasts in repair of the aged lung, we used the murine PNX model and compared alveolar regeneration in mice aged 12–16 weeks (hereafter referred to as ‘young mice’) with mice aged >40 weeks (hereafter referred to as ‘aged mice’). Consistent with previous studies, regeneration in aged mice is decreased compared alveolar regeneration in young mice (hereafter referred to as ‘young mice’) with mice aged >40 weeks (hereafter referred to as ‘aged mice’). We have previously shown that myofibroblast and matrix fibroblast orchestrate alveolar regeneration in young mice and that myofibroblast co-expressed PDGFRA (CD140) and CD29, whereas
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matrix fibroblast co-expressed PDGFRα (CD140) and CD34.15 Young mice actively regenerate their lung after PNX, with increased proliferation of CD326+ epithelial cells compared with young sham mice. While epithelial proliferation (CD326) is not changed in aged mice after PNX, proliferation of CD140/CD34 matrix fibroblast is reduced in aged mice compared with young mice (p=0.011). However, there was no significant difference in proliferation in CD140/CD34 matrix fibroblasts between regenerating young and regenerating RA pretreatment of aged mice (p>0.99), suggesting a restored proliferation of CD34- positive fibroblasts (figure 1C–E). Overall, the number of PDGFRA-expressing cells was similar between young and aged lungs. These data suggest that matrix fibroblast proliferation plays an important role in alveolar repair.

RNA-seq expression data, both PDGFRα and PDGFA were significantly upregulated in fibroblasts and epithelial cells in the lungs of RA-pretreated mice compared with aged lungs. Changes in gene expression patterns between PDGFRα+ fibroblasts and epithelial cells were strikingly different. In aged PDGFRα+ fibroblasts, a large subset of genes are altered by PNX, while RA pretreatment prevented these changes (online supplemental figure 1A). In contrast, in the epithelial cells, RA pretreatment induced a large subset of genes that were not changed by PNX (online supplemental figure 1B). These data suggest that PNX in aged lungs induces ‘misdirected’ gene activation and inactivation of PDGFRα+ fibroblasts that result in failure to regenerate. RA prevents these ‘misdirected’ gene changes, allowing regeneration. In the epithelium of aged lungs, PNX does not induce gene changes, but RA pretreatment promotes gene changes that result in regeneration.

Functional enrichment analysis of the ‘misdirected’ gene changes in PDGFRα+ fibroblasts shows that RA pretreatment prevented the induction of genes associated with apoptosis,
chromatin remodelling and inflammation while simultaneously preventing the loss of genes associated with matrix organisation, mesenchymal cell differentiation, lung development, stem cell differentiation and regulation of epithelial cell proliferation (online supplemental figure 1C). In contrast, functional enrichment analysis of the gene changes in epithelial cells shows that RA pretreatment inhibited the expression of genes associated with inflammation and cytokine production, while genes associated with epithelial development, branching, proliferation and differentiation were induced (online supplemental figure 1D). These data demonstrate that in both PDGFRα+ fibroblasts and epithelial cells, inflammation suppression coupled with induction of cell-type-specific differentiation is required to allow for alveolar regeneration.

To identify gene networks associated with alveolar regeneration in PDGFRα+ fibroblasts, upstream driver analysis was performed using the gene changes observed between RA pretreatment and aged PDGFRα+ fibroblasts (online supplemental table 3). Active PDGFA signalling and inactive PDGF-BB signalling were predicted as important regulators of gene changes that allow realveolarisation (figure 1F). Similarly, in young mice compared with aged mice (online supplemental table 4), inactivation of PDGF-BB signalling and alteration of PDGFA-AA were predicted to be primary mediators of the alveolar regeneration process (figure 1G). Both PDGFA and PDGFB are expressed in the respiratory epithelium, suggesting paracrine signalling from the epithelium to the resident interstitial fibroblasts. RNA-Seq analysis of EPCAM-sorted cells identified a significant 5.9-fold increase in PDGFA expression in aged PNX+RA compared with aged PNX. RT-PCR was used to assess levels of Pdgfa in epithelial cells isolated from sham, young, and aged RA pretreatment lungs but showed no statistical difference due to small sample size (online supplemental tables 1 and 2; online supplemental figure 1E). Taken together, these data suggest that increased reciprocal PDGFA signalling is essential for alveolar regeneration after PNX.

To test the role of PDGFA signalling in supporting alveolar regeneration and epithelial differentiation, an epithelial–mesenchymal mixed cell organoid system was used. Lung organoids were generated by combining primary EPCAM+ lung epithelial cells with primary PDGFRα+ fibroblasts isolated from young, aged and RA-pretreated murine lungs. Alveolar epithelial cell differentiation was assessed by immunofluorescence staining with SFTPc (AT2 cells), HOPX and AGER (AT1 cells) and quantified by morphometry (figure 2A,B). SFTPc, AGER and HOPX expression in primary lung epithelial cells from aged mice was increased when co-cultured in organoids with young or RA-pretreated mouse lung PDGFRα+ fibroblasts, compared with organoids co-cultured with PDGFRα+ fibroblasts from aged mice. Independent of age and RA pretreatment, epithelial cells showed no difference in AT2/AT1 differentiation when cultured with young PDGFRα+ fibroblasts. While RNA-Seq demonstrated that epithelial cells from young and RA-pretreated mice expressed higher levels of Pdgfa, their ability to differentiate into AT2 or AT1 cells was impaired when cultured with aged PDGFRα+ fibroblasts. When organoid cultures from aged PDGFRα+ fibroblasts were supplemented with PDGFA ligand, epithelial AT2/AT1 differentiation was restored as the expression of AT1 cell markers, HOPX and AGER, was increased (figure 2B). Taken together, these data demonstrate that the ‘aged’ PDGFRα+ cells have lost the ability to promote epithelial differentiation in organoid culture and that ‘aged’ epithelial cells still retain the potential to differentiate into AT2/AT1 cells.

IMR90 human fetal lung fibroblasts express PDGFRα. To determine the role of epithelial PDGFA-positive paracrine feedback loop on Pdgfa expression and matrix fibroblast differentiation, IMR90 cells were treated with PDGFA ligand. PDGFA treatment increased the expression of CD248, WNT5A, FGF1 and PDGFRα, previously identified matrix fibroblast signature genes, and reduced the expression of WNT2 and ACTA2, previously identified myofibroblast signature genes (figure 2C). These data support the concept that epithelial PDGFA signalling induces a positive paracrine feedback loop with PDGFRα+ fibroblasts and promotes matrix fibroblast differentiation, which in turn support epithelial AT2/AT1 differentiation.

IPF is associated with advanced age, failure of alveolar repair and impaired AT1 differentiation. Based on the role of PDGFRα+ fibroblast in AT1 differentiation, we therefore examined PDGFA signalling in IPF. Single-cell analyses of the IPF and donor epithelium identified subsets of epithelial cells that produce high levels of PDGFB and PDGFA, with PDGFB signalling only being detected in IPF epithelial cells.6 Due to altered PDGFA expression in IPF lungs, we hypothesised that the interstitial lung fibroblast population would be altered in IPF lungs. To assess the fibroblast populations’ composition changes in patients with IPF, peripheral tissue of age-matched donor lungs and non-fibrotic peripheral areas of IPF lungs were digested into single-cell suspensions and subjected to flow cytometry. Flow cytometry revealed a loss of 90% of CD140+ matrix fibroblasts in IPF (online supplemental figure 2A). RNA-Seq of CD140+ fibroblasts from non-fibrotic areas of IPF lungs revealed changes in gene expression related to ‘extracellular matrix organisation’, ‘response to wounding’ and altered ‘chromatin assembly’, when compared with donor lung CD140+ fibroblasts (online supplemental figure 2B,C and online supplemental table 5). Furthermore, upstream regulator analysis of differentially expressed genes in IPF-derived CD140+ fibroblasts predicted the activation of PDGFB-BB ligand homodimer signalling and reduced PDGFAA ligand homodimer signalling in IPF, which is consistent with the finding in aged PNX-injured mouse lungs (online supplemental figure 2D). Together, these data suggest a loss of normal interstitial matrix fibroblast as an additional pathological feature of IPF.

Recent proteomics analysis of young and aged murine lungs33 described downregulation of collagen XIV, which integrates collagen bundles by binding to collagen I fibrils and decorin.34 Changes in extracellular matrix composition and distribution have been previously suggested in aged and IPF lungs.35 To assess whether the loss of matrix fibroblast in non-fibrotic areas of the IPF lungs also results in changes in the extracellular matrix, we performed SHG resonance imaging to visualise collagen structures and immunofluorescent analysis of smooth muscle actin and AT2 cell distribution in alveolar regions of normal (n=3) and IPF (n=3) lungs (figure 3A). Normal lungs showed an intricate collagen network spanning the lung parenchyma and supporting alveolar structures.

We determined non-fibrotic areas with apparently normal alveolar structures in IPF lungs by H&E and performed SHG imaging on adjacent sections (online supplemental figure 2). SHG revealed that the intracollagen collagen structure found in donor lungs was amorphous, suggesting impaired support of alveolar structures. Dense collagen structures and considerable smooth muscle actin replace alveolar structures in fibrotic foci (figure 3A). These data suggest that the loss of matrix fibroblast function in the IPF lung contributes to destruction of the collagen network, revealing a possible underlying early pathological change within the extracellular matrix in IPF lungs.
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IPF is characterised by the loss of AT1 cells, but it remains unclear whether this is due to a defect of AT2 cells to differentiate and replenish chronically stressed AT1 cells. Our data showed the loss of matrix fibroblasts in IPF and suggest that matrix fibroblasts are required for AT1 differentiation (figure 3 and online supplemental figure 2). To test the hypothesis that

Figure 2  RA pretreatment and PDGFA treatment support alveolar differentiation in mouse lung organoids. (A) Immunofluorescence analysis of SFTPC, HOPX and AGER in mouse lung organoids generated with young epithelium and aged PNX fibroblast or aged epithelial and aged fibroblast treated with PDGFA after 21 days in culture. (B) Quantification of total area masked by staining compared with total area of DAPI (AGER) or total cell counts as a percentage of total DAPI (HOPX and SFTPC) for the different combinations of mouse lung organoids generated demonstrates increased AGER and HOPX expression in organoids generated with young or aged RA-pretreated fibroblast and PDGFA-treated organoids generated with aged fibroblasts. SFTPC expression was increased in organoids generated with RA-pretreated aged fibroblasts. *** indicates p<0.0001 compared with organoids generated with aged fibroblasts. (C) Quantification of RNA expression changes from IMR90 cells treated with PDGFA ligand demonstrated increased matrix fibroblast differentiation. PDGFA, platelet-derived growth factor subunit A; PNX, partial pneumonectomy; RA, retinoic acid.

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loss of AT1 cells in IPF is due, at least in part, to loss of matrix fibroblasts, we assessed AT1 cell differentiation in human/mouse organoids. Organoids were generated by combining human CD326+ epithelial cells isolated from normal donor and IPF lungs with murine young, aged or RA-pretreated PDGFRα+ fibroblasts. We assessed AT2/AT1 differentiation in human donor or IPF epithelial cells with immunofluorescence analysis of HOPX, AGER and SFTPC. While characteristic morphological features of AT2 and AT1 cells were not readily visible, protein expression of cell-type-specific markers was discernible. Only young and RA-pretreated fibroblasts supported AT2/AT1 differentiation in organoids generated from both donor and IPF epithelial cells (figure 3B, D). Organoids generated with IPF CD326+ cells expressed reduced AGER, HOPX and SFTPC.

Figure 3  IPF lungs contain abnormal fibroblast populations. (A) SHG resonance imaging (green) showing the structure of collagen in donor, ‘less severe’ region of IPF and ‘severe’ region of IPF lungs co-stained with immunofluorescence labelling of ABCA3+ AT2 cells (red) and αSMA+ smooth muscle (white), in 250 µm thick lung sections. Scale bars indicate 50 µm. RA pretreatment and PDGFA treatment enhance the expression of alveolar epithelial cell differentiation in human lung organoids. (B–C) Immunofluorescence analysis of AGER, HOPX and SFTPC demonstrates increased expression of IPF epithelial cells cultured with aged mouse fibroblasts or treated with PDGFA. (D) Quantification of area imaged with AGER expression as normalised to total DAPI area. HOPX-positive and SFTPC-positive cells are normalised to total DAPI nuclei. Organoids generated with IPF epithelium and young mouse fibroblasts expressed less SFTPC (p=0.0003), AGER (p=0.007) and HOPX (p=0.031) compared with organoids generated with normal donor epithelial cells. *** denotes p<0.0001 compared with organoids generated with aged fibroblasts. IPF, idiopathic pulmonary fibrosis; PDGFA, platelet-derived growth factor subunit A; RA, retinoic acid; SHG, second harmonic generation.

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compared with organoids generated with donor CD326+ cells co-cultured with young CD140+ cells. Other epithelial markers (P63, MUC5B, acetylated tubulin and CCSP) were comparable among all combinations of fibroblast and epithelial cells, indicating there was no shift in the bronchoalveolar/bronchial organoid ratio (online supplemental figure 4).

PDGFA treatment of organoids generated from aged murine fibroblasts and human IPF epithelial cells enhanced AT1 (HOPX and AGER) and AT2 (SFTPC) cell differentiation to similar levels as organoids generated with RA-pretreated or young fibroblasts (figure 3C,D). These data suggest that epithelial cells from patients with IPF have not entirely lost the ability to differentiate into AT1 cells and that fibroblasts with active PDGFA/PDGFRA signalling are required for AT1 cell differentiation in IPF.

To further test the importance of PDGFA signalling, aged mouse epithelial cells were co-cultured with young, aged or RA-pretreated PDGFRA+ fibroblasts and either treated with PDGFA-neutralising antibody to directly inhibit PDGFA activity or treated with nintedanib, a pan tyrosine kinase inhibitor, to block other aberrant epithelial signalling pathways (mTOR, YAP, TGFβ) identified in IPF.4 38 Several recent clinical trials (phases 2 and 3) have continued along this path, working to block other aberrant epithelial signalling pathways (mTOR, YAP, TGFβ) identified in IPF.4 38 However, perhaps a return to the molecular and cellular aspect of the diseased fibroblast would promote the identification of new targetable treatment options.39 Fibroblasts support alveolar regeneration, and previous findings demonstrated RA pretreatment enhanced alveolar regeneration in animal models,26 27 but RA treatment did not enhance alveolar repair in clinical trials treating COPD.46 Similarly, direct treatment of mouse organoids with RA, by Ng-Bleichfeldt et al, resulted in smaller organoid size with reduced epithelial cell differentiation, whereas inhibition of RA increased organoid size and increased epithelial cell proliferation via activation of YAP and FGF signalling.47 In our study, organoid cultures were not treated with RA, but mice were administered RA 2 weeks prior to PNX surgery and cell harvest. Together, these data suggest RA has a positive preconditioning effect but is inhibitory during active regeneration. RA treatment of patients with IPF would be

**DISCUSSION**

**Potential therapeutic advantage of PDGFA ligand treatment**

The current focus of IPF research has revolved around correcting alveolar regeneration caused by chronic epithelial cell injury which is believed to be an underlying cause of the disease. The US Food and Drug Administration (FDA) has approved two drugs, pirfenidone and nintedanib, a broad tyrosine kinase inhibitor that acts on several targets including PDGFA and PDGFBR. Thus far, these FDA-approved treatments slow loss of forced vital capacity but have not significantly enhanced life expectancy following diagnosis.36 37 Several recent clinical trials (phases 2 and 3) have continued along this path, working to block other aberrant epithelial signalling pathways (mTOR, YAP, TGFβ) identified in IPF.4 38 However, perhaps a return to the molecular and cellular aspect of the diseased fibroblast would promote the identification of new targetable treatment options.39 Fibroblasts support alveolar regeneration, and previous findings demonstrated RA pretreatment enhanced alveolar regeneration in animal models,26 27 but RA treatment did not enhance alveolar repair in clinical trials treating COPD.46 Similarly, direct treatment of mouse organoids with RA, by Ng-Bleichfeldt et al, resulted in smaller organoid size with reduced epithelial cell differentiation, whereas inhibition of RA increased organoid size and increased epithelial cell proliferation via activation of YAP and FGF signalling.47 In our study, organoid cultures were not treated with RA, but mice were administered RA 2 weeks prior to PNX surgery and cell harvest. Together, these data suggest RA has a positive preconditioning effect but is inhibitory during active regeneration. RA treatment of patients with IPF would
be after disease diagnosis and onset; thus, it is very unlikely to be effective. RA is an epigenetic modulating agent that is extensively used in reprogramming fibroblast to induced pluripotent stem cells. With advanced age, epigenetic modifications increase. Understanding the cellular and molecular mechanisms of induction of RA-mediated regeneration in aged lungs could be central for effective translation into elderly patients with lung disease and may reveal novel insights into the pathogenesis of alveolar disease and senescence. In this study, we used RA pretreatment prior to PNX injury to understand the downstream mechanisms that result in regenerative activation of fibroblasts, leading to alveolar repair with advanced age.

A large number of gain-of-function and loss-of-function mutations in PDGF and PDGFR genes have been generated and demonstrated that the PDGF/PDGFR plays important roles in the development and that PDGF overexpression or misexpression may induce pathological responses. While loss of PDGFA signalling during lung development resulted in failure to form secondary septa, misexpression of PDGFA in the pulmonary epithelium resulted in overproliferation of mesenchymal cells compromising the expansion of distal airspaces. PDGFA and PDGFC are potent inducers of mesenchymal proliferation, whereas PDGFB is more pleiotropic and induces an inflammatory response. PDGFA binds PDGFRα, which is mainly expressed in mesenchymal cells in the lung and has been detected in subtypes of mesenchymal progenitors in lung, skin and intestine, in a homodimer or PDGFA/PDGFB heterodimer. PDGFRβ is expressed in mesenchyme, particularly in vascular smooth muscle cells and pericytes. Pulmonary hypertension, lung cancer and lung fibrotic diseases have been linked with elevated PDGF signalling. Murine models of lung fibrosis indicated elevated PDGFB signalling as the major source of inflammation and fibrotic response with the role of PDGFA signalling not been extensively studied.

Our current findings support the notion that the activation of PDGFA signalling mediated by RA treatment before injury resulted in gene expression changes in fibroblasts that promote epithelial regeneration. In vitro treatment of primary rat lung fibroblasts with RA increased the expression of Pdgfra. We found a similar increase in Pdgfra expression in lung fibroblasts of RA-pretreated mice. We found that the epithelium of RA-pretreated mice produces increased Pdgfa ligand after injury. Bronchoalveolar organoids generated with aged mouse CD140+ fibroblasts fail to differentiate both AT2 and AT1 cells in vitro regardless of the types of epithelial cells used. However, treatment of these organoids with PDGFA ligand supports alveolar epithelial cell marker expression, suggesting PDGFA is sufficient to induce alveolar differentiation. PDGFA ligand appears to activate a cascade of epithelial–mesenchymal crosstalk, likely through enhanced fibroblast differentiation into beneficial matrix fibroblasts, which promotes alveolar regeneration. Further studies identifying the underlying cause of loss of the PDGFRα+ matrix fibroblast population in aged and IPF lungs could provide future therapies for patients with IPF in need of efficacious treatment.

The ageing lung
Age is associated with increased risk of several diseases including IPF. Single-cell RNA-Seq analysis of aged murine lungs revealed altered transcriptional signalling that was associated with epigenetic changes in multiple cell types. RA is known to place or remove epigenetic marks on histones and DNA. In this study, we demonstrate that RA pretreatment prevented many gene expression changes associated with aged fibroblasts and induced epithelial cell gene expression.

It has been previously shown that monocyte recruitment is important for reaerolisation after partial PNX. Based on single-cell RNA-Seq analysis, aged fibroblasts have an increased proinflammatory signature. Functional enrichment analysis in this study shows that RA pretreatment prevented the induction of genes involved in inflammation in both fibroblasts and epithelial cells. Taken together, these data suggest that aged lungs are predisposed to inflammation and that inflammation induced by PNX needs to be controlled to allow regeneration.

This study demonstrates that pulmonary fibroblast plays an important role in alveolar repair, which is lost with age, and highlights the ability of aged epithelium or epithelium from patients with IPF to differentiate into AT1 cells in the presence of regenerative matrix fibroblast. Our flow cytometry and RNA-Seq data demonstrate that in IPF the PDGFRα+ fibroblast population is reduced and shifted toward myofibroblast differentiation; to our knowledge, similar flow cytometry data have not been published. Single-cell seq data on IPF lungs support the reduced presence and expression of PDGFRα in lung fibroblasts (http://www.1PFCellatlas). Our in vitro data in IMR90 cells demonstrate that PDGFA supplementation shifted the PDGFA-expressing cells towards the matrix functional stage. Similar shift to matrix function has been shown by activation of PDGFA signalling by a constitutive PDGFRα mutation. With age, PDGFA signalling is reduced in mouse epithelium. Direct supplementation of PDGFA in IPF human/mouse organoids can induce IPF epithelial cell AT1 cell differentiation. upstream regulator analysis of disease-related gene changes predicted increased PDGF-BB ligand homodimers and loss of PDGFA ligands in IPF fibroblasts driving disease progression, consistent with previous observations.

In support of its putative role as a driver of IPF pathogenesis, PDGF-BB ligand homodimers increase fibrosis in other injury models, and blocking PDGF-BB ligand homodimers reduces fibrosis in bleomycin-injured mouse lungs. These findings, coupled with our PDGFA-neutralising antibody and nintedanib treatment data, suggest that there is a balance between the fibrotic response induced by PDGFB ligand homodimers and the alveolar repair supported by PDGFA signalling. FDA-approved nintedanib is a broad target tyrosine kinase inhibitor that blocks both PDGFA and PDGFRB signalling cascades. Our data suggest that specific activation of PDGFA ligand and specific inhibition of PDGFB ligand may further enhance alveolar repair, supporting a balance between PDGFA and PDGFB signalling cascades. Future therapies may rely on a combination of blocking aberrant pathways (PDGFRB) while supporting the activity of essential pathways for alveolar repair (PDGFA).

Organoid model system
Explanted lungs are end-stage disease that provide a very valuable source of live disease tissue that can be used in the organoid model to identify molecular events that occur during the pathogenesis of IPF and to test potential therapeutic targets (ie, abnormal AT2–fibroblast crosstalk). We have focused on the fibroblast stage that supports proper AT2/AT1 differentiation in this study. We evaluated primary alveolar epithelial cells and fibroblasts isolated directly from patients with IPF; we studied live cells in an environment permitting them to communicate with and influence each other, and we identified age-dependent differences in promoting AT2/AT1 cell differentiation.
The present RNA analysis demonstrates that epithelial and fibroblast interactions are required for alveolar regeneration. Both human normal donor and IPF CD326+ epithelial cells have the potential to differentiate into AT2 and AT1 cells, although IPF epithelial cells have reduced ability, suggesting the underlying mechanism for repair is still present in the IPF epithelium. This work suggests that the fibroblast activates PDGFA ligand in the epithelium to promote alveolar regeneration, a finding consistent with other reports showing that the fibroblast plays a key role in alveolar differentiation. In previous studies, PDGFA-GFP fluorescent label was used to isolate MANCs, the subset of WNT2+/PDGFA-GFP+ intersitial fibroblasts that interact with alveolar epithelial cells to support repair and development of the alveolar region.1 We have previously published that the PDGFA-GFP and PDGFA expression detected by the CD140 antibody does not perfectly overlap.14 15 In this study, we investigate murine and human PDGFA-expressing cells by isolating them using the PDGFA antibody (CD140) and not the PDGFA-GFP fluorescent label; we also do not separate them by expression of Axin2 and/or Wnt2. We and others have previously demonstrated that these PDGFA+ fibroblasts are a mixed population of three functionally distinct fibroblast stages, myofibroblasts, matrix fibroblasts and lipofibroblasts, with increased population of one or the other subtypes depending on the context of age, regeneration and homeostasis.15 10 11 14 15 20 21 Previoulsy published studies22–24 and our current study demonstrate that age and injury promote a myo subtype and that in IPF the fibroblast function is impaired. Myofibroblasts are characterised by the expression of PDGFA/CD29/aSMA, and matrix fibroblasts are characterised by the expression of PDGFA/CD34/elastin.14 Increased PDGFA signalling promotes myo to matrix transition.14 54 RA pretreatment increased PDGFA and PDGFA expression in lung fibroblasts and AT2 cells, resulting in a positive paracrine feedback loop between fibroblasts and AT2 cells. Organoids made from PDGFA+ fibroblasts isolated from aged mice are predominantly myofibroblasts and support organoid formation lacking alveolar type 1 (AT1) cell marker expression. Organoids made from PDGFA+ fibroblasts isolated from young or RA-pretreated mice are predominantly matrix fibroblasts and induce increased PDGFA expression in co-cultured lung epithelial cells, supporting alveolar organoid formation and AT1 cell differentiation. IPF, idiopathic pulmonary fibrosis; PDGFA, platelet-derived growth factor subunit A; PDGFA, platelet-derived growth factor receptor A; RA, retinoic acid.

**Limitations**

Our studies were limited by sample numbers. Pneumonectomy is a survival surgery; thus, the number of mice used in each study was adjusted to reduce stress on the animals. Access to patient samples and patient diagnosis was limited to IPF for RNA-Seq and to usual interstitial pneumonia/non-specific interstitial pneumonia and IPF for organoid generation. We used CD326+ selection for our epithelial isolation as others and our previous work demonstrate distal lung epithelial cells do not express ‘normal’ AT2 cell markers. We performed CD326+ isolation on murine lungs as well to be consistent between human and mouse.12 57 58 RNA-Seq analysis was unable to assess the signalling mechanism from the mesenchyme to the epithelium that supported AT1 cell expression.

Other study designs and experimental set-ups could overcome this limitation and identify that potential crosstalk mechanisms or alteration of matrix stiffness is possible to support AT1 cell differentiation.7 39 66 Our analysis demonstrates that PDGFA is increased in the epithelium, and addition of PDGFA to the organoid culture system supports matrix fibroblast gene expression. Other cellular compartments as a source of PDGFA such as the endothelium were not investigated in this study.61 62 Due to the simplified structure of organoids resulting in abnormal cell shape or by artefacts of the staining process caused by the presence of Matrigel, some immunohistochemical staining
appears abnormal. For example, AGER is usually expressed in the membrane of AT1 cells, and while the staining of AGER in the organoids appears specific, it is expressed in the cytoplasm of some organoids. We used organoids generated with aged mouse lung epithelium as a negative control to set expression thresholds that detected specific protein expression to determine increased protein expression. Herein, mouse fibroblast–human epithelial cell organoids were used to generate organoids in which the fibroblasts were RA-treated prior to isolation. However, mouse human organoids may not fully recapitulate ageing in humans; therefore, further optimisation of the organoid systems in the future will allow for a more rigorous testing of the role of ageing in humans.

Taken together, our data suggest a model of paracrine signalling leading to alveolar repair in the aged lung, by which RA pretreatment of the fibroblast induces signalling to the epithelium that activates epithelial PDGFA secretion. Production of PDGFA by alveolar epithelial cells then activates pulmonary matrix fibroblast differentiation, which in turn leads to restoration of alveolar differentiation after injury (Figure 5).”

Correction notice This article has been corrected since it was published Online First. A minor modification has been made to the abstract.

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Data availability statement Data are available in a public, open access repository. GEO: GSE157440.

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