Single-cell RNA sequencing identifies G-protein coupled receptor 87 as a basal cell marker expressed in distal honeycomb cysts in idiopathic pulmonary fibrosis

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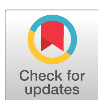
To the Editor:

Idiopathic pulmonary fibrosis (IPF) is a devastating and life-threatening lung disease characterised by epithelial reprogramming and increased extracellular matrix deposition leading to loss of lung function. Prominent histopathological structures in the distal IPF lung include honeycomb cysts in the alveolar space [1]. These are heterogeneous bronchiolised areas that feature clusters of simple epithelium with keratin (KRT)5^{+} basal-like cells interspersed with pseudostratified epithelium containing differentiated, hyperplastic epithelial cells, as well as aberrant ciliated cells [2–5]. Recent single-cell RNA sequencing studies of whole lungs from IPF and donor tissue revealed cellular subtypes unique to IPF, including basaloid KRT5^{-}/KRT17^{+} cells present in the distal lung [6–10]. However, IPF distal bronchiole KRT5^{+} basal cell subtypes still remain poorly characterised and their disease contribution remains under-investigated. Here, we report *G-protein coupled receptor (GPR) 87* as a marker of distal bronchioles and KRT5^{+} basal-like cells in IPF. We generated single cell transcriptomes from EpCAM^{+} cells isolated from parenchymal lung tissue from three IPF patients and three age-matched healthy donors. In short, fresh non-fixed human lung tissue from de-identified healthy donors and explants from IPF patients with end-stage disease was received from National Jewish Hospital/UC Health University of Colorado Hospital (Denver, CO, USA) (COMIRB 11–1664). Right lower or middle lobes of healthy donor (n=3, two males both aged 66 years, and a 68-year-old female) and IPF patient tissue (n=3, two males aged 45 and 65 years, and a 68-year-old female), respectively, were used. All tissues were obtained from non-smokers. Human lung tissue was homogenised and tissue was digested by dispase/collagenase (collagenase: 0.1 U·mL^{-1}; dispase: 0.8 U·mL^{-1}; Roche). Samples were successively filtered through nylon filters (100 µm and 20 µm) followed by a percoll gradient and CD45 MACS sorting (Miltenyi Biotec). After FACS, EpCAM^{+}/DAPI^{-} live single epithelial cell suspensions were used for single-cell RNA sequencing (scRNAseq). Detailed single cell methodology and data processing and analysis is reported in the GitHub repository (https://github.com/KonigshoffLab/GPR87_IPF_2022). The raw data have been deposited in NCBI's Gene Expression Omnibus with accession number GSE190889. Using the 10x Genomics platform, we generated a dataset of 46 199 cells and found nine distinct cell clusters, including main progenitor cell types of the alveolar region and distal airways as well as rare cell types, such as suprabasal cells, recently reported in the healthy lung (figure 1a) [11]. Cells from both conditions were found in all clusters with differentially distributed clusters between healthy and IPF (figure 1b). In line with previous single cell data [6–8], ciliated cells were predominantly found in IPF while ATII cells were largely present in non-diseased lungs, further suggesting a loss of ATII cells and distal bronchiolisation in IPF. Honeycomb cysts are an important histopathological criteria for the diagnosis of IPF; however, mechanistic insight in the process of bronchiolisation and remodelling of the terminal bronchiole in IPF remains scarce. To shed light into cell populations potentially contributing to honeycomb cysts, we analysed differentially expressed genes in all epithelial clusters and found cytokeratins such as *KRT6A*, *KRT5*, *KRT17*, and *KRT15* among the most upregulated genes in IPF (figure 1c). KRT5 is a well-characterised marker of basal and suprabasal cells, and KRT5^{+} cells strongly accumulate in distal IPF lung tissues, mostly in areas of honeycombing [3, 4, 12]. To further identify cellular surface markers and potential pharmacological targets that might be expressed in KRT5^{+} cells, we analysed transmembrane signalling receptors (GO:0004888) in all epithelial cells and found *GPR87*, a G-protein coupled receptor with unknown function in IPF, to be



Shareable abstract (@ERSpublications)
Bronchiolisation and honeycombing are features of IPF. ScRNA sequencing identified GPR87 as a novel marker of basal cells in IPF, enriched in honeycomb cysts. GPR87 overexpression resulted in aberrant airway cell differentiation. https://bit.ly/3i4dXeT

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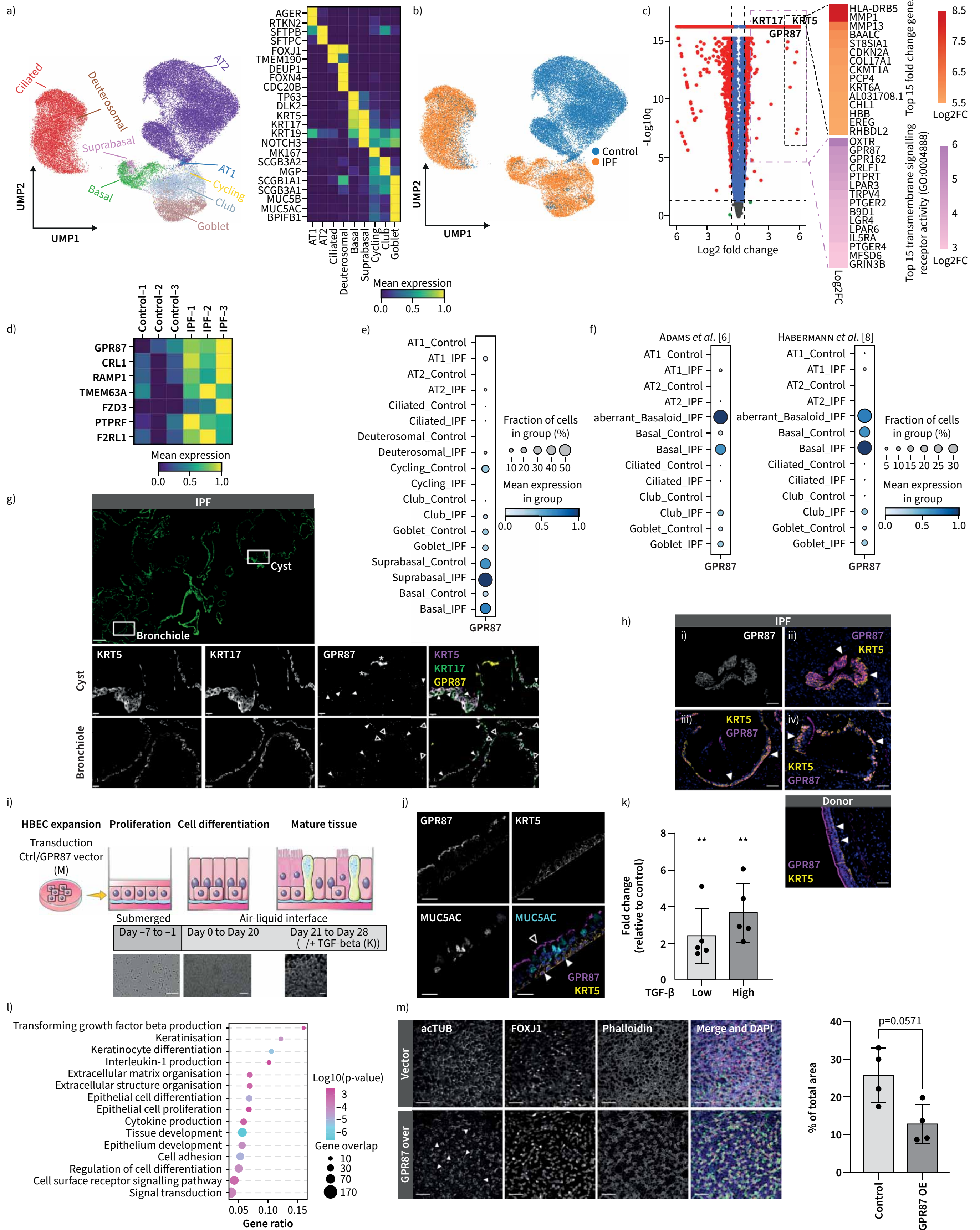

FIGURE 1 Expression of GPR87 in accumulated basal progenitor cells within the idiopathic pulmonary fibrosis (IPF) lung. a) Uniform manifold approximation and projection (UMAP) visualisation shows unsupervised transcriptome clustering, revealing nine distinct cell clusters. Heatmap shows the highest expressed marker genes of each cluster. b) UMAP visualisation showing distribution of healthy donor and IPF cells to different clusters. c) Volcano plot of differentially expressed genes (red, log2 fold change (FC) >0.6, *q*<0.05) in IPF EpCAM$^+$ epithelial cells compared with donor samples, zooming in gene sets with top-15 fold change and top-15 fold change genes related to transmembrane signalling receptor activity (GO:0004888). d) Heatmap of transmembrane signalling receptor genes robustly regulated in the (supra)basal cell population across all individual tissue samples. Dotplots show GPR87 expression in our (e) and another two publicly available datasets [6, 8] (f), respectively. g) GPR87 mRNA was detected by RNAscope (Advanced Cell Diagnostics, number 471861) and combined with fluorescent immunolabelling of basal cell markers KRT5 and KRT17 in IPF lung sections (n=6) (KRT5: Biolegend 905901; KRT17: Abcam ab51056) Top row: lower magnification of a distal lung area with several remodelled airways (scale bar: 200 µm). Higher magnification of a representative cyst and a bronchiole are presented below in higher magnification (scale bar: 20 µm). KRT5$^+$/KRT17$^+$/*GPR87*$^+$ cells are indicated by arrowheads. KRT5$^−$/KRT17$^+$/*GPR87*$^+$ cells are indicated by open triangles. Non-cellular staining is marked by an asterisk. h) Lung tissue sections of IPF (n=3) and healthy donor (n=2) were co-immunolabelled for GPR87 (Novus Biologicals NBP2-16728) and KRT5. Nuclei are visualised by DAPI staining. Protein expression of GPR87 alone is shown in subpanel (i) and co-immunolabeled with KRT5 in (ii). Two more representative areas of remodelled airways with merged protein expression are shown in (iii) and (iv). Representative double positive cells for respective markers are indicated by arrowheads. Scale bar: 50 µm. i) Scheme of primary human bronchial epithelial cell (HBEC) isolation and air–liquid interface (ALI) culture (reproduced from Servier Medical Art (smart.servier.com) with permission). HBECs were isolated from healthy donors (n=3) and cultured on rat-tail collagen type I under submerged conditions, either transduced with lentivirus (empty vector (Origene, PS100092) or human GPR87 ORF (Origene, RC218486L3)) (m), and/or directly transferred and cultured on collagen type IV membranes, airlifted (day 0) and differentiated to a mature epithelium within 21 days. Transforming growth factor (TGF)-β treatment (R&D, 240-B-002, 2 or 4 ng·mL$^{−1}$) was performed at day 21 and every other day till day 28 (four times in total) (k). Shown are phase contrast images for dish cultured cells and early ALI (left, middle; scale bars: 250 µm, 100 µm), and a confocal image of acetylated tubulin (acTub) (Abcam ab24610) to visualise late ALI (mature epithelium, right; scale bar: 25 µm). j) Vertical membrane sections of mature ALI cultured HBECs were immunolabeled for GPR87, basal cell marker KRT5 and secretory cell marker MUC5AC (Abcam ab3649) (n=2). Representative double positive cells for respective markers are indicated by arrowheads. Scale bar: 25 µm. (We also observed cilia staining, as indicated by open triangles, and based on our single cell dataset ciliated cells might also express GPR87.) k) Airlifted donor HBECs were stimulated with low (2 ng·mL$^{−1}$) and high (4 ng·mL$^{−1}$) concentrations of TGF-β, as described in (i). GPR87 gene expression was assessed by qPCR in five independent donor cell lines. GAPDH was used as an housekeeper gene control (huGPR87-fw (ACCTATGCTGAACCCACGC), -re (CCGTGCAGCTCGTTATTTGG); huGAPDH-fw (ACTAGGCGCTCACTGTTCTC), -re (AATACGACCAAATCCGTTGACTC)). Two-tailed Mann–Whitney test was performed to determine statistical significance. **: p<0.01. n=5. l) Functional annotation enrichment analysis of GPR87 positive correlated genes reveals several categories of airway remodelling. m) HBECs were transduced with lentivirus containing the full ORF of GPR87 to generate a stable overexpression of GPR87 (GPR87-over). Empty backbone-vector alone was used as a control (vector). Cells were cultured on ALI till day 21 and co-immunolabelled for acTub and FOXJ1 (Invitrogen 14-9965-82). DAPI and phalloidin stainings were performed to visualise nuclei and cellular integrity. Cells with no/shortened cilia are indicated by arrowheads. Representative images of n=4 are shown. Scale bar: 25 µm. Areas covered by cilia were quantified with ImageJ [17]. Two-tailed Mann–Whitney test was performed to determine statistical significance.

one of the highest regulated transcripts (figure 1c). Importantly, when we analysed transmembrane signalling receptors specifically in the (supra) basal cell population across individual tissue samples, we observed a strong and robust increase of *GPR87* (figure 1d). A limitation of our scRNASeq dataset is the small sample size used for scRNASeq (n=3 each); thus, we further confirmed upregulation of *GPR87* in (supra) basal cells in comparison to other epithelial cells not only in our own (figure 1e) but in two additional independently published datasets (figure 1f) [6, 8]. Notably, GPR87 showed further enrichment in basaloid KRT5$^−$/KRT17$^+$ cells, a cell type which we did not detect in our dataset (figure 1f).

We focused on GPR87 for our subsequent studies for several reasons: First, it belongs to the class of G-protein coupled receptors, which are intensively studied drug targets with attractive pharmacological accessibility. Second, although classified as an orphan receptor, profibrotic ligands have been discussed, such as lysophosphatidic acid [13]. Third, GPR87 has been linked to aberrant cell cycle control [14, 15], which is a feature of epithelial reprogramming and bronchiolisation/honeycomb cyst development in IPF [1]. Thus, we aimed to investigate GPR87 expression within the distal IPF lung and its potential contribution to airway cell differentiation and bronchiolisation in IPF.

We confirmed GPR87 epithelial cell expression and distribution within the IPF lung *in situ* using fluorescent immunolabelling and RNAscope of human tissue section as previously described [4, 16]. RNAscope detected *GPR87* RNA in KRT5$^+$ cells in areas of bronchiolisation and honeycomb cysts in distal IPF tissue sections, respectively (figure 1g; arrowheads). The *GPR87* RNA was also found in KRT5$^−$/KRT17$^+$ cells (figure 1g; open triangles). In addition, GPR87 protein was observed in clusters of KRT5$^+$ basal cells in IPF lungs as well as in some KRT5$^+$ cells in non-diseased lungs (figure 1h; arrowheads). GPR87 function was further investigated in an air–liquid interface (ALI) cell culture model of primary human bronchial epithelial cells (HBECs), mimicking *in vivo*-like differentiation of basal cells to

more mature cell types, including ciliated and secretory cells (figure 1i) [4, 16]. GPR87 was expressed in KRT5$^+$ basal cells of our human ALI culture (figure 1j). Transforming growth factor (TGF)-β treatment, inducing fibrotic epithelial reprogramming, led to increased *GPR87* expression in mature ALI cultures (figure 1k). This was consistent with the functional annotation enrichment analysis of our scRNAseq data, which revealed tissue development, keratinocyte differentiation and extracellular matrix remodelling, as well as TGF-β production; all indicative of altered epithelial airway differentiation and integrity, to be correlated with GPR87 (figure 1l). Moreover, GPR87 overexpressing HBECs cultured at ALI displayed impaired differentiation of KRT5$^+$ cells into mature airway cells evidenced by altered epithelial structure and a decrease in cilia coverage (mean±SD 27.65±6.21% for the control, compared to 12.90±4.47% for the GPR87 overexpression) (figure 1m).

Our data suggest that overexpression of GPR87 leads to impaired airway cell differentiation of KRT5$^+$ basal cells, and thus support the hypothesis that GPR87 might contribute to bronchiolisation and honeycomb cyst formation. It will be important to further study the functional consequences of GPR87 expression in basal cells *in vivo* and to analyse whether inhibition of GPR87 would be able to revert impaired airway cell differentiation and prevent TGF-β induced fibrotic reprogramming, thus serving as a potential therapeutic target.

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