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Early View

Research letter

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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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Idiopathic Pulmonary Fibrosis (IPF) is a devastating and life-threatening lung disease characterized by epithelial reprogramming and increased extracellular matrix deposition leading to loss of lung function. A prominent histopathological structure in the distal IPF lung includes honeycomb cysts in the alveolar space [1]. These are heterogeneous bronchiolized 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 the lung epithelium shed further light into 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 characterized 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 patient 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, age and sex (m,f): 66 (m), 66 (m), 68 (f) years) and IPF patient tissue (n = 3, age and sex (m,f): 45 (m), 64 (m), 68 (f) years), respectively, were used. All tissues were obtained from non-smokers. Human lung tissue was homogenized and 4 g of tissue were digested by dispase/collagenase (Collagenase: 0.1U/mL, Dispase: 0.8U/mL, 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 9 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 [11] (A). Cells from both conditions were found in all clusters with differentially distributed clusters between healthy and IPF (B). 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 bronchiolization in IPF. Honeycomb cysts are an important histopathological criteria for the diagnosis of IPF, however, mechanistic insight in the process of bronchiolization and remodeling of the terminal bronchiole in IPF, remains scarce. To shed light into cell populations potentially contributing to honeycomb cysts, we analyzed differentially expressed genes in all epithelial clusters and found cytokeratins such as *KRT6A*, *KRT5, KRT17,* and *KRT15* among the most upregulated genes in IPF (C). KRT5 is a well-characterized 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 analyzed transmembrane signaling receptors (GO:0004888) in all epithelial cells and found *GPR87*, a G-protein coupled receptor with unknown function in IPF, to be one of the highest regulated transcripts (C). Importantly, when we analyzed transmembrane signaling receptors specifically in the (supra) basal cell population across individual tissue samples, we observed a strong and robust increase of *GPR87* (D). 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 cell epithelial cells not only in our own (E) but in two additional independently published datasets [6, 8] (F). Notably, GPR87 showed further enrichment in basaloid cells, a cell type, which we did not detect in our dataset (F).

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 bronchiolization/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 bronchiolization in IPF.

We confirmed GPR87 epithelial cell expression and distribution within the IPF lung *in situ* using fluorescent immunolabeling and RNAscope of human tissue section as previously described [4, 16]. RNAscope detected *GPR87* RNA in $KRT5^+$ cells in areas of bronchiolization and honeycomb cysts in distal IPF tissue sections, respectively (G, arrowheads). The *GPR87* RNA was also found in $KRT17^+$ $KRT5^-$ cells (G, 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 (arrowheads) (H). 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 (I) [4, 16]. GPR87 was expressed in $KRT5^+$ basal cells of our human ALI culture (J). TGF-β treatment, inducing fibrotic epithelial reprogramming, led to increased *GPR87* expression in mature ALI cultures (K). This was consistent with the functional annotation enrichment analysis of our scRNAseq data, which revealed tissue development, keratinocyte differentiation, extracellular matrix remodeling, as well as TGF-β production, all indicative of altered epithelial airway differentiation and integrity, to be correlated with GPR87 (L). 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 (27.65% ± 6.21% for the control compared to 12.90% ± 4.47% (mean ± SD) for the GPR87 overexpression) (M). 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 bronchiolization and honeycomb cyst formation. It will be important to further study the functional consequences of GPR87 expression in basal cells *in vivo* and to analyze 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.

Figure Legends

FIGURE 1 Accumulated basal progenitor cells highly expressing GPR87 localize within the IPF lung. A) Uniform manifold approximation and projection (UMAP) visualization shows unsupervised transcriptome clustering, revealing 9 distinct cell clusters. Heatmap shows the highest expressed marker genes of each cluster. **B)** UMAP visualization showing distribution of healthy donor and IPF cells to different clusters. **C)** Volcano plot of differentially expressed genes (red, log2FC > 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 Signaling Receptor Activity (GO:0004888). **D)** Heatmap of transmembrane signaling 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 visualized by RNAscope (Advanced Cell Diagnostics, # 471861) and combined with fluorescent immunolabeling 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 remodeled airways (bar size: 200 µm). Higher magnification of a representative cyst (C) and a bronchiole (B) are presented below in higher magnification (bar size: 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-immunolabeled for GPR87 (Novus Biologicals NBP2-16728) and KRT5 (see above). Nuclei are visualized by DAPI staining. Protein expression of GPR87 alone is shown in subpanel (a) and co-immunolabeled with KRT5 in (b). Two more representative areas of remodeled airways with merged protein expression are shown in (c) and (d). Representative double positive cells for respective markers are indicated by arrowheads. Bar size: 50 µm; **I)** Scheme of HBEC isolation and ALI culture (reproduced and modified 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. TGF-β treatment (R&D, 240-B-002, 2 or 4 ng/ml) 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; bar sizes: 250 µm, 100 µm), and a confocal image of acTUB to visualize late ALI (mature epithelium, right, bar size: 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. Bar size: 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) and high (4 ng/ml) 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 remodeling. **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 d21 and co-immunolabeled for acetylated tubulin (acTub) (Abcam ab24610) and FOXJ1 (Invitrogen 14-9965-82). DAPI and phalloidin stainings were performed to visualize nuclei and cellular integrity. Cells with no/shortened cilia are indicated by arrowheads. Representative images of an n=4 are shown. Bar size: 25 µm. Areas covered by cilia were quantified with ImageJ [17]. Two-tailed Mann-Whitney Test was performed to determine statistical significance.

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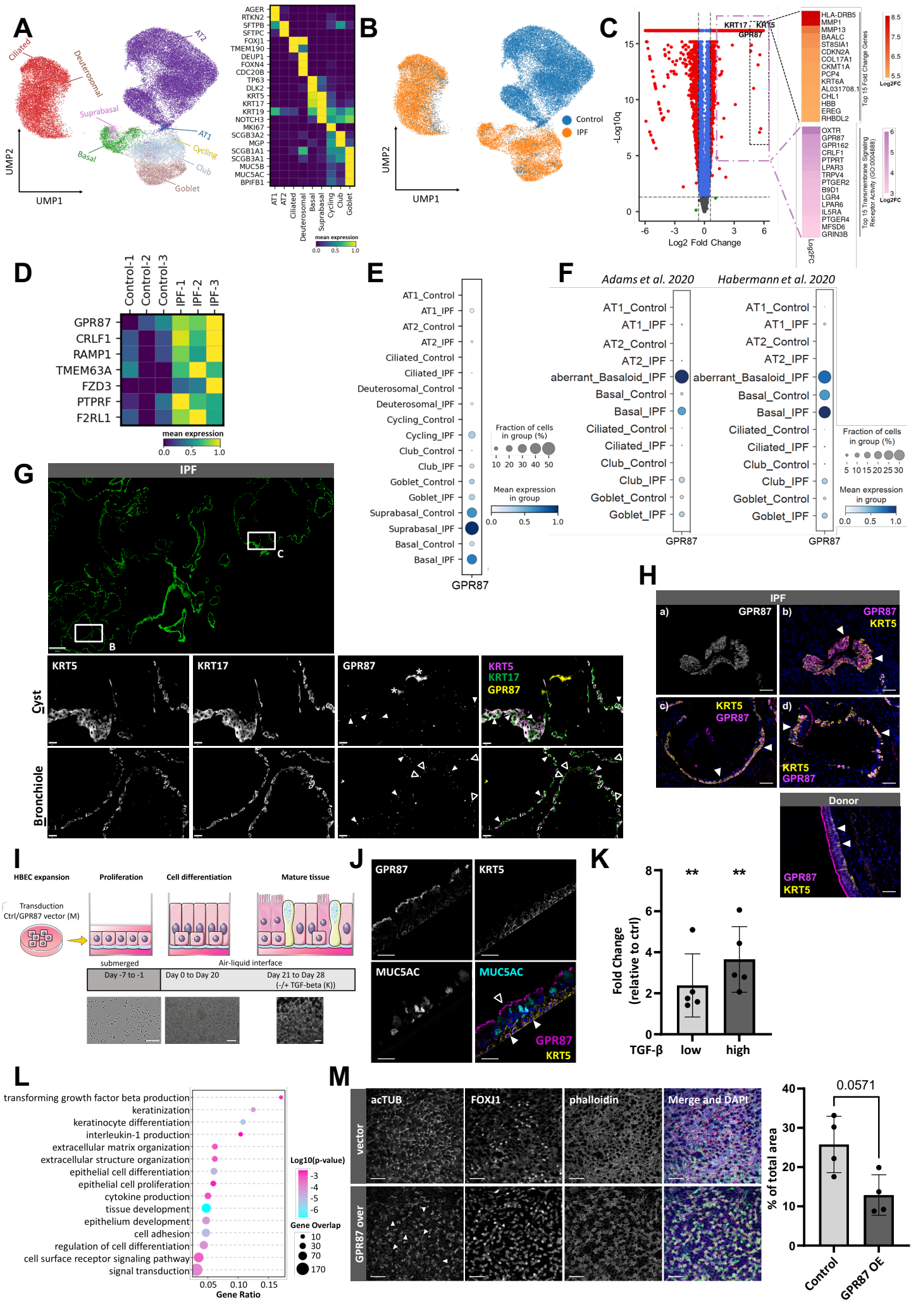
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TMEM190
DEUP1
FOXN4
CDC20B
TP63
DLK2
KRT5
KRT17
KRT19
NOTCH3
MKI67
SCGB3A2
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SCGB1A1
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MMP1
MMP13
BAALC
ST8SIA1
CDKN2A
COL17A1
CKMT1A
PCP4
KRT6A
AL031708.1
CHL1
HBB
EREG
RHBDL2
Top 15 Fold Change Genes
Log2FC
OXTR
GPR87
GPR162
CRLF1
PTPRT
LPAR3
TRPV4
PTGER2
B9D1
LGR4
LPAR6
IL5RA
PTGER4
MFSD6
GRIN3B
Top 15 Transmembrane Signaling Receptor Activity (GO:0004888)
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Control-1
Control-2
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GPR87
CRLF1
RAMP1
TMEM63A
FZD3
PTPRF
F2RL1
mean expression
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AT1_Control
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Ciliated_Control
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Cycling_Control
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Club_Control
Club_IPF
Goblet_Control
Goblet_IPF
Suprabasal_Control
Suprabasal_IPF
Basal_Control
Basal_IPF
GPR87
Fraction of cells in group (%)
10 20 30 40 50
Mean expression in group
0.0 0.5 1.0
F
Adams et al. 2020
Habermann et al. 2020
AT1_Control
AT1_IPF
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AT2_IPF
aberrant_Basaloid_IPF
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Basal_IPF
Ciliated_Control
Ciliated_IPF
Club_Control
Club_IPF
Goblet_Control
Goblet_IPF
GPR87
GPR87
Fraction of cells in group (%)
5 10 15 20 25 30
Mean expression in group
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G
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Cyst
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b)
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c)
KRT5
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d)
KRT5
GPR87
Donor
GPR87
KRT5
I
HBEC expansion
Proliferation
Cell differentiation
Mature tissue
Transduction
Ctrl/GPR87 vector (M)
submerged
Air-liquid interface
Day -7 to -1
Day 0 to Day 20
Day 21 to Day 28
(-/+ TGF-beta (K))
J
GPR87
KRT5
MUC5AC
MUC5AC
GPR87
KRT5
K
Fold Change
(relative to ctrl)
**
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TGF-β
low
high
L
transforming growth factor beta production
keratinization
keratinocyte differentiation
interleukin-1 production
extracellular matrix organization
extracellular structure organization
epithelial cell differentiation
epithelial cell proliferation
cytokine production
tissue development
epithelium development
cell adhesion
regulation of cell differentiation
cell surface receptor signaling pathway
signal transduction
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Log10(p-value)
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-4
-5
-6
Gene Overlap
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30
70
170
M
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GPR87 over
acTUB
FOXJ1
phalloidin
Merge and DAPI
0.0571
% of total area
Control
GPR87 OE