Title

TGF-β activation impairs fibroblast ability to support adult lung epithelial progenitor cell organoid formation

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Running title

Fibroblast TGF-β signaling and lung organoids

Abstract (181 words)

Transforming growth factor-β (TGF-β)-induced fibroblast-to-myofibroblast differentiation contributes to remodeling in chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis, but whether this impacts the ability of fibroblasts to support lung epithelial repair remains little explored. We pre-treated human lung fibroblasts (primary [phFB] or MRC5 cells) with recombinant human TGF-β to induce myofibroblast differentiation, then co-cultured them with adult mouse lung $EpCAM^+$ cells to investigate their capacity to support epithelial organoid formation *in vitro*. While control phFB and MRC5 lung fibroblasts supported organoid formation of mouse $EpCAM^+$ cells; TGF-β-pre-treatment of both phFB and MRC5 impaired organoid-supporting ability. We performed RNA sequencing of TGF-β treated phFB, which revealed altered expression of key Wnt signaling pathway components and Wnt/β-catenin target genes, and modulated expression of secreted factors involved in mesenchymal-epithelial signaling. TGF-β profoundly skewed the transcriptional program induced by the Wnt/β-catenin activator CHIR99021 (CHIR). Supplementing organoid culture media recombinant hepatocyte growth factor (HGF) or fibroblast growth factor 7 (FGF7) promoted organoid formation when using TGF-β pre-treated fibroblasts. In conclusion, TGF-β-induced myofibroblast differentiation results in Wnt/β-catenin pathway skewing, and impairs fibroblast ability to support epithelial repair likely through multiple mechanisms including modulation of secreted growth factors.

Keywords

Lung stem cells, lung regeneration/repair, mesenchymal-epithelial signaling, TGF-β, Wnt/β-catenin signaling

Introduction

Aberrant mesenchymal-epithelial signaling contributes to remodeling and failure of epithelial repair in chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF). Signaling interactions between mesenchymal cells and epithelial progenitors are critical for lung development and adult lung maintenance and repair (27, 66), and lung mesenchymal cells support the regenerative function of adult distal lung epithelial progenitors *in vitro* and *in vivo* (10, 35, 46, 62, 67, 76). Fibroblasts, the major mesenchymal cell type in the adult distal lung, are often situated *in vivo* in direct contact with alveolar type 2 (AT2) cells, progenitors of the gas-exchanging alveolar epithelium (10, 56). The impact of pathological mesenchymal signaling on lung epithelial repair has been little explored.

Fibroblast activation by transforming growth factor (TGF)-β is crucial for normal repair in various adult tissues, whereas excessive TGF-β signaling is a feature of numerous chronic diseases (21). TGF-β is a pleiotropic cytokine that exerts diverse transcriptional effects via interactions with TGF-β receptors type I and II, and subsequent phosphorylation and nuclear translocation of Smad2/3 (39). Acute tissue injury typically induces TGF-β expression, which activates local fibroblasts to synthesize matrix to provide a substrate for re-epithelialization, while inducing a subset of fibroblasts to transition into myofibroblasts, which express contractile proteins that enable force generation and wound closure (21). Whereas in physiological wound repair TGF-β-induced myofibroblasts are cleared via apoptosis, TGF-β activity is elevated in chronic inflammatory diseases, leading to myofibroblast persistence, fibrotic scarring and compromised tissue function (70). TGF-β-induced myofibroblasts contribute to small airways remodeling and airflow obstruction in COPD (31), and are a constituent of fibroblastic foci in IPF (28). COPD and IPF are both characterized by aberrant epithelial repair, which may be due to defective fibroblast-epithelial crosstalk (53); TGF-β activation can also interfere with pro-repair signaling pathways including Wnt/β-catenin signaling (45). TGF-β has previously been shown to impair the ability of mouse lung stromal cells to support lung epithelial colony formation *in vitro* (41). However, the impact of TGF-β-induced myofibroblast differentiation on the ability of human lung fibroblasts to support lung epithelial repair, and the consequences of TGF-β pathway activation on activity of regenerative signaling pathways in human lung fibroblasts, are poorly understood.

We investigated the hypothesis that TGF-β-induced myofibroblast differentiation impairs ability of human lung fibroblasts to support epithelial repair. We used an organoid assay, in which freshly isolated $EpCAM^+$ epithelial cells from mouse lung are co-cultured with human lung fibroblasts *in vitro*. Pretreating phFB or MRC5 with TGF-β to induce myofibroblast differentiation impaired subsequent organoid-supporting ability. Using transcriptome analysis, we show that TGF-β induces a wide range of

transcriptional effects including alteration of Wnt/β-catenin signaling, and highlight modulation of secreted growth factor expression as a potential mechanism to in part explain impaired organoid-supporting ability by myofibroblasts. This study highlights aberrant fibroblast-epithelial interactions as a possible future therapeutic target for correcting epithelial repair in chronic lung diseases.

Methods

Mouse epithelial cell isolation

Epithelial ($EpCAM^+$) cells were isolated from lungs of adult wild type mice with microbeads as previously described (43, 71). Briefly, lungs of pathogen-free wild type C57BL/6N mice (>8 weeks of age) were flushed through the heart with PBS, instilled with dispase (BD Biosciences, Oxford, UK #354235) and low-melt agarose (Sigma Aldrich, Poole, UK #A9414), and incubated at room temperature for 45 minutes. Trachea and extrapulmonary airways were removed, and remaining lobes were homogenized in DMEM with DNase1 (Applichem, Germany #A3778). The resulting suspension was passed through nylon filters, incubated with microbeads conjugated to antibodies for CD45 (Miltenyi Biotec, Teterow, Germany #130-052-301) and CD31 (Miltenyi, #130-097-418), and passed through LS columns (Miltenyi #130-091-051). The $CD31^-/CD45^-$ suspension was then enriched for epithelial cells by positive selection using EpCAM (CD326) microbeads (Miltenyi #130-105-958). $EpCAM^+$ cells were resuspended in DMEM with 10% FBS. All protocols were approved by the University of Groningen animal experimentation committee under CCD license AVD105002015303.

Fibroblast cell culture and treatments

MRC5 human lung fibroblasts (CCL-171; ATCC, Wesel, Germany) were cultured in Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 1% amphotericin B (Gibco) at 37°C with 5% CO_2. For RNA sequencing experiments, primary human lung fibroblasts (phFB) obtained from the CPC-M bioArchive in Munich, Germany that were isolated from adult human donor lung tissue were used (N=4). For organoid experiments, adult human phFB isolated from histologically normal regions of lung tissue specimens obtained at UMCG, Groningen, Netherlands from 4 patients undergoing resections for suspected tumor, or from lung tissue specimens from 4 COPD patients (1 GOLD stage III and 3 GOLD stage IV) undergoing lung transplantation, were used (N=8 total). Patient details are in Table 1. All phFB were cultured in DMEM/Ham's F12 (1:1) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 1% amphotericin B (Gibco) at 37°C with 5% CO_2. For organoid experiments,

MRC5 or phFB were grown to confluence in 6-well culture plates and serum deprived in medium supplemented with 0.5% FBS, L-glutamine and antibiotics (serum deprivation medium) for 24 hours. Cells were then incubated with either vehicle, recombinant human TGF-β_1 (R&D Systems, Oxford, UK #240-B), CHIR99021 (CHIR; Axon Medchem, Groningen, The Netherlands #1386), or CHIR+TGF-β (added together); cells were incubated with treatments added to serum deprivation medium for 48 hours. Cells were washed 3 times with warm PBS and proliferation-inactivated by incubation in mitomycin C (10μg/ml, Sigma #M4287) for 2 hours, followed by 3 washes in warm PBS and trypsinization prior to mixing with $EpCAM^+$ cells.

Organoid culture

The organoid assay is based on published protocols with slight modifications (10, 42, 62). 20,000 $EpCAM^+$ cells were mixed with 20,000 fibroblasts (MRC5 or phFB) in 100 μl growth factor-reduced Matrigel (Fisher Scientific, Landsmeer, The Netherlands #11523550) diluted 1:1 with DMEM/F12 containing 10% FBS and seeded into transwell inserts for 24-well plates (Thermo Fischer Scientific, Waltham, USA #10421761). Cultures were maintained in DMEM/F12 with 5% (v/v) FBS, 2 mM L-glutamine, antibiotics, insulin-transferrin-selenium (1x, Gibco #15290018), recombinant mouse EGF (0.025μg/ml, Sigma #SRP3196), bovine pituitary extract (30μg/ml, Sigma #P1476), and freshly added all-trans retinoic acid (0.01μM, Sigma #R2625) at 37°C with 5% CO_2. Y-27632 (10 μM, Tocris, Oxford, UK #1254) was added for the first 48 hours of culture. Media was refreshed every 2-3 days.

For organoid treatment experiments, organoid culture media were supplemented from day 0 with recombinant proteins FGF10 (R&D Systems #345-FG-025), FGF7 (R&D Systems #251-KG-010), HGF (Sigma #H9661), Wnt5a (R&D Systems #645-WN-010) or vehicle, as indicated.

To quantify colony forming efficiency, the total number of organoids per well was counted manually 7 days after seeding using a light microscope at 20x magnification. Organoid diameter was measured 14 days after seeding with a light microscope connected to NIS-Elements software. For immunofluorescence, organoid cultures were fixed with ice-cold acetone/methanol (1:1) for 12 minutes at -20^{o}C, then blocked in PBS with 5% (w/v) bovine serum albumin (BSA, Sigma). Cultures were incubated with primary antibodies diluted in PBS with 0.1% (w/v) BSA and 0.1% Triton-X100 at 4^{o}C overnight, then washed 3 times in PBS (>1 hour between washes) and incubated with secondary antibodies at 4^{o}C overnight. Cultures were excised from inserts and mounted on glass slides with mounting media containing DAPI (Abcam #ab104139) and glass coverslips. Immunofluorescence was visualized using a Leica SP8 confocal microscope (Wetzlar, Germany), and images obtained with Leica LAS software.

Library preparation and RNA sequencing

PhFB were incubated with vehicle, TGF-β, CHIR, or CHIR+TGF-β for 24 hours, as in previous studies we found this time point to be optimal for detecting gene expression changes after treatment with GSK-3 inhibitors and TGF-β (6, 59). Cells were lysed and homogenized in 500 µL of TRIzol™ Reagent (Invitrogen, #15596026) and total RNA was isolated according to the manufacturer's instructions. Total RNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer. 1 µg of total RNA was used for library preparation. Subsequently, a purification step was included to isolate pure, intact messenger RNA (mRNA) through magnetic bead separation, using NEXTflex™ Poly(A) Beads (Bioo Scientific, #512980), according to the manufacturer's instructions. Samples were then prepared for directional, strand-specific RNA libraries for Illumina sequencing, using the NEXTflex® Rapid Directional qRNA-Seq™ Kit (Bioo Scientific, #5130-01D), according to the manufacturer's instructions. Sequencing was performed on an Illumina NextSeq 500 system with an average sequencing depth of 10 million sequencing reads per sample. Sequencing data was aligned to human genome reference GRCh38 (with gene annotation from Ensembl database release 88, http://www.ensembl.org) using STAR version 2.5.3a (19). PCR duplicates were filtered using unique molecular identifiers as recommended by kit manufacturer. The full dataset is available as Supplemental Material.

Data analysis and statistics

For the sequencing analysis, genes with an average (across all samples) expression level exceeding 10 reads per million were included in the analysis using software package edgeR and using paired-sample analysis with TGF-β treatment, and CHIR99021 treatment as factors (40). In the differential expression analyses, differentially expressed genes with a minimum of 2-fold change and a false discovery rate (FDR) <0.01 were included in the pathway analyses. Heatmaps were generated with R or with Heatmapper (7). Venn diagrams were generated with GeneVenn (http://genevenn.sourceforge.net/). Post-hoc analyses of RNASeq data were performed with PANTHER (www.Pantherdb.org) (44) with PANTHER pathways analysis (48), and STRINGDB (https://string-db.org/) (60). Functional data were analyzed with GraphPad Prism 5.0. Data are presented as mean ± SEM, or median (interquartile range). N refers to number of independent experiments starting from an independent $EpCAM^+$ isolation, and n refers to number of organoids. The statistical tests used are stated in the Figure legends. Differences at a value for p of < 0.05 were considered significant.

Results

TGF-β-induced myofibroblast differentiation impaired ability of human lung fibroblasts to support epithelial organoid formation

PhFB or MRC5 require 48 hours pre-treatment with TGF-β (2ng/ml) to induce myofibroblast differentiation (6), whereas the use of alveolar progenitor cells in the organoid assay requires immediate processing of freshly obtained lung tissue. To investigate the ability of human lung fibroblasts and myofibroblasts to support epithelial repair, we therefore modified existing organoid assay protocols (10, 42, 62) and co-cultured adult mouse lung epithelial ($EpCAM^+$) cells with either primary human lung fibroblasts (phFB) isolated from resected adult tissue, or MRC5 human lung fibroblasts, in Matrigel (Figure 1A). Organoids did not form in the absence of fibroblasts (data not shown); however, both phFB and MRC5 cells were able to support adult mouse lung $EpCAM^+$ organoid formation with similar efficiencies (1.1±0.2% with phFB, and 0.89±0.1% with MRC5 cells, p=0.28; Figure 1B). Efficiencies were comparable to our previous studies using cultures with CCL206 mouse lung fibroblasts (~1%)(47). By day 14, epithelial organoids cultured with phFB or MRC5 cells had either an alveolar (pro surfactant protein C, $proSFTPC^+$), or airway (acetylated alpha-tubulin, ACT^+) phenotype, while very few organoids had a mixed alveolar/airway phenotype ($proSFTPC^+/ACT^+$; Figure 1C). When co-cultured with phFB, alveolar organoids had a more mature morphology, and airway organoids appeared to contain more ACT^+ cells, when compared to MRC5 cultures (Figure 1C). Quantification showed organoids from both phFB and MRC5 were predominantly alveolar; phFB tended to give rise to a higher proportion of $proSFTPC^+$ organoids than MRC5 (organoids were 74.4±1.9% $proSFTPC^+/ACT^-$ with phFB, and 61.3±4.6% $proSFTPC^+/ACT^-$ with MRC5, p=0.12; Figure 1D), with a concomitant lower proportion of organoids that expressed neither marker; however, these differences did not reach statistical significance (organoids were 20.3±1.9% $proSFTPC^-/ACT^-$ with phFB, and 35.2±4.0% $proSFTPC^-/ACT^-$ with MRC5, p=0.07; Figure 1D). As with organoid forming efficiencies, organoid differentiation in cultures with MRC5 and phFB was comparable to our studies using cultures with CCL206 mouse lung fibroblasts (47).

Pre-treatment of MRC5 with TGF-β significantly reduced the number of resulting epithelial organoids (vehicle 178.6±19, TGF-β 102.9±15, p<0.001; Figure 1E), and significantly decreased median organoid diameter (vehicle 53.1(39-78)µm, TGF-β 39.1(31-55)µm, p<0.0001; Figure 1F). Quantitative immunofluorescence for proSFTPC and ACT showed a non-significant increase in the proportion of organoids that were $proSFTPC^+/ACT^-$ (vehicle 61.3±5%, TGF-β 71.6±3%, p=0.12), which may reflect selective inhibition of airway organoid formation (Figure 1G).

Pre-treatment of phFB with TGF-β led to a reduction in the number of resulting epithelial organoids measured at day 7 (vehicle 353.1±67, TGF-β 243.3±54, p<0.01; Figure 1H). Initial analysis revealed similar variability between COPD and non-COPD phFBs, but no clear effect of disease status on

either baseline organoid forming efficiency, or on the effect of TGF-β1 (Figure 1E), so data from both COPD and non-COPD phFB lines were pooled for subsequent analysis. Pre-treatment of phFB with TGF-β caused a small but significant increase in median organoid diameter measured at day 14 that is unlikely to be biologically relevant ($p<0.05$, Figure 1I). Quantitative immunofluorescence for proSFTPC and ACT showed that neither alveolar nor airway differentiation were affected by pre-treating phFB with TGF-β (Figure 1J).

RNA-sequencing analysis reveals perturbation of Wnt/β-catenin signaling induced by TGF-β in primary adult human lung fibroblasts

To investigate mechanisms of impaired organoid-forming ability by TGF-β, phFB were incubated with TGF-β for 24 hours, and bulk RNA-Sequencing (RNA-Seq) was performed (Figure 2A). TGF-β induced differential expression of 3795 genes with a false discovery rate (FDR) of <0.01 compared to vehicle control (Figure 2B). Of these, 1792 were upregulated, and 2003 were downregulated compared to vehicle (Figure 2B). STRING-based analysis was used to identify networks of co-regulated genes that are likely to have functional relevance based on known protein-protein interactions (60). Analysis of the top 200 significantly up-regulated genes revealed 4 distinct TGF-β-induced transcriptional hubs encoding 1) contractile proteins (e.g. *TPM1*, *ACTA2*), 2) matrix proteins (e.g. *FB1*, *COL1A1*), 3) RNA synthetases (e.g. *AARS*, *LARS*), and 4) heat shock proteins (e.g. *HSPA5*, *HSPA9*) (Figure 2C, D).

Next, PANTHER-based gene ontology (GO) analysis was used to identify molecular pathways overrepresented within TGF-β-modulated genes (1026 genes with FDR <0.01 and >2 fold change compared to vehicle control) in phFB. Interestingly, the top 2 pathways overrepresented in TGF-β-modulated genes were Integrin signaling (34 genes, $p=1.24x10^{-11}$), and Wnt signaling (24 genes, $p=8.04x10^{-3}$) (Figure 3A). Since recent studies implicate Wnt signaling in lung fibroblasts in regulating epithelial progenitor cell function in the adult lung (35, 76), and since in our previous work we found dysregulated Wnt signaling in chronic lung disease (29, 30, 58), we analyzed this pathway in further detail. Numerous components of the Wnt/β-catenin signaling pathway were significantly differentially expressed following TGF-β activation. *WNT5A*, *WNT5B*, and *WNT2*, which encode Wnt ligands, and *FZD6*, *FZD2* and *FZD8*, which encode Wnt receptors, were increased in fibroblasts upon TGF-β treatment, whereas *WNT2B* and *FZD1* were decreased by TGF-β (Figure 3B). Notably, *TCF7*, *LEF1*, and *TCF7L1*, which encode T-cell factor (TCF)/Lymphoid-enhancer factor (LEF) transcriptional co-activators critical for β-catenin-dependent transcription, were all significantly decreased following TGF-β treatment in phFB, whereas *TCF7L2*, which encodes TCF4, showed a non-significant increase by TGF-β (Figure

3C). These data support the idea that TGF-β perturbs the capability of the Wnt/β-catenin pathway to mediate gene expression (6).

We next asked whether TGF-β activation affects transcription of Wnt/β-catenin target genes. First, a phFB-specific Wnt/β-catenin target gene signature was generated by incubating phFB with the Wnt/β-catenin signaling activator CHIR99021 (CHIR, 2μM) for 24 hours followed by RNA-Seq. CHIR inhibits the intracellular kinase glycogen synthase kinase (GSK)3, leading to β-catenin accumulation and nuclear translocation, thus activating the Wnt/β-catenin signaling pathway (11). CHIR led to differential expression of 4817 genes with a FDR of <0.01 compared to vehicle control (Figure 4A). Of these, 2226 were upregulated, and 2591 were downregulated compared to vehicle. As expected, upregulated genes included many known Wnt/β-catenin target genes such as *NOTUM*, *NKD1*, *NKD2*, *GREM2*, *AXIN2* and *FRZB* (Figure 4B). GO analysis revealed several biological processes were overrepresented following CHIR treatment in phFB including 'Developmental processes' (120 genes, $p=1.43x10^{-20}$), in line with the described role for Wnt/β-catenin signaling in early morphogenesis (37) (Figure 4C). Additional GO analysis revealed Wnt signaling to be the top pathway overrepresented in CHIR-modulated genes (26 genes, $p=6.63x10^{-3}$), in accordance with numerous Wnt signaling pathway genes being direct targets of the Wnt/β-catenin pathway (37) (Figure 4D). A comparison of the TGF-β- and CHIR-induced transcription profiles revealed that of 2226 genes upregulated by CHIR treatment, 273 were decreased by TGF-β (Figure 4E), whereas of 2591 genes downregulated by CHIR treatment, 254 were increased by TGF-β (Figure 4E). These data suggest that TGF-β activation may alter the Wnt/β-catenin target gene program in phFB with consequent effects on expression of a large set of genes.

We previously showed that GSK3β inhibition prevented TGF-β-induced myofibroblast differentiation in MRC5 cells (3). To investigate whether inhibition of TGF-β-induced myofibroblast differentiation by CHIR could rescue organoid formation, MRC5 cells or phFB were pre-treated with vehicle, CHIR alone, TGF-β alone, or CHIR added together with TGF-β (CHIR + TGF-β) and ability to support organoid formation was investigated. In both MRC5 cells and phFB, pre-treatment with CHIR alone did not influence organoid number. However, in MRC5 cells, CHIR + TGF-β prevented TGF-β-induced reduction in organoid formation (TGF-β 90.0±12%, CHIR+TGF-β 136.0±14%, $p<0.05$; Figure 5A). In contrast, pre-treatment of phFB with CHIR + TGF-β did not prevent reduction in organoid formation compared to TGF-β alone (Figure 5B). These data suggest that in phFB, TGF-β-induced transcriptional changes impair their capability to respond to GSK3 inhibition and subsequent Wnt/β-catenin activation.

To investigate this further, RNA-Seq was performed on phFB incubated with TGF-β + CHIR. Analysis of selected myofibroblast-associated genes confirmed partial inhibition of TGF-β-induced myofibroblast differentiation by combination with CHIR in phFB (*ACTA2*, *TPM1*, *MYH11*; Figure 5C).

The transcriptional profile of TGF-β + CHIR-treated phFB was compared to the TGF-β, CHIR and vehicle-treated phFB RNA-Seq profiles, and interaction analysis was performed. 181 genes showed a statistically significant interaction effect (FDR <0.01) of CHIR + TGF-β versus either treatment alone (Figure 5D, Supplemental figure 1). Unsupervised clustering revealed several distinct interaction patterns, which we categorized accordingly (Supplemental figure 1): type 1, genes downregulated by CHIR, TGF-β, and CHIR + TGF-β, compared to vehicle alone (55 genes); type 2, genes downregulated by CHIR, which is partially prevented by CHIR + TGF-β (24 genes, Figure 5E); type 3, genes highest expressed after CHIR + TGF-β compared to CHIR, TGF-β or vehicle alone (10 genes); type 4, genes downregulated by CHIR alone, but upregulated by CHIR + TGF-β (11 genes, Figure 3F); type 5, genes highest expressed after TGF-β compared to vehicle, CHIR, or CHIR + TGF-β (26 genes); and type 6, genes upregulated by CHIR, which is prevented by CHIR + TGF-β (55 genes, Figure 3G). Of these, types 2, 4 and 6 interactions are consistent with TGF-β distorting the CHIR-induced Wnt/β-catenin-transcriptional program (Figures 3E-G).

Within genes corresponding to types 2, 4 and 6 interactions, we identified numerous genes encoding signaling molecules (e.g. *SEMA3D*), and transcription factors (e.g. *RUNX2, MSX1, ETV5*). Notably, expression of genes for Wnt pathway components *FZD4*, *LGR4*, *TCF7* and *FRZB* exhibited significant interactions between CHIR and TGF-β (Figure 5E-G, Supplemental figure 1). Furthermore, GO analysis revealed that components of the Wnt/β-catenin pathway were slightly overrepresented among genes with significant CHIR-TGF-β interactions (8 genes, p=0.005, Figure 5H).

Altogether, our data suggest that TGF-β may modulate Wnt pathway component expression, and distort the Wnt/β-catenin target gene program. In light of recent reports that lung mesenchymal cells serving as niche cells for epithelial progenitor cells are characterized by expression of the Wnt/β-catenin signaling target Axin2, and of the Wnt co-receptor Lgr6 (35, 76), future investigations into TGF-β-mediated Wnt/β-catenin distortion in fibroblasts and its relevance to epithelial repair are warranted.

TGF-β perturbs the profile of secreted factors from fibroblasts that support organoid formation

In our culture conditions, fibroblast co-culture is essential for epithelial organoid formation, and organoids form only if fibroblasts are in the Matrigel itself and not when cultured underneath the insert (data not shown), indicating the involvement of direct contact or short-range secreted factors in their supportive role. Therefore, we hypothesized that perturbation of secreted factors could contribute to the observed effects induced by TGF-β. Interestingly, among the top processes overrepresented in response to TGF-β using GO analysis was mesenchymal-epithelial signaling (5.26 fold enriched, $p=2.68x10^{-3}$, Figure 6A), which includes genes for secreted growth factors involved in lung development such as *HGF*

(hepatocyte growth factor), *WNT5A*, *WNT2B* and *FGF10* (fibroblast growth factor 10). Furthermore, we identified 31 genes differentially expressed by TGF-β that encode proteins with known roles as secreted signaling molecules according to published literature (Figure 6B). Thus, TGF-β may modulate expression of secreted growth factors/signaling molecules required for organoid formation.

To investigate this hypothesis, we supplemented culture media with recombinant signaling proteins in the organoid assay. We focused on FGF2, WNT5A, CTGF, WNT2, SEMA3C and SEMA7A, which were significantly increased by TGF-β in phFB. We additionally focused on HGF and FGF10, which were significantly decreased by TGF-β in phFB (Figure 6C). FGF7 was also selected due to its previously described function as a mesenchyme-secreted factor that regulates lung development and adult lung epithelial cell growth (38, 50); *FGF7* showed a non-significant trend to decreased expression in phFB by TGF-β (Figure 6C).

FGF2 treatment from day 0 caused a significant increase in organoid formation (1.15±0.01 fold of vehicle, $p<0.05$; Figure 6D). In contrast, WNT5A treatment caused a significant decrease in organoid formation (0.93±0.003 fold of vehicle, $p<0.05$; Figure 6D), partially mimicking the effect of fibroblast TGF-β pre-treatment. Addition of CTGF, WNT2, SEMA3C or SEMA7A did not affect organoid number (Figure 6D). FGF2 treatment led to increase in organoid size (vehicle 38.5(31-49)µm, FGF2 41.5(34-59)µm, $p<0.01$; Supplementary Figure 2A). CTGF, WNT2, WNT5A, SEMA3c or SEMA7A did not affect the size of the resulting organoids (Supplementary Figure 2A,B).

Pre-treatment of MRC5 with TGF-β led to a significant reduction in the number of resulting epithelial organoids (0.13±0.02 fold of non pre-treated fibroblasts, $p<0.001$; Figure 6E). When added to cultures containing TGF-β pre-treated fibroblasts, HGF increased organoid formation compared to TGF-β pre-treatment alone (0.23±0.02 fold of non pre-treated fibroblasts, $p<0.01$ compared to TGF-β pre-treated control; Figure 6E). FGF7 dramatically increased organoid formation compared to TGF-β pre-treatment alone (0.96±0.001 fold of non pre-treated fibroblasts, $p<0.001$ compared to TGF-β pre-treated control; Figure 6E). In contrast, FGF10 did not affect organoid formation (0.16±0.01 fold of non pre-treated fibroblasts, $p>0.05$ compared to TGF-β pre-treated control; Figure 6E).

TGF-β pre-treatment alone led to a significant reduction in median organoid diameter compared to non pre-treated fibroblasts (vehicle 38.5(31-49)µm, TGF-β 30.2(28-33)µm, $p<0.001$; Supplemental Figure 2C). HGF and FGF7 both caused a significant increase in organoid diameter compared to TGF-β pre-treatment alone (HGF 32.5(29-37)µm, FGF7 42.3(37-63)µm, both $p<0.001$ compared to TGF-β pre-treatment alone; Figure 6G). FGF10 did not affect organoid diameter (29.9(28-33)µm, $p>0.05$; Supplemental Figure 2C).

Discussion

Prolonged local TGF-β activation leading to persistent fibroblast-to-myofibroblast differentiation, for example due to oxidative stress arising from inhalation of pollutants, is a major contributor to fibrotic remodeling in chronic lung diseases (28, 31, 70), yet the impact of TGF-β on fibroblast ability to support epithelial repair is poorly understood. Using an adult lung organoid assay in which primary adult human lung fibroblasts (phFB) or MRC5 cells are co-cultured with adult mouse lung epithelial cells *in vitro*, we found that TGF-β-induced myofibroblast differentiation impairs epithelial organoid-supporting ability. Transcriptome analysis of TGF-β activation in phFB revealed alterations in Wnt/β-catenin signaling. Furthermore, TGF-β altered expression of secreted factors that functionally contribute to lung organoid growth *in vitro*. TGF-β-induced myofibroblast differentiation may thus contribute to failure of lung epithelial repair in adult chronic lung diseases such as COPD and IPF via aberrant mesenchymal-epithelial crosstalk.

We performed transcriptome analysis of TGF-β-treated phFB to investigate downstream mechanisms of impaired organoid-supporting ability. As expected, we found induction of TGF-β targets including genes for myofibroblast-associated contractile proteins (*ACTA2*, *CNN1* and *TPM1*) and ECM proteins (*FBN1*, *COL1A1* and *COL4A2)*, and genes required for transcription and translation including RNA synthetases (e.g. *AARS*, *LARS* and *TARS*), and Hsp70 family heat shock proteins (e.g. *HSPA5*, *HSPA9* and *HSPA13*) (61). Interestingly, transcriptome analysis of TGF-β-treated phFB revealed altered expression of Wnt/β-catenin signaling pathway components, and modulated expression of Wnt/β-catenin target genes both at baseline and following treatment with the Wnt/β-catenin activator CHIR. Wnt signaling is mediated by secreted Wnt ligands that interact with cell-surface Frizzled receptors, which together with LRP5/6 co-receptors inactivate the intracellular 'destruction complex' to allow β-catenin accumulation and nuclear translocation; β-catenin then induces transcription via interaction with DNA-bound TCF/LEF transcriptional co-activators (4, 37, 57). Wnt pathway components significantly differentially expressed by TGF-β in phFB included genes encoding Wnt ligands (*WNT5A*, *WNT5B*, *WNT2*, *WNT2B*), receptors (*FZD6*, *FZD2*, *FZD8*, *LRP5*), and negative Wnt pathway regulators (*DKK3*, *NKD2*, *FRZB*). Moreover, expression of *TCF7* (encodes TCF1), *TCF7L1* (encodes TCF3), and *LEF1*, was significantly decreased by TGF-β in phFB. LEF1 mediates gene activation by Wnt/β-catenin, whereas TCF3 typically represses transcription, and TCF1 and TCF4 may either activate or repress transcription (13). TCF1, 3, 4 and LEF1 exhibit partially non-overlapping genome-wide chromatin occupancy and may engage with distinct cofactors (13). Altered expression of TCF/LEF family members by TGF-β could thus alter the set of genes activated by Wnt/β-catenin signaling. Future studies investigating TCF/LEF protein

abundance and genome-wide DNA binding patterns following TGF-β-induced myofibroblast differentiation would be informative.

Although the importance of epithelial Wnt/β-catenin signaling for adult lung maintenance and repair is well recognized (46, 74), increasing evidence implicates mesenchymal Wnt/β-catenin activation as critical for regulating growth and differentiation in developing and adult lung. During mouse lung development, mesenchymal Wnt/β-catenin signaling regulates airway smooth muscle lineage specification (32) and mesenchymal proliferation (16, 51, 55), and studies using reporter mice revealed Wnt/β-catenin pathway activation in sub-epithelial lung mesenchyme in a temporally and spatially-restricted pattern (2, 17). Alveolar fibroblasts isolated from adult mouse lung expressing leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) instructed alveolar differentiation of adult lung epithelial progenitors *in vitro*, whereas $Lgr6^+$ airway smooth muscle cells promoted airway differentiation (35). Lgr5 and Lgr6 are receptors for R-Spondin, which by inhibiting Rnf43 and Znrf3-mediated endocytosis of Frizzled receptors, potentiate Wnt ligand-driven Wnt/β-catenin signaling (18). Another, possibly overlapping alveolar fibroblast type co-expressing the Wnt/β-catenin target gene Axin2 and PDGFRα also preferentially supported lung epithelial organoid formation *in vitro* (76). The precise role of mesenchymal Wnt/β-catenin signaling in regulating adult lung epithelial repair remains to be clarified. In the context of these studies, from our data it is tempting to speculate that distorted Wnt/β-catenin signaling by TGF-β activation in lung fibroblasts may contribute to impaired ability to support epithelial repair, however, further studies are clearly needed to determine the relevance of TGF-β-induced distorted fibroblast Wnt/β-catenin signaling to repair.

We previously showed that in MRC5 cells, pharmacological GSK3 inhibition completely prevented TGF-β-induced myofibroblast differentiation via CREB phosphorylation (3). In the current study, CHIR did not completely prevent myofibroblast differentiation in phFB: TGF-β-induced expression of *ACTA2*, *TPM1* and *MYH11* was only partially inhibited by addition of CHIR. Full versus partial inhibition of myofibroblast differentiation may explain the ability of CHIR to rescue organoid formation after TGF-β-pre-treatment in the fetal MRC5 cell line but not in phFB. A possible reason is that TGF-β elicits divergent responses in adult compared to fetal lung fibroblasts, as the repertoire of genes regulated by Smad3 is influenced by the presence of other transcription factors and the chromatin environment, which vary with developmental stage (22). In support of this idea, phFB TGF-β pre-treatment gave rise to slightly larger organoids with no effect on differentiation, whereas MRC5 TGF-β pre-treatment resulted in smaller organoids, with a higher proportion expressing SFTPC. MRC5 cells may also respond differently to CHIR compared phFB, as Wnt/β-catenin signaling is known to exert highly developmental-stage-specific transcriptional effects (37). In the future, comparative analyses of transcriptional and functional responses to CHIR and TGF-β in adult and fetal human lung fibroblasts may help elucidate these issues.

Aberrant Wnt/β-catenin pathway activation contributes to TGF-β-induced fibrotic remodeling in numerous adult fibrotic diseases including IPF (1, 15, 25, 30). Our study revealed different types of transcriptional interaction between Wnt/β-catenin activation by CHIR and TGF-β in phFB, providing evidence that TGF-β activation disturbs the Wnt/β-catenin target gene program. Signaling crosstalk between Wnt/β-catenin and TGF-β pathways is well described and interactions may arise through several different mechanisms (reviewed in (24)), including direct interaction between Smads and TCF/LEF or upstream Wnt/β-catenin signaling components (34, 68), and altered expression of Wnt/β-catenin pathway components by TGF-β (1). Thus, although TGF-β leads to accumulation of active β-catenin (6, 59), these pathway interactions may alter the set genes induced by β-catenin in phFB, possibly explaining the type 2, 4 and 6 interactions we observed which are consistent with TGF-β distorting the Wnt/β-catenin gene program. Future clarification of molecular mechanisms of Wnt/β-catenin-TGF-β crosstalk in phFB may aid development of pharmacological approaches to restore regenerative processes in chronic lung disease.

Using our culture protocol, adult distal lung epithelial progenitors form organoids when co-cultured directly with fibroblasts in Matrigel and not in the absence of fibroblasts, suggesting a requirement for fibroblast-derived secreted signals in organoid initiation; the precise identity of such factors remains to be determined (9). Our analysis revealed TGF-β-induced alterations in mesenchymal-epithelial signaling as a possible contributor to impaired organoid-supporting ability. TGF-β downregulated *FGF10* in phFB, as previously described in mouse lung stromal cells (41); TGF-β also downregulated *HGF* and *FGF7* in phFB, which encode mesenchyme-produced factors implicated in adult lung repair (42, 49, 67, 72, 73). Supplementation with HGF and FGF7 to organoid cultures containing TGF-β-pre-treated phFB rescued organoid number and significantly increased organoid size. Interestingly, recent reports describe fibroblast-free culture of adult human lung airway organoids with media containing FGF10 and FGF7 (26, 78), and of mouse alveolospheres with several factors including Fgf7 (54), suggesting that fibroblast-secreted factors activating epithelial FGFR2b are critical for organoid initiation.

TGF-β also induced expression of *WNT5A* and *WNT5B* in phFB. Addition of recombinant WNT5A to cultures with non-pre-treated fibroblasts reduced organoid number, supporting the idea that *WNT5A* gene induction may contribute to impaired organoid-supporting ability by TGF-β. WNT5A and WNT5B can induce cellular changes independently of β-catenin, and so their induction by TGF-β may represent a shift from β-catenin-dependent to -independent (non-canonical) Wnt pathways; TGF-β-induced WNT5A could thus antagonize epithelial Wnt/β-catenin signaling required for lung repair (5, 46, 64, 75). TGF-β-induced WNT5A expression in phFB may explain increased WNT5A protein previously observed in COPD lung tissue samples (5), and could be relevant to lung growth/repair *in vivo* as WNT5A inhibition led to increased lung repair in adult mice with emphysema following chronic cigarette smoke (5).

Moreover, TGF-β induced expression of *FGF2*, *CTGF*, *WNT2*, *SEMA3C* and *SEMA7A*. FGF2 was recently demonstrated to inhibit proliferation of adult mouse lung basal cells, progenitor cells of the proximal airways(8). Surprisingly, we found that FGF2 significantly increased both organoid number and size. The reasons for this discrepancy are unclear but may relate to differences in response to FGF2 between basal cells and the distal epithelial population used in our study, or could reflect indirect effects of FGF2 on fibroblasts in our culture system. Connective tissue growth factor, CTGF, is a secreted matricellular protein implicated in tissue fibrosis (36). Studies in mice revealed central roles for WNT2 and WNT2B in early lung development, although only WNT2 is expressed in adult lung (23). *SEMA3C* and *SEMA7A* encode members of the semaphorin family, secreted molecules with key roles in nervous system development and angiogenesis (69). SEMA3A was implicated in alveolar development (64), whereas SEMA7C was implicated in lung inflammation following acute injury (52). We failed to find any effect of CTGF, WNT2, SEMA3A or SEMA7C on organoid number or size, suggesting increased expression by TGF-β is unlikely to contribute to impaired organoid formation. Nonetheless, it is possible that persistent TGF-β-induced semaphorin expression may contribute to vascular abnormalities observed in chronic lung diseases such as COPD/emphysema (65).

A potential limitation to our study is the use of the GSK3 inhibitor CHIR to activate the Wnt/β-catenin pathway. GSK3 regulates other signaling pathways including PI3K and Hedgehog pathways, and thus we cannot exclude Wnt/β-catenin-independent transcriptional effects of CHIR treatment in phFB. However, in support for a predominant effect on Wnt/β-catenin activation, Wnt signaling was identified as the top signaling pathway overrepresented in genes differentially expressed after CHIR treatment, consistent with Wnt pathway feedback control (37). Another potential limitation is that we isolated adult mouse lung $EpCAM^+$ cells for the organoid assay, previously characterized as ~91% $SFTPC^+$ cells, and ~4% CC10 positive cells (data not shown, (43, 71)). The population thus likely contains numerous organoid-forming epithelial progenitor types including club cells, $SFTPC^+$ alveolar type 2 cells, and distal basal-like cells with both alveolar and airway lineage potential, which may account for our observations of alveolar, airway, and mixed alveolar/airway organoid phenotypes (10, 14, 33, 74). In the future it would be of interest to investigate if TGF-β fibroblast activation impacts crosstalk with specific lung epithelial progenitor types. Of note, organoid-forming efficiency at baseline was similar in cultures containing either non-COPD or COPD phFB, and there was no clear effect of disease status on response to TGF-β. Differences between phFB from COPD and non-COPD patients have previously been reported; in one study, impaired contractile and migratory ability, and decreased alpha smooth muscle actin induction by TGF-β, were observed in phFB from COPD patients compared to non-COPD controls (63), whereas our group has previously reported increased accumulation of active β-catenin after TGF-β stimulation in COPD compared to non-COPD phFB lines (6, 59). COPD fibroblasts have been shown to exhibit altered

expression of intercellular adhesion molecule-1 after cytokine stimulation (75), and to be less proliferative and more senescent in culture with higher pro-inflammatory cytokine secretion (77). Although we failed to find an effect of COPD disease status on fibroblast ability to support epithelial repair in the organoid assay, due to the low sample numbers and high variability in both non-COPD and COPD cultures in our study, further studies are needed to clarify this.

In summary, TGF-β-induced myofibroblast differentiation impaired fibroblast ability to support epithelial organoid formation *in vitro*, suggesting that persistent mesenchymal TGF-β activation in chronic lung diseases such as COPD and IPF may contribute to defective epithelial repair. Excessive, persistent TGF-β activation is a major feature of numerous other chronic inflammatory diseases including of the kidneys and liver (12, 20). Thus, targeting defective mesenchymal-epithelial signaling induced by mesenchymal TGF-β activation may help restore epithelial repair in diverse adult chronic diseases.

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Disclosures

All authors have no competing interests to declare, financial or otherwise.

Figure Legends

Figure 1 – Fibroblast TGF-β activation impairs epithelial organoid supporting ability

A) Schematic of experimental setup

B) Organoid-forming efficiency of $EpCAM^+$ cells cultured with either phFB or MRC5. Box and whisker plots representing min and max. Unpaired t-test, N=3, n.s. non-significant.

C) Representative whole-mount immunofluorescence images of day 14 epithelial organoids generated from co-culture with phFB (top row) or MRC5 (bottom row), stained for pro-surfactant protein C (proSFTPC, green), acetylated tubulin (ACT, red) with DAPI (blue) as counterstain. Both fibroblast types gave rise to organoids with alveolar ($proSFTPC^+/ACT^-$, left), airway ($proSFTPC^-/ACT^+$, middle) and mixed ($proSFTPC^+/ACT^+$, right) characteristics. $proSFTPC^-/ACT^-$ organoids were also observed (not shown). Scale bars = 20 µm.

D) Quantification of day 14 organoids co-stained for pro-SFTPC and ACT, showing proportion of total organoids exhibiting one, both, or neither marker. Unpaired t-tests were performed on corresponding groups between phFB and MRC5 cultures, N=3, mean ± SEM.

E-J) Quantification of organoid number measured at day 7 (E, H), size measured at day 14 (F, I) and proportion expressing pro-SFTPC or ACT at day 14 (G, J), after co-culture with MRC5 (E-G) or phFB (H-J), pre-treated with vehicle or TGF- β. (E) Unpaired t-test, N=6. (F) Mann Whitney test, n>162 organoids from N=2. (G, J) Unpaired t-tests were performed on corresponding groups between vehicle and TGF-β cultures, G) N=2, J) N=3. (H) Paired t-test, N=8 donors. Empty circles = non-COPD phFB, black circles = COPD phFB, showing lack of disease-associated effect; non-COPD and COPD lines were pooled for the statistical analysis. (I) Mann Whitney test, n>501 organoids in each group from N=4 donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. non-significant. (F, H and I) horizontal line represents the median. (G, J) mean ± SEM.

Figure 2 – TGF-β-induced myofibroblast differentiation involves discrete transcriptional networks defined by gene product interactions

A) Schematic of experimental setup

B) Unsupervised clustering heatmap of TGF-β or vehicle treated phFB showing significantly differentially expressed genes according to false discovery rate < 0.01 and >2 fold change cutoff.

C) STRING-based analysis of top 200 significantly TGF-β upregulated genes ranked by FDR. Different colours represent k-means clustering, which led to 4 discrete clusters (labeled 1-4). FDR = false discovery rate.

D) Expression of selected genes within hubs 1-4. RPM values were normalized to the mean RPM in vehicle control for each gene. White bars = vehicle control, black bars = TGF-β. RPM = reads per million.

Figure 3 – Pathways analysis reveals TGF-β modulates Wnt signaling components and Wnt/β-catenin target genes

A) Top 20 pathways overrepresented in differentially expressed genes after TGF-β activation in phFB (DE, > 2FC), according to GO analysis. P values calculated using Fisher's exact test.

B) Unsupervised clustering of Wnt pathway component genes significantly differentially expressed following TGF-β activation in phFB.

C) Expression of TCF/LEF family members following TGF-β activation in phFB.

Figure 4 - Transcriptional profile of Wnt/β-catenin pathway activation in phFB

A) Unsupervised clustering heatmap of CHIR- or vehicle-treated phFB showing significantly differentially expressed genes according to false discovery rate < 0.01 and >2 fold change cutoff.

B) Expression of selected Wnt/β-catenin-target genes significantly upregulated by CHIR. RPM values were normalized to the mean RPM in vehicle control for each gene. White bars = vehicle control, black bars = CHIR. RPM = reads per million.

C) Top GO biological processes overrepresented in differentially expressed genes after CHIR in phFB (DE, > 2FC), according to GO analysis. P values calculated using Fisher's exact test.

D) Top 20 pathways overrepresented in differentially expressed genes after CHIR in phFB (DE, > 2FC), according to GO analysis. P values calculated using Fisher's exact test.

E) Venn diagrams showing overlap between genes significantly upregulated by CHIR and significantly downregulated by TGF-β, or significantly downregulated by CHIR and significantly upregulated by TGF-β. Number of genes in each list, and the overlap, are given within the plots.

Figure 5 – TGF-β skews the Wnt/β-catenin program by CHIR in phFB

A-B) Quantification of organoid number measured at day 7 after co-culture with MRC5 (A) or phFB (B) pre-treated with Vehicle, CHIR, TGF-β or CHIR + TGF-β for 48 hours prior to organoid assay. Repeated measures one-way ANOVA with Bonferonni post-test, A) N>4 per group, B) N=8 per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. non-significant.

C) Expression of selected myofibroblast-associated genes in phFB treated with Vehicle, CHIR, TGF-β or CHIR + TGF-β.

D) Unsupervised clustering heatmap showing genes with significant interaction between CHIR and TGF-β treatment. Different types of interactions are labeled

E-G) Examples of genes exhibiting interaction effects consistent with distortion of Wnt/β-catenin-signaling by TGF-β.

H) Top 10 pathways pathways overrepresented in genes with significant CHIR-TGF-β interaction effects in phFB according to GO analysis. P values calculated using Fisher's exact test.

Figure 6 – Secreted factors may contribute to TGF-β effect

A) Top 20 GO biological processes overrepresented in differentially expressed genes after TGF-β in phFB (DE, > 2FC), according to GO analysis.

B) Unsupervised clustering heatmap of TGF-β or vehicle treated phFB showing significantly differentially expressed genes according to false discovery rate < 0.01with described roles as secreted signaling molecules.

C) Gene expression of selected secreted molecules (from RNA-Seq) taken further for functional analysis.

D, E) Quantification of organoid number measured at day 7 after co-culture with MRC5, showing effect recombinant FGF2 (100 ng/ml), WNT5A (100 ng/ml), CTGF (100 ng/ml), WNT2 (50 ng/ml) SEMA3C (250 ng/ml) and SEMA7A (250 ng/ml) added to culture media in vehicle pre-treated MRC5 (D) or of recombinant HGF (50 ng/ml), FGF7 (50 ng/ml) and FGF10 (100 ng/ml) added to organoid cultures with TGF-β pre-treated MRC5 (E). Data presented relative to vehicle pre-treated controls (dotted line). One-way ANOVA with Dunnet's post-test (D,E). N=3-4, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. non-significiant.

Supplementary Figure 1 – Heatmap of CHIR-TGF-β interaction effects

Complete unsupervised clustering heatmap showing genes with significant interaction between CHIR and TGF-β treatment with all genes annotated. Plots show selected genes representing each type of interaction.

Supplementary Figure 2 – Effect of recombinant proteins of secreted factors on organoid size

Quantification of organoids size measured at day 14, using same cultures as in Figure 6 D&E. (A) Kruskall Wallis with Dunn's post test, n>623 organoids from N=3, (B) Kruskall Wallis with Dunn's post test, n>428 organoids from N=4, (C) Kruskall Wallis with Dunn's post test, n>280 organoids from N=3. *** $p<0.001$, n.s. non-significiant.

Table 1 – Patient information for phFB used in organoid experiments (N=8 total)

	Gender (F/M)	Age	Pack years	FEV1 (L)	FEV1/FVC ratio	COPD GOLD stage
Non-COPD	2/2	61±5.1	32.1±7	90.6(68.7-95.3)	76.8(75.6-80.6)	NA
COPD	3/1	55±2.3	34.3±3	18.8(15.4-39.6)**	25.9(22.2-45.0)***	I(1), IV(3)

Clinical information was available for all 8 patients. ** $p<0.01$, *** $p<0.001$ unpaired t-test COPD vs non-COPD. NA = not applicable.

Supplemental Material: 1602_Gosens_RNAseq.expression.genelevel.v75.htseq.txt

Complete processed RNA Sequencing dataset containing all raw read counts per sample. Samples were primary human lung fibroblasts obtained from 4 donors, each treated with 1 of 4 conditions (Vehicle, CHIR99021, TGFβ, CHIR99021 + TGFβ) for 24 hours prior to RNA isolation and processing for RNA sequencing (see text for details).

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