Modeling pulmonary fibrosis: Impacts of glutaredoxin on differentiation in tracheosphere development

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Modeling pulmonary fibrosis: Impacts of glutaredoxin on differentiation in tracheosphere development

A Thesis Presented

by

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a deadly disease whose pathology involves improperly differentiated airway epithelia present in alveolar regions. Previous work from our group has shown decreased activity of the enzyme glutaredoxin (GLRX) in the lungs of IPF patients, and demonstrated reversal of lung fibrosis in mice via administration of exogenous GLRX protein. We modeled airway epithelial tissue using 3D cell culture of wild type and GLRX-/- airway epithelial basal cells, both in monoculture and in coculture with fibroblasts. We present data from qPCR and immunohistochemistry which suggest that GLRX is essential for the proper differentiation of airway epithelia in the presence of fibroblasts. These findings suggest a role for GLRX in the attenuation of fibrosis via the support of healthy epithelial differentiation.

Introduction

IPF patients have poor clinical prognoses.

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive disease characterized by the buildup of scar-like tissue in the lungs, and is one of several diseases falling in the category of interstitial lung disease\(^1\). In the United States, IPF affects between 10 and 60 people per 100,000, with a disproportionate impact on the elderly\(^1\). The most significant symptom of IPF is chronic exertional dyspnea, or shortness of breath, as the disease stiffens the lung and prevents normal breathing\(^1\). Prognosis for IPF patients is bleak, as the disease tends to progress significantly during pre-symptomatic and pre-diagnostic periods\(^2\). Rates of patient decline are variable, but median survival time is 2.5-3.5 years post diagnosis\(^2\), with FDA-approved drugs Nintedanib and Pirfenidone capable of slowing, but not resolving IPF\(^3\).
Several genetic variations are known to confer risk for IPF.

Mutations in genes implicated in telomere maintenance, including TERT, TERC, PARN, and RTEL1 have known associations with increased IPF risk.¹ Risk for IPF is also associated with variation in genes implicated in cell adhesion, integrity, and mechanotransduction, including DSP, AKAP13, CTNNA, and DPP9.¹ Mucin 5B is a protein involved in airway clearance, which is overexpressed in small airway epithelia in IPF.¹ No mechanism linking mucin 5B overexpression to IPF has been established, but a SNP in the promoter region of the MUC5B gene, which codes for mucin 5B, predisposes individuals to IPF.¹ The microbiome may also play a role in IPF, as mucin 5B is required for innate immune responses to bacteria, and variation in TOLLIP, a gene associated with responses to microbes, increases IPF risk.¹ GLRX, a gene of interest in this thesis, has no known association with IPF risk.

Excessive fibroblast activation can lead to fibrosis.

Fibrosis develops when extracellular matrix (ECM) accumulates and disrupts the physiology of a tissue⁴. Often, fibrosis occurs in response to repeated tissue insult, where chronic, unresolved fibroblast activation leads to excessive ECM production and secretion⁴. Fibroblasts are a mesenchymal cell type playing a key role in healthy tissues, as well as in the wound repair process⁴. Fibroblasts become activated during wound repair in response to intercellular signals including transforming growth factor β (TGF-β)⁴. When exposed to TGF-β, fibroblasts upregulate their production of extracellular matrix (ECM) proteins, such as collagen, and transdifferentiate into myofibroblasts⁴. Myofibroblasts are a cell type similar to fibroblasts, which are also involved in wound healing⁵. They are distinguished from fibroblasts by expression of the protein α-smooth muscle actin (α-SMA), lending to more contractile capability than fibroblasts and contributing to increased tissue stiffness⁵. Myofibroblasts secrete collagen at a higher rate than fibroblasts and are known to contribute to the pathology of fibrosis⁵.
In IPF, myofibroblasts secrete unorganized but dense collagen into the lung interstitium\(^6\). They adhere to ECM and irreversibly contract, increasing tissue stiffness\(^6\). This stiffening process is subject to positive feedback, as myofibroblast ECM synthesis itself is upregulated by contact with stiffer ECM\(^6\). A key distinguishing histological feature of the IPF lung is the presence of fibroblastic foci\(^6\). These are regions of lung tissue characterized by clustered fibroblasts and myofibroblasts adjacent to epithelia, and serve as sites of massive ECM generation\(^6\). Lung fibrosis impairs normal gas exchange by interfering with alveolar physiology and compromises respiration by stiffening the lung, leading to eventual patient death\(^6\).

**Abnormal epithelial differentiation is a key component of IPF pathogenesis.**

Many cell types must communicate properly during the development and maintenance of the lung\(^7\). A key step leading to fibroblast activation in the pathogenesis of IPF is aberrant differentiation of alveolar epithelia\(^7\). Single cell RNA sequencing (scRNA-seq) data has shown disrupted epithelial cell differentiation patterns in IPF\(^7\). In addition, scRNA-seq data on IPF lung epithelia has shown atypical expression of Maternally Expressed Gene 3 (MEG3), which encodes a long noncoding RNA that is a marker of indeterminate and atypical basal cell phenotypes\(^7\). MEG3 is known to inhibit genes implicated in normal basal cell differentiation. Recent studies have also reported progenitor-like cells displaying dysregulated differentiation markers present around myofibroblast foci in the IPF lung\(^7\). In keeping with these findings of suppressed epithelial differentiation in IPF, increased numbers of keratin 5-positive basal cells have been found in the IPF lung\(^7\). These aberrant epithelia secrete a wide variety of intercellular signals, including TGF-\(\beta\), which can induce fibroblast activation\(^7\).

While aberrant epithelia can stimulate fibroblast activation, healthy epithelia can suppress fibroblast activation\(^8\). Bone morphogenetic protein (BMP4) is secreted by healthy epithelia and has been shown to block fibroblast activation using organoid models\(^8\). When epithelia are damaged, BMP4 secretion declines, and fibroblast activation is derepressed\(^8\). Exogenous BMP4 has been
shown to maintain fibroblast repression in organoids with damaged epithelia, and blocking BMP4 signaling has been shown to prevent fibroblast repression in healthy organoids\textsuperscript{6} (Figure 1).

![Figure 1. Key components of currently understood IPF pathology are compared to normal lung signaling.](image)

While healthy epithelia can suppress fibroblast activation via BMP\textsubscript{4}, aberrant epithelia can stimulate fibroblast activation via TGF-\textbeta\ and other signaling molecules. Activated fibroblasts lead to fibrosis via transdifferentiation to myofibroblasts and ECM secretion.

**Airway epithelial basal cells differentiate into secretory and ciliated cells.**

The origin of aberrantly differentiated epithelial cells in IPF alveoli is not clear, but migration of more proximal airway epithelia has been proposed as one explanation\textsuperscript{7}. In recent studies, airway basal cells have been found aberrantly in alveolar regions of IPF lung, suggesting a role for airway basal cells in IPF pathology\textsuperscript{9}. For this reason, this thesis will focus on proximal lung epithelia, in order to study the potential contribution of migrated airway epithelial cells to IPF development in the alveolus.
The proximal lung epithelium has the function of purifying inhaled air before gas exchange is to occur in the alveoli. In this tissue, secretory cells secrete mucus that lines the airway, trapping inhaled pathogens and toxic materials. Ciliated cells also populate the epithelium, flushing mucus in the proximal direction. Both ciliated and secretory cells are produced by the differentiation of progenitor basal cells, which maintain the epithelium via proliferation.

**Oxidative stress is implicated in lung fibrosis.**

Oxidative stress has been shown to be increased in lung fibrosis, but the mechanism by which oxidative stress may lead to fibrosis is unclear. Oxidative stress occurs when reactive oxygen species (ROS) are produced during oxidative phosphorylation at a higher rate than they can be scavenged by antioxidants. ROS are also produced normally via other mechanisms for the purpose of cellular signaling, and the effects of ROS in aging are complex and context dependent. Cell senescence is one key component of tissue aging known to be driven by ROS, and senescence of epithelial cells is known to contribute to pulmonary fibrosis. Mice that are naturally aged have been shown to be more sensitive to fibrosis induced by environmental stimuli and injuries than younger mice. Senescence of aged epithelia likely contributes to IPF pathogenesis via two mechanisms: firstly, permanent arrest of the cell cycle prevents senescent cells from performing self-repair or tissue repair upon injury; secondly, senescent cells secrete cytokines, including TGF-β, which induce fibroblast activation.

**GLRX administration reverses induced lung fibrosis in mice.**

Because of the implication of oxidative stress in fibrotic disease, our group is interested in studying the roles of redox signaling in IPF. Glutathione (GSH) is a tripeptide which is attached to proteins in order to prevent their oxidation, and plays a key role in cellular redox signaling. Protein-S-glutathionylation (PSSG) is the process by which GSH gets covalently attached to proteins, and may play a role in signaling mechanisms. GSH is known to be depleted in the lung-lining fluid of IPF patients. Glutaredoxin (GLRX) is an enzyme which removes glutathione
from proteins, reversing PSSG\textsuperscript{12}. The Janssen-Heininger research group has previously shown decreased GLRX enzymatic activity in the lungs of human IPF patients and used bleomycin-induced pulmonary fibrosis mouse models to study the effects of GLRX protein\textsuperscript{12}. Bleomycin is a cancer drug known to cause DNA damage and lipid peroxidation, whose toxicity is especially potent in lung tissue, and leads to the development of fibrosis\textsuperscript{13}. Work with these mouse models in our lab demonstrated reversal of lung fibrosis via administration of exogenous GLRX protein\textsuperscript{12}. In the same study, the group also showed that mice lacking GLRX were more susceptible to bleomycin-induced lung fibrosis, and mice overexpressing GLRX were less susceptible\textsuperscript{12}. These findings suggest an important role for GLRX during lung fibrosis. It remains unclear whether GLRX activity important for lung fibrosis occurs intracellularly or extracellularly.

3D culture provides a valuable model for the study of tissue morphogenesis.

Three-dimensional (3D) cell culture allows for the ex-vivo growth of cells in a condition that better mimics the in-vivo environment than do traditional, 2D culture dishes\textsuperscript{14}. When cultured in a 3D hydrogel, cells with stem-like properties undergo cell division, differentiation and self-organization, and are capable of reproducing important structures in mammalian anatomy\textsuperscript{14}. This model system enables researchers to manipulate and closely analyze tissue morphogenesis without the restrictions of using live animal models, and also shows promise for personalized medical care\textsuperscript{14}. 3D culture is rising in prominence in the study of lung development and disease. Organoids have been successfully grown from airway epithelial basal cells (tracheospheres), with ciliated and secretory cell types present after development\textsuperscript{15}. This model system presents a powerful tool with which diseases like IPF can be studied.

Hypothesis

In this thesis, I examine the hypothesis that GLRX is essential for normal airway basal cell differentiation into ciliated or secretory cells. If airway epithelial cells deficient in GLRX display aberrant differentiation, this could lead to fibroblast activation and ECM secretion in the
pathogenesis of IPF, and help to explain our group’s previous findings that GLRX can resolve bleomycin-induced lung fibrosis in mice.

**Methods**

**GLRX Knockout**

A GLRX -/- mouse line was generated using a C57Bl/6J background. This yielded mice lacking GLRX and containing a NEO cassette. Genotyping was performed for GLRX and NEO to confirm gene knockout. GLRX knockout conferred no observable developmental lethality, and GLRX -/- mice survived to adulthood with no observable defects.

**Cell culture**

Mouse tracheal epithelial cells (mTECs) were isolated from euthanized wild-type or GLRX -/- mice by removing the tracheas and submerging them overnight at 4 °C in OptiMEM with 0.1% protease type 14. The following day, the tracheas were flushed with DMEM/F12 supplemented with 10% FBS into a 50 mL tube, centrifuged at 500 x g for 5 minutes at 4 °C, and the resulting pellet was resuspended in culture media and placed in T75 flasks pre-coated with 7.5 mL polymerized rat tail collagen. mTECs were expanded in 2D culture for 14 days, after which the collagen gel was digested with 400 µg/mL collagenase and cells were freed by incubation with 0.05% trypsin. Cells were then centrifuged at 500 x g for 5 minutes at 4 °C, resuspended in culture medium and then centrifuged again. The resulting pellet was resuspended in Pneumacult ALI medium from Stemcell Technologies (Vancouver, Canada) and used to seed 3D culture experiments.

Normal human lung fibroblasts (NHLF) were purchased from Lonza Group (Basel, Switzerland) and expanded in Lonza Fibroblast Growth Medium in T75 flasks. Cells were then trypsinized, centrifuged at 500 x g for 5 minutes at 4 °C, resuspended in Pneumacult medium and used to seed 3D culture experiments.
For 3D cultures of either mTECs alone or mTECs cocultured with NHLF, each well in 12 well transwell plates was coated with 100 µL 50% GFR Matrigel from Corning Inc. (Corning, NY) (1:1 volumetrically of Matrigel to Pneumacult medium) and placed in a 37 °C, 5% CO₂ incubator for 20 minutes to allow hydrogel polymerization. 50,000 mTECs (and in some experiments, 50,000 NHLF) were suspended in 200 µL Pneumacult medium and then combined with 200 µL Matrigel (resulting in 50% Matrigel) and seeded on top of the polymerized layer of Matrigel in the well. Cell suspensions were immediately placed in a 37°C, 5% CO₂ incubator for 30 minutes to allow hydrogel polymerization, after which 1 mL Pneumacult medium was added to the lower chamber. Medium was then changed every 48 hours and cultures were allowed to grow for 28 days.

**Immunohistochemistry/Immunofluorescence**

Tracheospheres were processed for paraffin embedding by fixing in 4% PFA overnight at 4 °C, rinsing with PBS and then embedding in Histogel. Histogel-embedded samples were then fixed in 10% neutral buffered formalin for 4 hours at 4°C, and then placed in 70% ethanol overnight at 4°C and then paraffin embedded. Paraffin blocks were then cut into 5µm sections and mounted on slides for subsequent staining.

For hematoxylin and eosin staining, slides were deparaffinized, rehydrated, incubated in hematoxylin and eosin, de-stained with tap water, dehydrated and mounted using Permount. For immunofluorescence staining, slides were deparaffinized, rehydrated, subjected to heat-induced epitope retrieval and incubated in blocking buffer (10% normal goat serum from Jackson Immunoresearch Laboratories, West Grove, PA) for 30 minutes at room temperature. After blocking, slides were incubated with primary antibodies at 4 °C overnight, rinsed with PBS and then incubated with Alexafluor secondary antibodies for 1 hour at room temperature. Slides were then rinsed with PBS and mounted using Aqua Polymount.
**Imaging**

Brightfield imaging was performed on an Olympus IX70 inverted microscope with a Zeiss AxioCam 202 Mono camera, with images being processed using Zeiss Zen software. Confocal imaging was performed on a Nikon A1R-ER confocal microscope powered by a LUNV laser launch and driven by NIS-Elements software.

**Antibodies**

For immunofluorescence staining, all antibodies were purchased from commercial vendors and diluted in 5% goat serum blocking buffer. Anti-cytokeratin 5 rabbit antibody ab24647 from Abcam (Cambridge, UK) was used at a dilution of 1:200. Anti-cytokeratin 8 rabbit antibody ab53280 from Developmental Studies Hybridoma Bank (Iowa City, IA) was used at a dilution of 1:200. Anti-acetylated tubulin mouse antibody T7461 from Sigma-Aldrich (St. Louis, MO) was used at a dilution of 1:300. Anti-pan-cytokeratin mouse antibody C2562 from Sigma-Aldrich was used at a dilution of 1:400. Alexafluor 488 and Alexafluor 594 secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA).

**RT-qPCR**

To obtain RNA from organoids in 3D culture, cells were first separated from the Matrigel by incubating cultures in Corning Cell Recovery Solution for 20 minutes at 4 °C, then centrifuged at 500 x g for two minutes at 4 °C. Supernatant was removed and pellet was rinsed by resuspending in ice-cold PBS, then centrifuged at 500xG for 2 minutes 4 °C. The pellet was again rinsed with ice-cold PBS, and then resuspended in Qiagen RLT buffer supplemented with 0.01% β-mercaptoethanol. RNA was then extracted from samples using Qiagen’s RNEasy kit, then converted to cDNA using MMLV transcriptase. Sample cDNA was then used in SYBR green (Sigma) qPCR assays using the following primers:

Cytokeratin 5: F: 5’-AGGATGTTGGATGCTGCCCTAC-3’

R: 5’-CCATGGAAGGACCACAGAT-3’
Mouse-specific binding of primers for Cytokeratin 5, FoxJ1 and Scgb1a1 had previously been verified (Supplemental Figure 3). CT values were read by the thermocycler via fluorescence of SYBR green, and were then standardized against GAPDH expression in each culture to produce ΔCT values. These were then standardized against the average ΔCT value for WT in each condition to produce ΔΔCT values. Fold change was calculated according to the following equation:

\[
\text{Fold change} = 2^{-\Delta\Delta CT}
\]

Results

GLRX is not essential for mouse tracheal basal cells to form tracheospheres in 3D culture.

Research from our group has shown that oropharyngeal administration of GLRX protein in fibrotic mouse lungs has the effect of reversing existing pulmonary fibrosis. Airway basal cell gene signatures have recently been observed in alveolar areas of IPF lung, suggesting that basal cells play a role in IPF pathology. To better understand the roles played by GLRX during the
morphogenesis and injury repair of lung tissues, we harvested mouse tracheal basal epithelial cells (mTECs) from both wild type (WT) and GLRX -/- (KO) mice and cultured them in 3D conditions, which allowed for their differentiation into secretory or ciliated epithelia (Figure 2A). After 25 days of culture in 3D hydrogel, both WT and GLRX KO mTECs had developed spherical, fluid-filled organoids (tracheospheres) and light microscopy showed no obvious differences in organoid appearance between the two conditions (Figure 2B).

**Figure 2.** WT and GLRX KO mTECs develop spherical organoids in 3D culture. **A.** Graphical image depicting 3D cell culture of WT or GLRX KO mTECs. **B.** Both WT (left panel) and GLRX KO mTEC (right panel) cultures were able to form fluid-filled organoids when cultured for 25 days in 3D conditions.

Gene expression data obtained via qPCR verified a lack of GLRX expression in the GLRX KO condition, as compared to WT controls (Figure 3). Additionally, elevated expression of basal cell marker keratin 5, as well as lowered expression of ciliated cell marker FoxJ1 and secretory
cell marker Scgb1a1, were found in the GLRX KO condition, as compared to WT, yet these differences were not statistically significant (Figure 3).

**Figure 3.** WT and GLRX KO mTEC tracheospheres do not show significantly different differentiation patterns. While not statistically significant, expression of basal cell marker keratin 5 was slightly higher, while ciliated cell marker FoxJ1 and secretory cell marker Scgb1a1 were slightly lower in GLRX KO tracheospheres, as compared to WT tracheospheres. Presence of GLRX in the WT condition and absence in the GLRX KO condition are confirmed. Each data point represents a biological replicate.

GLRX KO mTECs cocultured with NHLFs in 3D develop a phenotype characterized by aberrant differentiation and lumenal keratin deposition.

Fibroblasts are a key cell type in the wound healing process, interacting with multiple other types of cells and secreting ECM to resolve damaged tissues. During IPF, chronic alveolar epithelial cell injury results in sustained wound healing processes that lead to fibrotic scarring in the lung. To more closely mimic the in vivo lung environment, we next cocultured WT or GLRX KO mTECs with normal human lung fibroblasts (NHLF) in 3D conditions (Figure 4A). Markedly greater differences were seen between the WT and GLRX KO mTECs when they were cocultured with NHLFs than without them. After 25 days of coculture in 3D hydrogel, both WT and GLRX KO mTECs had again developed spherical organoids, yet the appearance of the organoids differed between the two conditions. Light microscopy showed a normal translucent phenotype in organoids grown from WT mTECs with NHLFs, while organoids grown from GLRX KO mTECs with NHLFs were abnormal in appearance and did not permit light to pass through (Figure 4B).
Figure 4. Tracheospheres from GLRX KO mTECs display abnormal morphogenesis when cocultured with NHLFs. A. Graphical image depicting 3D cell coculture of NHLF with WT or GLRX KO mTECs. B. WT mTECs cocultured with NHLFs form spherical organoids after 25 days of development in 3D culture, while GLRX KO mTECs cocultured with NHLFs form abnormal structures that are too dense for light to penetrate.

Using qPCR with species-specific primers (as mTECs came from mouse tissue and NHLFs came from human tissue), GLRX expression was again validated in the WT mTECs, while no expression was found in the GLRX KO mTECs. Basal cell marker keratin 5 was significantly higher, while ciliated cell marker FoxJ1 was significantly lower, in GLRX KO mTECs cocultured with NHLFs, as compared to WT mTECs with NHLFs (Figure 5). Secretory cell marker Scgb1a1 was elevated in the GLRX KO condition, but this result was not significant (Figure 5).
Figure 5. GLRX KO mTEC + NHLF tracheospheres display suppressed differentiation compared to WT mTEC + NHLF tracheospheres. Basal cell type marker keratin 5 was elevated in the GLRX KO condition. Ciliated cell type marker FoxJ1 was lowered in the GLRX KO condition. Secretory cell type marker Scgb1a1 was not significantly different in the two conditions. Presence of GLRX in the WT condition and absence in the GLRX KO condition are confirmed. Each data point represents a biological replicate.

The aberrant phenotype seen in organoids grown from GLRX KO mTECs with NHLFs suggested a critical role for GLRX in the differentiation of basal cells in the presence of fibroblasts found in the lung. To further understand this differentiation defect, we next used hematoxylin and eosin staining on the organoids grown from mTECs and NHLFs, which showed a high frequency of protein buildup in the interior lumenal space of organoids from GLRX KO mTECs, as compared to WT (Figure 6A). Immunofluorescence staining showed a lack of a lumenal, differentiated cell layer lining the inner side of the organoid epithelium in the GLRX KO condition, with both lumenal cell marker cytokeratin 8 (Figure 6B) and ciliated cell marker acetylated tubulin (Figure 7A) absent in most GLRX KO mTEC + NHLF organoids.
Figure 6. When cocultured with NHLFs, GLRX KO mTEC organoids show aberrant development. A phenotype characterized by protein buildup in the organoid interior was present with high frequency in the GLRX KO condition (A). Lumenal cell marker cytokeratin 8 (CK8) was largely absent from organoids with in the GLRX KO condition. Basal marker cytokeratin 5 (CK5) was present around the exterior of all organoids (B).

Quantification of the organoids showed a significant difference in acetylated tubulin expression (Figure 7B). While the protein buildup did not stain positive for keratin 5 or 8, a pan-cytokeratin staining showed the presence of high levels of keratin in the protein buildup in the aberrantly developed organoids, now dubbed “keratin bodies” (Figure 8A). When cultured in the presence of human lung fibroblasts, GLRX KO mTEC organoids had a significantly higher frequency of keratin bodies than did WT mTEC organoids. Organoids grown from GLRX KO
mTECs + NHLFs also had a significantly higher frequency of keratin bodies than organoids grown from either WT or GLRX KO mTECs alone. Coculture with NHLFs did not, however, produce a higher frequency of keratin bodies in WT mTEC organoids (Figure 8B).

Collectively, these results demonstrate the importance of GLRX during basal cell differentiation and tracheosphere morphogenesis in the presence of lung fibroblasts, and suggest a key role for GLRX in epithelial-fibroblast crosstalk during wound repair.

Figure 7. When cocultured with NHLFs, GLRX KO mTEC organoids lack a ciliated cell layer significantly more frequently than WT mTEC organoids. A. Immunofluorescence staining depicting ciliated cell marker acetylated tubulin largely absent from organoids in the GLRX KO mTEC + NHLF condition, as compared to WT controls. Basal cell marker cytokeratin 5 (CK5) was present around the exterior of all organoids. B. Quantification of mTEC organoids grown in the presence of NHLFs showing that the presence of acetylated tubulin was significantly less frequently found in the GLRX KO mTEC condition than the WT mTEC condition. Data were not available for the GLRX KO mTEC monoculture condition due to damage to samples during antibody staining. Each data point represents a biological replicate.
Figure 8. Keratin(s) comprise the protein buildup found in lumens of GLRX KO mTEC + NHLF organoids. A. Immunofluorescence imaging of pan-cytokeratin showing cytokeratin protein buildup in the lumenal spaces of the aberrant GLRX KO mTEC + NHLF organoids. B. Both GLRX KO and NHLF coculture conditions increase the frequency of these keratin bodies. Among organoids grown from mTECs alone, GLRX KO organoids had a significantly higher frequency of keratin bodies than did WT organoids. Among GLRX KO mTEC organoids, those grown from mTECs + NHLFs had a significantly higher frequency of keratin bodies than did those grown from mTECs alone. Each data point represents a biological replicate.

Discussion

Role of GLRX in normal airway basal cell differentiation

Previous studies have demonstrated airway epithelial cells abnormally populating alveolar regions of the lung in IPF patients\(^9\). Additionally, research from our group has shown that GLRX can reverse induced pulmonary fibrosis in mice\(^{12}\). In this study, we set out to identify the roles of GLRX in airway basal cells by isolating them from WT or GLRX KO mice and culturing them in 3D conditions that would allow for proper differentiation. Interestingly, GLRX was not, as we hypothesized, essential for normal differentiation into ciliated or secretory cells. GLRX KO mTECs grew organoids without significant difference in appearance from WT mTEC organoids (Figure 2), and expressed basal, ciliated and secretory cell markers at levels not significantly different
from WT mTEC organoids (Figure 3). However, as epithelia work in concert with other cell types during *in vivo* lung development, maintenance and disease, we next proposed that GLRX played a role in mTEC function in the presence of fibroblasts. To address this, we 3D cocultured mTECs with commercially-obtained normal human lung fibroblasts. Using this model, GLRX indeed appeared to be essential for the proper differentiation of airway basal cells into ciliated and secretory cells. When grown with NHLFs, GLRX KO mTEC organoids displayed a dense morphology (Figure 4), and failed to display normal differentiation into the normal, luminal ciliated and secretory cell types, as shown via qPCR data (Figure 5) and immunofluorescence data (Figures 6B and 7).

The process of removing mTECs from the mouse and seeding them in 3D culture is similar to wounding, where normal tissue architecture is interrupted, and the basal cells are left to reform functional structures. While the mechanisms through which GLRX supports proper basal cell differentiation are by no means clear yet, these data suggest that the signals provided by fibroblasts are abnormally received in GLRX KO mTECs. It is quite plausible that GLRX could alter signaling from fibroblasts to epithelia. For example, by reversing PSSG, GLRX liberates GSH molecules. So, if fibroblasts are placing epithelia under oxidative stress, increased cytosolic GSH levels due to GLRX activity could neutralize oxidants, protecting normal epithelial physiology. The idea that GSH is depleted in IPF is not new, as lower levels of GSH in fluid of the lung lining has been reported in IPF patients\(^\text{12}\). In addition to being an important antioxidant, GSH is also an important signaling molecule\(^\text{16}\). GLRX could therefore alter signaling from fibroblasts to airway basal cells either by liberating GSH to bind signaling proteins, or by deglutathionylating signaling proteins.

**Significance of luminal keratin in tracheospheres**

The keratin body tracheosphere phenotype observed in our experiments (Figures 6A, 8A) is dramatic in the degree to which the luminal space is filled with keratin protein. If a pulmonary
airway were similarly filled with keratin, massive airflow blockage would occur, and respiration would be inhibited. However, a real human airway would be unlikely to become obstructed in this way, due to the constant flow of gas through the region, as well as the constant removal of waste via cilia beating. In the alveoli, however, blockage by keratin is conceivable, given their near-spherical shape and lack of ciliated cells. Obstruction of gas exchange in the lung is a known cause of respiratory failure, as seen in chronic obstructive pulmonary disease (COPD)\textsuperscript{17}, but so far IPF research has provided little documentation of this kind of airway obstruction. Ultimately, our keratin body finding is most likely significant as a clue about the aberrant epithelia which are secreting keratin, rather than as a model of pathology in IPF.

**Implications for IPF pathology**

It is well established in the literature that aberrant epithelial differentiation plays a key role in the development of IPF\textsuperscript{7}. In addition, recent work has shown that airway basal epithelial cells are aberrantly located in alveolar regions of the IPF lung\textsuperscript{9}. Here, we present evidence that, in GLRX-deficient airway basal cells, the presence of fibroblasts leads to improper differentiation in 3D culture. Taken together with previous research showing airway cells abnormally located in alveolar regions of the IPF lung\textsuperscript{7}, and research showing that GLRX deficiency is implicated in IPF\textsuperscript{12}, our data add novel insight into the roles of GLRX in airway basal cell differentiation in IPF (Figure 9). Reasons for airway epithelial cell migration to the alveoli remain unknown, as do the specifics of how GLRX affects signaling from fibroblasts to airway basal cells. Our findings suggest, however, that airway basal cells rely on GLRX for their proper differentiation in the presence of lung fibroblasts and, given that GLRX expression is lowered in IPF, raise interesting questions about the status of GLRX expression in human IPF basal cells.
Figure 9. Fibroblasts suppress differentiation of GLRX-deficient epithelial basal cells. In our experiments, mTECs required GLRX to differentiate properly into ciliated and secretory epithelial cell types when cocultured with NHLFs. This, together with findings that aberrant epithelial differentiation plays a role in IPF pathology, and findings that GLRX is deficient in IPF, suggests that suppression of GLRX-deficient epithelial cell differentiation by fibroblasts plays a role in IPF pathology.

Future Directions

Further analysis of present 3D culture models

Understanding the mechanism through which differentiation was altered in the GLRX KO mTECs cocultured with NHLF will depend on a more detailed picture of gene expression. It is not obvious why the GLRX KO mTECs would be producing so much excess keratin, but a first step will be determining *which* keratin protein is secreted. To do this, we will be performing mass spectrometry on laser-microdissected keratin bodies from slides of the cocultures of GLRX KO mTECs and NHLF. Once the keratin body composition is characterized, a future experiment may use qPCR or SDS-PAGE/Western blot to examine the expression of the
particular keratin gene at different timepoints during tracheosphere development. It will also be important to explore links between the transcriptional regulation of genes required for production and secretion of this keratin protein, and genes required for differentiation to ciliated and secretory cell types.

In order to further examine the effects of GLRX in our 3D culture model system, we will be performing immunohistochemical stains for PSSG on our samples. By comparing our conditions, we will aim to examine where in our organoids GLRX is impacting PSSG levels, and how this is altered by the presence of fibroblasts.

Our analysis of qPCR data to determine expression of cell type markers relied on comparison to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH has traditionally been used as a control gene for RT-PCR, as it is expressed in all cells. Recent work, however, has shown that GAPDH expression levels can vary significantly in different tissues. In the past decade, research has identified a list of genes, including glucose-6-phosphate isomerase (GPI), whose expression levels are especially constant across tissues and conditions. If we ran our qPCR experiments again, using GPI as a housekeeping gene instead of GAPDH, we may obtain more accurate measures of gene expression for our genes of interest.

Our qPCR and immunohistochemistry data gave an indication of how cells had differentiated in our organoids, but only provided information on the expression of a few genes. Spatial transcriptomic data would provide a more complete picture of organoid development in our 3D culture conditions. A variety of spatial transcriptomic approaches exist, with the common aim of obtaining data on the expression of different genes in different regions of a tissue sample. These approaches generally involve imaging a tissue sample, assigning a barcode to a tissue region or even a single cell, and performing RNA sequencing on individual regions or cells from the sample. Performing such experiments would allow us to identify a wide variety
of changes in gene expression in GLRX KO mTEC + NHLF organoids, beyond a single cell type marker.

**Effects of fibroblast activation on suppression of epithelial differentiation**

Having shown that NHLFs suppress GLRX KO mTEC differentiation, we will next examine how fibroblast activity modulates this effect. Future experiments may include mTECs cocultured with NHLFs of varying concentrations, NHLFs that have been incubated in TGF-β, and NHLFs that have been incubated in BMP4. We will aim to determine whether myofibroblast transdifferentiation or ECM secretion are important to the differentiation-suppressing effect NHLFs displayed towards GLRX KO mTECs. Fibroblast activation could upregulate a signaling mechanism that leads to the effects seen in our experiments, or it could simply lead to fibroblast proliferation and resultant increase in fibroblast number. Either way, a causal link between NHLF activation and the suppression of GLRX KO mTEC differentiation could have major implications for our understanding of IPF.

**Effects of mTECs on fibroblast activation**

It has been shown that healthy epithelia suppress fibroblast activation⁶, and that aberrant epithelia induce fibroblast activation⁷, yet we do not know whether these effects are present in our 3D cocultures of NHLFs with WT and GLRX KO mTECs. In the future we will analyze the expression of α-SMA to determine whether the WT and GLRX KO mTECs differ in their ability to suppress myofibroblast activation. Data on α-SMA expression will be specific to NHLFs and their progeny, as we cocultured cell types from different species, (mTECs from mice and NHLFs from humans). These data, along with data from cocultures of WT and GLRX KO mTECs with TGF-β- and BMP4-incubated NHLFs will allow us to develop more informed hypotheses about the nature of epithelium-fibroblast crosstalk in our organoids and in IPF.
Hypothesized positive feedback mechanism

A hypothesis that I find quite intriguing is that the activation of NHLFs by aberrant mTECs and the suppression of mTEC differentiation by active NHLFs could act in a mutually reinforcing manner. Validation of this hypothesis will depend on the results of the experiments suggested above, but could uncover a potent positive feedback mechanism driving IPF (Supplemental Figure 1). In this hypothetical mechanistic model, GLRX-deficient epithelium is present in the damaged alveolar region of IPF lung, possibly as a result of epithelial cell migration from the airway as described above. Signaling from fibroblasts in the alveolus suppresses differentiation of this epithelium, yielding aberrant epithelium. This aberrant epithelium then activates those fibroblasts via TGF-β and other signaling molecules, thereby increasing the suppression of epithelial differentiation. As fibroblasts become increasingly activated, they transdifferentiate into myofibroblasts, which secrete large amounts of ECM stiffening the lung tissue and disrupting alveolar gas exchange. This model could explain the ability of GLRX to resolve bleomycin induced lung fibrosis in mouse models, as blocking the differentiation-suppressive signal from fibroblasts to epithelia could halt the mechanism of fibrosis generation.
Supplemental Figure 1. A hypothesized model of positive feedback between aberrant epithelial differentiation and fibroblast activation. In this model, aberrant epithelium fails to block fibroblast activation via BMP and other signaling molecules, and instead activates fibroblasts via TGF-β and other signaling molecules. Activated fibroblasts transdifferentiate into myofibroblasts and secrete ECM, producing fibrosis. They also suppress epithelial differentiation in the absence of GLRX, producing more aberrant epithelium.

**Conditional Knockout of GLRX**

In order to more accurately identify the roles of GLRX during mTEC differentiation, our lab has also generated a tamoxifen inducible GLRX conditional deletion mouse line. We recently performed 3D culture of mTECs which had LoxP sites inserted around the GLRX gene, and a Cre recombinase gene inducible by the drug tamoxifen via the keratin 5 promoter sequence. We grew these cultures in 3D with tamoxifen doses of 0nM, 100nM, 250nM, and 500nM. Interestingly, despite confirming GLRX knockout with all non-zero tamoxifen doses, only the 500nM dose produced an increase in basal cell marker keratin 5 (Supplemental Figure 2). It is suspected that this may be due not only to the absence of GLRX, but to the presence of high levels of tamoxifen itself, or possibly Cre recombinase. In order to control for the effects of tamoxifen and isolate the
effects of GLRX, we will repeat the experiment, this time using two additional mTEC cell lines. The first will be normal mTECs, and the second will express Cre recombinase in response to tamoxifen, but will not have any LoxP sites around its GLRX gene. These three experiments run in parallel will allow us to separate the effects of tamoxifen, Cre recombinase, and GLRX expression. In addition to verifying knockout of GLRX via qPCR, we will also run western blots for organoids at different time points during development, in order to determine how long glutaredoxin protein remains present in the cell after tamoxifen in introduced. If successful, this model system will allow us to examine the effects of GLRX knockout in mTECs, independent of any departures from the wild type phenotype that occur as a result of GLRX absence during mouse development.

Supplemental Figure 2. Tamoxifen-induced conditional knockout of GLRX increases expression of basal cell marker keratin 5 (K5) at a dosage of 500 nM. In mTEC organoids, expression of basal marker keratin 5 was increased at 500 nM compared to other doses. Expression of mitotic marker Ki67 was not significantly different at increasing tamoxifen doses. GLRX presence is shown with 0 nM tamoxifen and absence is shown at 100, 250 and 500 nM.
Appendix

Supplemental Figure 3. Primers for keratin 5 (K5), FoxJ1 and Scgb1a1 do not amplify cDNA from NHLFs. cDNA quantity fold change for keratin 5, FoxJ1 and Scgb1a1, using amplification primers specific to mouse genes, is shown for WT mTEC + NHLF cocultures alongside NHLF monocultures.

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References


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