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Transcription factor FOXF1 identifies compartmentally distinct mesenchymal cells with a role in lung allograft fibrogenesis

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ABSTRACT

In this study, we demonstrate that Forkhead Box F1 (FOXF1), a mesenchymal transcriptional factor essential for lung development, is retained in a topographically distinct mesenchymal stromal cell population along the bronchovascular space in an adult lung and identify this distinct subset of collagen-expressing cells as a key player in lung allograft remodeling and fibrosis. Utilizing Foxf1_tdTomato BAC (Foxf1_tdTomato) and Foxf1_tdTomato;Col1a1_GFP mice, we show that Lin⁻Foxf1⁺ cells encompass the Sca1⁺CD34⁺ subset of collagen I-expressing mesenchymal cells (MCs) with capacity to generate colony forming units and lung epithelial organoids. Histologically, Foxf1-expressing MCs formed a three-dimensional network along the conducting airways; FOXF1 was noted to be conspicuously absent in MCs in the alveolar compartment. Bulk and single-cell RNA sequencing confirmed distinct transcriptional signatures of Foxf1⁺/neg MCs, with Foxf1-expressing cells delineated by their high Gli1 and low Integrin a8 expression, from other collagen-expressing MCs. Foxf1⁺Gli1⁺ MCs demonstrated proximity to Sonic hedgehog (Shh) expressing bronchial epithelium, and mesenchymal Foxf1/Gli1 expression was found to be dependent on the paracrine Shh signaling in epithelial organoids. Utilizing a murine lung transplant model, we show dysregulation of the epithelial mesenchymal Shh/Gli1/Foxf1 crosstalk and expansion of this specific peri-bronchial MC population in chronically rejecting fibrotic lung allografts.

Words: 197
INTRODUCTION

It is increasingly recognized that characterizing mesenchymal cell (MC) populations within distinct anatomic locations is key to understanding local homeostatic and regenerative processes as well as delineating unique pathogenic mechanisms of diverse fibrotic diseases. Lung is a vital organ with anatomically and functionally distinct conducting and gas-exchange units, which harbor histologically identifiable, specialized cell populations. The presence of resident mesenchymal progenitors in the human adult lungs was highlighted by our finding of donor-derived multipotent mesenchymal stromal cells in sex-mismatched allografts (1). Subsequent studies utilizing transgenic mice and single-cell RNA-seq have shed novel light on various MC subpopulations residing within the lung (2-4). However, despite identification of a plethora of genes, characterization of anatomically and functionally distinct collagen-expressing MC populations remains limited. Furthermore, majority of studies focus on interstitial pulmonary fibrosis utilizing the bleomycin injury models with lack of investigations into other fibrotic processes such as the conducting airway fibrosis which marks chronically rejecting lung allografts.

Here we utilize the expression of FOXF1, a key transcription factor expressed in developing lung mesenchyme, to identify a unique subset of Col1+ MCs which reside within a specific peri-bronchial niche in an adult lung and contributes to allograft fibrogenesis. FOXF1, is a Shh/Gli1 target expressed in lateral mesoderm during embryogenesis, essential for branching morphogenesis and lung development (5, 6), and unique among embryonic transcription factors in that its expression persists in adult lungs (5, 7). We have previously demonstrated that mesenchymal stromal cells derived from human adult lung allografts (lung-MSCs) express FOXF1 (1, 7, 8). However, whether persistent expression of FOXF1 in adulthood marks all or a subset of collagen-expressing MCs is unknown, and the in-situ niche of these cells remained to be
determined. Present studies of Foxf1-tdTomato BAC and bi-transgenic Foxf1_tdTomato;Colla1_GFP mice reveal that FOXF1 expression demarcates a distinct collagen-expressing MC population with a unique transcriptional profile and anatomic localization. The demonstration of an in-situ peri-bronchial sub-epithelial niche of Foxf1+Gli1+ cells, their in-vitro ability to support epithelial cell organoids, and their in-vivo expansion in response to airway-centric injury post-lung transplantation reflects their participation in homeostatic/fibrotic responses within this specific compartment.
RESULTS AND DISCUSSION

Embryonic transcription factor FOXF1 is retained in the Sca1⁺CD34⁺ mesenchymal stromal cells subset of collagen 1-expressing cells in the adult lung. To characterize the Foxf1 expressing lung-resident MC population, C57BL/6J-Tg(Foxf1_tdTomato) (Foxf1_tdTomato) mice were generated utilizing a modified bacterial artificial chromosome (BAC) in which exon 1 of the Foxf1 gene was replaced with the coding sequence of tdTomato (Supplemental Figure 1A). A distinct tdTomato fluorescent population was identified in the adult transgenic mouse by FACS analysis with the viable sorted cells demonstrating higher expression of Foxf1 and collagen1α1 (Figure 1A). The tdTomato expression faithfully recapitulated endogenous expression of FOXF1 with tdTomato⁺ cells demonstrating lack of expression of epithelial marker EpCAM and immune marker CD45 (Figure 1B). As expected, the tdTomato⁺ population encompassed CD31⁺ endothelial cells which are known to arise from embryonic lung mesenchyme and express FOXF1 in adulthood (9). Immunostaining demonstrated similar staining pattern for endogenous FOXF1 protein and tdTomato fluorescence driven by BAC_Foxf1 (Supplemental Figure 1B). The Lin(CD45/CD31/EpCAM)negFoxf1_tdTomato⁺ population uniformly expressed the mesenchymal surface marker-platelet derived growth factor receptor alpha (PDGFRα), and the progenitor cell markers-stem cell antigen 1 (Sca1) and CD34 (Figure 1C, 1D). A majority of Lin⁻Foxf1_tdTomato⁺ cells also stained positive for CD44 while CD73 staining was limited to approximately one third of the cells (Figure 1D). Mesenchymal phenotype of Lin⁻Foxf1_tdTomato⁺ cells was further confirmed by staining for vimentin and prolyl-4-hydroxylase (Supplemental Figure 1C).

To evaluate if FOXF1 is expressed in all lung-resident Col1⁺ MCs or represents a MC sub-population, Foxf1_tdTomato mice were crossed with Collagen1α1 promoter-driven
GFP(Col1GFP) mice. FACS revealed two distinct Col1GFP populations demarcated by the presence or absence of Foxf1_tdTomato in Foxf1_tdTomato;Col1a1_GFP bi-transgenic adult lungs (Figure 1E). FOXF1 protein expression was restricted to the Foxf1_tdTomato+ subset of collagen 1-expressing MCs (Figure 1F). The Foxf1+Col1+ MCs formed significantly more colony forming units (CFUs) (Figure 1G) and demonstrated higher Sca1 and CD34 expression than the Foxf1−Col1+ subpopulation (Figure 1H). The Lin−Sca1+ population has been demonstrated to mark multipotent mesenchymal progenitors in an adult lung (10). Flow cytometry in adult Foxf1_tdTomato BAC mice demonstrated that Lin−Sca1+ cells uniformly express FOXF1 (Figure 1I). Transient overexpression of Foxf1 in murine lung fibroblasts induced Sca1 expression, further suggesting positive correlation between these markers in MCs (Figure 1J). Together, these data indicated that expression of embryonic lung mesenchyme associated transcription factor, FOXF1, is restricted to a subset of collagen-expressing MCs in an adult lung and overlaps with progenitor cell marker, Sca1.

**Topographic confinement of FOXF1 to three-dimensional mesenchymal cell network along the conducting airways.** Next, to investigate the localization of the FOXF1-expressing MC population in an adult lung, FOXF1 staining was performed in Col1GFP transgenic mice. As can be appreciated by zooming into the Scan Large Image (Nikon Elements, USA), co-localization of FOXF1 staining (red) with intrinsic Collagen 1α1-GFP (green) was noted to be confined to spindle cells in the bronchovascular areas, in association with bronchi and vessels, and interspersed in the adjoining loose interstitium (Figure 2A). Foxf1+Col1GFP+ MCs lay in close apposition to club-cell secretory protein (CCSP)-expressing bronchial epithelial cell (white), suggesting that these cells are important components of the airway progenitor cell niche (Figure 2B, Supplemental
Figure 2A). This network of cells extended in a three-dimensional manner from the proximal to the distal airways (Figure 2B, insets 1-3). Sub-epithelial MCs in the lung airways have been identified histologically as distinct intermeshed spindle-shaped cells; the unique appearance of this layer of thin MCs, with its large surface area and close proximity to the epithelial/environmental interface, lead it to be termed the attenuated fibroblast sheath with similar cells in the gut identified as the peri-cryptal fibroblast sheath (11). Interestingly, along with finding that FOXF1 marks the MCs in this anatomically distinct location in the lung, our Foxf1_tdTomato BAC mice revealed expression of FOXF1 in the peri-cryptal MCs in the gut (data not shown).

Co-expression of FOXF1 was notably absent in the Col1GFP+ cells in the alveoli where FOXF1 positivity was restricted to endothelial cells (Figure 2B, inset 4; Supplemental Figure 2B). Confocal imaging and co-staining of type 1 and type 2 pneumocytes with aquaporin 5 and TTF-1 was utilized to confirm the absence of FOXF1 expression in MCs of the alveolar niche (Supplemental Figure 2C). On quantitation, FOXF1 nuclear staining was evident in 76.4% of Col1GFP+ cells within the bronchovascular unit, while 93.9% of MCs in the alveolar space were demonstrably negative for FOXF1 nuclear expression (Figure 2C).

The transgenic PDGFRαEGFP mouse, where PDGFRα promoter drives the expression of the H2B-eGFP fusion gene, was utilized to corroborate these findings. In the PDGFRαEGFP adult lung, FOXF1 staining co-localized with endogenous PDGFRα-GFP nuclear expression in MCs near the airways but was absent in the PDGFRα+ alveolar MCs (Figure 2D). Immunofluorescence staining in human adult lungs confirmed the peri-bronchiolar niche of the FOXF1-expressing MCs where FOXF1 expression was noted in MCs interspersed between the smooth muscle and bronchial epithelium (Figure 2E).
Bulk and single-cell RNA sequencing confirm distinct transcriptional signatures of Foxf1+ and Foxf1− resident Coll1+ MC populations. The transcriptome profiles of freshly sorted Col1GFP+/Foxf1_tdTomato pos/neg MCs were compared by Affymetrix assay. Coll1−/Foxf1+ endothelial cells were included as controls (Supplemental Figure 3A). Principal component analysis and unsupervised clustering indicated that the two mesenchymal populations have unique gene signatures (Figure 3A; Supplemental Figure 3B). Consistent with their unique anatomic niches, comparison of biological processes gene ontology (GO) terms related to epithelium demonstrated statistically significant differences between the two populations (Supplemental Table 1). A previous study classified two distinct matrix fibroblast cell populations based on Coll13a1 and Coll14a1 expression (3). Foxf1_tdTomato+Col1GFP+ cells were found to express a Coll14a1 matrix fibroblast signature (Coll14a1, Pi16, Cygb, Rtp4, Rbp4, Meg3), while Foxf1_tdTomato−Coll1GFP+ cells exhibited a Coll13a1 matrix fibroblast signature (Coll13a1, Itga8, Npnt, Cxcl14, Cdh11, Tcf21) (Figure 3B). In addition to Foxf1, SHH signaling gene Gli1, a transcriptional promoter of Foxf1 expression (12), was upregulated in Col1GFP+Foxf1_tdTomato+ as compared to Col1GFP+Foxf1_tdTomato− MCs (Figure 3B). This was further investigated by utilizing tamoxifen-treated Gli1CreERT2/WT;RosaEYFP/WT adult mice. Flow cytometry analysis revealed that similar to Foxf1_tdTomato+ MCs, >80% of Gli1CreERT2-EYFP labeled cells are Sca1+ (Supplemental Figure 3C). Immunofluorescence staining demonstrated nuclear FOXF1 expression in Gli1-GFP expressing sub-epithelial peri-bronchial MCs; no significant GLI1 expression was noted in the alveoli (Figure 3C).

Affymetrix analysis identified cell surface molecule integrin alpha 8 (Itga8) to be highly upregulated in Col1GFP+Foxf1_tdTomato− cells (Figure 3B). Immunofluorescence staining of Col1GFP+ adult lungs confirmed ITGA8 co-staining in Col1GFP+Foxf1− cells in the alveolar
space and its absence in Col1GFP⁺Foxf1⁺ cells of the peri-bronchiolar space (Figure 3D). FACS sorting of lung MCs based on ITGA8 expression confirmed lower mRNA expression of Foxf1/Gli1 in ITGA8⁺ MCs (Supplemental Figure 3D, 3E). Freshly sorted Lin−PDGFRα⁺ITGA8pos/neg cells were studied by time-lapse microscopy and high content analyses of live-cell migration, proliferation, and morphology (Supplemental Figure 4). Very distinct phenotypes were observed with ITGA8⁻(Foxf1⁺Gli1⁺) MCs demonstrating higher migratory and proliferative capacity. The shapes and areas of the whole cells and nuclei between the two cell populations were significantly different, shown by single-cell linear discriminant analysis (Wilcoxon Rank-Sum test; p<0.0001).

We next performed single-cell RNA-sequencing (scRNA-seq) on CD45⁻CD31⁻ populations sorted from adult murine lungs. Using nonnegative matrix factorization and Louvain community detection, we identified 14 transcriptionally-distinct cell populations (Figure 3E; Supplemental Figure 5). Most cells belonged to two large clusters characterized by high expression of Coll1a1, with high expression of Foxf1 and Itga8; and we refer to them as Foxf1_MC and Itga8_MC, respectively (Figures 3E, 3F). Consistent with our results above, our scRNA-seq data indicated a significant correlation (Pearson ρ = 0.64, p < 2⁻¹⁶) between the expression of Foxf1 and Gli1 within the same cell (Figure 3G). Foxf1_MC and Itga8_MC clusters also distinguished themselves by the expression of discriminatory Col13a1 and Col14a1 genes previously identified on scRNA-seq analysis by Xie et al. (3) (Figure 3F). Foxf1_MC cluster encompasses sub-bronchial and both subpopulation of cuff Col1⁺ MCs described by Tsukui et al. (2). The RNA velocity vectors for the MC clusters revealed an interesting trend: the arrows point from Foxf1_MC → Itga8_MC, suggesting that transcriptional changes proceed primarily in this direction, rather than from Itga8_MC → Foxf1_MC or in random directions (Supplemental Figure 6). The continuous nature of the Foxf1 and Itga8 MC populations combined with the velocity
analysis further raises the intriguing possibility that there may be a spatial gradient of transcriptional variation among these cells.

**The Foxf1⁺Gli1⁺ positive mesenchymal cells as a significant contributor to airway fibrosis in an orthotopic mouse lung transplant model.** Together this work identified a Foxf1⁺Gli1⁺Itga8⁻Col1⁺ MC population residing in a unique niche associated with bronchovascular bundles (BVB-MCs). Bronchovascular bundles are also the primary site of the allogeneic insult in a transplanted lung with acute rejection marked by lymphocytic infiltration of blood vessels and airways, and chronic rejection presenting as peri-bronchiolar and peri-vascular fibrosis. Our human studies have revealed mobilization of Foxf1-expressing lung-MSCs during post-transplant injury period with an increase in their numbers early post-transplant and in chronically rejecting grafts (8). We have also recently demonstrated that Foxf1 acts as suppressor gene in human lung-MSCs with its downregulation promoting cellular proliferation and migration (13). As FOXF1 is a Shh/Gli1 target (6, 12), we hypothesized that Shh expression in bronchial epithelial cells is key in maintaining Gli/Foxf1 expression in BVB-MC and that its dysregulation marks airway fibrosis.

The proposed Shh/Gli/Foxf1 paracrine signaling between epithelial and mesenchymal components was first studied in-vitro by utilizing epithelial organoid formation. Col1GFP⁺Foxf1_tdTomato⁺ MCs promoted formation of organoids when co-cultured with freshly isolated bronchioalveolar stem cells (BASCs) from Rosa26<sup>miTm</sup>G mice in matrigel (47.5±3.29/well, n=6) (Figure 4A). Gene expression analysis confirmed the exclusive expression of Shh and Foxf1 in the sorted epithelial and mesenchymal component of the organoids, respectively (Figure 4B). The organoid-forming capacity of Col1GFP⁺Foxf1_tdTomato⁺ MCs was significantly higher than
of Col1GFP<sup>+</sup>Foxf1<sub>_tdTomato</sub><sup>−</sup> MCs (Supplemental Figure 7A, 7B). Foxf1<sub>_MCs</sub> in organoid co-cultures demonstrated higher Foxf1/Gli1 mRNA expression compared to those grown in Matrigel alone (Figure 4C). Presence of the Shh signaling/Smoothened antagonist LDE225, inhibited Foxf1/Gli1 mRNA expression in Foxf1<sub>_MCs</sub> grown in co-cultures compared to that in matrigel alone, demonstrating contribution of epithelial-derived Shh in maintaining expression of Foxf1/Gli1 in Foxf1<sub>_MCs</sub> (Figure 4C).

Next, we utilized a recently described F1→parent orthotopic murine lung transplant model of chronic lung allograft dysfunction to investigate if disruption of Shh/Gli1/Foxf1 crosstalk accompanies airway remodeling. This B6D2F1/J→C57BL6/J model of lung transplantation demonstrate evolution from marked lymphocytic infiltration with bronchial epithelium and endothelial injury to robust peri-bronchial and peri-vascular fibrosis (14). Affymetrix analysis of the whole lung lysate at various timepoints post-transplant showed persistent decrease in Shh and Foxf1 expression in the allografts, compared to isografts (Supplemental Figure 7C). To confirm their loss in specific cellular compartments, epithelial (CD45<sup>−</sup>CD31<sup>−</sup>P<sub>DGFRα</sub><sup>−</sup>EpCAM<sup>+</sup>) and mesenchymal (Lin<sup>−</sup>PDGFRα<sup>+</sup>) cells were sorted from single cell suspensions of grafts at day 28 post-transplantation. Loss of Gli1/Foxf1 mRNA expression in MCs and a concomitant decrease in Shh expression in the epithelial cells was seen in the allografts (Figure 4D). To track BVB-MCs undergoing a loss of Foxf1/Gli1 expression after lung transplantation, we elected to stably label these cells utilizing the Gli1<sup>CreERT2/WT;Rosa26<sup>mTmG</sup>/WT</sup> transgenic mouse (Figure 4E). By day 14, Gli1<sup>CreERT2/WT;Rosa26<sup>mTmG</sup>/WT</sup> donor lung allografts demonstrated a robust peri-bronchiolar and peri-vascular expansion of Gli1-labeled cells, with loss of CCSP-expressing epithelial cells and sub-bronchial fibrosis (Figure 4F). Day 28 post-lung transplant allografts demonstrated similar extensive expansion of Gli1<sup>+</sup> MCs (Supplemental Figure 7D, 7E). ITGA8 staining of these
allografts demonstrated continued lack of ITGA8 in Gli1+ airway MCs as well as no significant expansion or myofibroblast differentiation in ITGA8+alveolar MCs, suggesting that fibrotic activation is limited to BVB-MCs in rejecting allografts (Supplemental Figure 7F). Comparison of MC subsets (Lin−PDGFRαITGA8− BVB-MCs and Lin−PDGFRαITGA8+ alveolar-MCs) sorted from isografts and allografts confirmed upregulation of Col1a1 mRNA in BVB-MCs in allografts with no change noted in alveolar-MCs (Supplemental Figure 7G). Similarly, only BVB-MCs demonstrated upregulation of Autotaxin(Atx), a lysophospholipase D enzyme which produces pro-fibrotic mediator lysophosphatidic acid and has been shown to be key in autocrine fibrotic activation of lung MCs and allograft fibrogenesis (15). FOXF1 has been identified in our recent study as a transcriptional repressor of ATX in lung-MSCs (13), suggesting a unique fibrotic differentiation program in rejecting allografts with loss of FOXF1 in BVB-MCs leading to ATX upregulation and fibrotic expansion.

Taken together, our results suggest that anatomically localized Foxf1+Gli1+Itga8− BVB-MCs are the key contributors to fibrogenesis in a rejecting allograft. Our data on downregulation of Shh/Gli1/Foxf1 signaling during allograft fibrogenesis is consistent with prior studies where Shh was shown to regulate airway quiescence with its inhibition leading to peri-bronchial fibrosis (16). This combined with our recent demonstration of repressive functions of FOXF1 and its role as a brake on MC activation (13), suggests that FOXF1 is a likely mediator of Shh-induced quiescence of the airway compartment.

The simple cell surface markers identified for isolating phenotypically, transcriptionally, anatomically, and functionally distinct BVB- and alveolar-MCs in this study, lays the foundation for future work investigating specific mechanisms by which these cells support their respective epithelial niches and compartmentalized fibrotic injury. Our data also reinvigorates the interest in
mesenchymal progenitors by demonstrating the niche along the conducting airways of a Foxf1^{+}Sca1^{+} MC population with CFU potential and an ability to sustain epithelial organoids. RNA velocity analysis on scRNA-seq data supported the transcriptional changes proceeding in the direction from bronchovascular compartment localizing Foxf1_{MCs} to alveolar compartment residing Itga8_{MCs}. Further investigations are needed to ascertain the role of these cells as potential progenitors for other MC populations and for characterization of further specific subpopulations within this population.
METHODS

Refer to Supplemental Material for more details.

Study approval. All animal experiments were performed in accordance with protocols approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC).

Accession number. RNA-seq databases with accession no. GSE176476 for data presented in Figures 3A-B/Supplemental Figures 3A-B, and single-cell RNA-seq databases with accession no. GSE179034 for data presented in Figures 3E-H/Supplemental Figure 5, were deposited in the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/).
REFERENCES


FIGURE 1

A. C57BL/6 Wildtype C57BL/6 Foxf1 tdTomato

B. SS A

C. Lin Pac-Blue/DAPI Foxf1 tdTomato

D. Percent of Total

E. CD45-PerCP Col1a1

F. CD31-PacBlue FO XF1

G. Sca1-APC-Cy7 CD34-APC

H. CD45-PerCP Sca1-APC-Cy7

I. Percent Total

J. GAPDH FOXF1

TD+ TD- TD+ TD- TD+ TD-
Figure 1. Foxf1_tdTomato labels Sca1⁺ mesenchymal cells in the adult lung. (A) Flow cytometric analysis of single cell suspension from lungs of adult C57BL/6J-Tg(Foxf1_tdTomato) BAC mice. Real time PCR analysis in Foxf1_tdTomato⁺(TD⁺) or Foxf1_tdTomato⁻(TD⁻) sorted cells. (B) TD⁺ populations from (A) were analyzed for indicated markers. (C&D) Lin⁻(CD45⁻ CD31⁻ EpCAM⁻) TD⁺/neg cells from Tg(Foxf1_tdTomato) BAC mice were analyzed for shown cell surface markers. (E-H) Flow cytometric analysis of adult lungs from Foxf1_tdTomato;Col1GFP mice. Foxf1_tdTomato(TD)⁺/neg Col1⁺MCs were sorted and analyzed for FOXF1 expression by western blot (F), and Colony Forming Unit (CFU) assay (G; n=7). (H) Sca1 and CD34 expression in Lin⁻ Col1_GFP⁺PDGFRα⁺Foxf1_tdTomato(TD)⁺/neg cells. (I) Characterization of Lin⁻ Sca1⁺ population in adult Foxf1_tdTomato BAC mice by flow cytometry (n=7). (J) Sca1 cell surface expression in Foxf1-overexpressing murine lung fibroblasts. Values=means±SD. *p<0.05, ***p<0.001, Statistics: two-tailed unpaired t test (A,G).
FIGURE 2

A. Col1GFP

B. Col1GFP

C. Col1GFP

D. PDGFRα GFP

E. Human Lung

A. Bronchi Alveoli

B. Bronchi Alveoli

C. Bronchi Alveoli

D. Bronchi Alveoli

E. Bronchi Alveoli
**Figure 2. In situ niche of FOXF1-expressing Col1+ mesenchymal cells.** (A-C) Adult lungs of Col1_GFP mice stained with FOXF1(red) and CCSP (white). Representative scan large image of entire lung (A) and four locations along the bronchial tree (B; Scale bars:50 μm). Arrows demonstrate Foxf1*Col1*MCs along the bronchovascular bundle. (C) Quantification of nuclear FOXF1 intensity in Col1GFP+ cells in bronchovascular space (Bronchi) and the alveolar interstitium (Alveoli). n=3. (D) Confocal imaging and quantification of PDGFRα_GFP mouse lungs stained with anti-FOXF1 antibodies. Co-localization of FOXF1 (red) and PDGFRα (green) in peri-bronchial region (Cyan arrows) with no co-staining noted in alveoli. n=3; Scale bars:20 μm (E) Adult human lung stained with FOXF1 (green) and smooth muscle marker (α-SMA;red) Scale bars:50 μm. AW:airway; BV:blood vessel. Values=means±SD; *p<0.05, **p<0.01, ****p<0.0001. Statistics:one-way ANOVA (Bonferroni;C), unpaired t test (D).
FIGURE 3

A. Col1GFP+ TD' ECs

B. Col1GFP+ TD' MCs

C. Gli1CreERT2/WT;RosamTmG/WT

D. Collagen1α1 GFP

E. UMAP1

F. UMAP2
Figure 3. Bulk and single cell transcriptome analysis of Foxf1_MCs. (A) Principal component analysis plot from affymetrix analysis of MC populations sorted from adult Foxf1_tdTomato;Col1_GFP mice. (B) Heatmap comparing Foxf1_tdTomato positive (TD⁺) or negative (TD⁻) CD45-Col1⁺MCs for selected genes. (C&D) FOXF1 immunostaining in adult lungs of Gli1CreERT2/WT;Rosa26mTmG/WT (C) and Col1GFP (D) mice. n=3; Scale bars:50μm (inset:10 μm) (E) Uniform Manifold Approximation and Projection (UMAP) representation of 11,118 single-cell RNAseq profiles; each dot represents a single cell. Cells are colored to indicate their membership in one of 14 clusters. (F) UMAP plots of specific genes. The coordinates are same as in (E), but the color indicates the expression level of the gene within each cell. (G) Correlation plot from single-cell RNAseq analysis; Statistics:Pearson’s correlation.
FIGURE 4

A. **Brightfield**

Col1GFP⁺
Foxf1_TD⁺
MCs

*td_Tomato* BAsCs

Day 8 Organoid

B. **Day 8 Organoid**

Col1GFP⁺ Foxf1_TD⁺ MCs

**GFP**

**mRNA Expression**

C. **mRNA Expression**

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D. **Relative mRNA expression**

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E. **Tam Start**

**Tam Stop**

(Age of mouse)

Wildtype Recipient

Lung Transplant

F. **Gli1CreERT2/WT;Rosa26mTmG/WT**

*αSMA* GFP CCSP

*GFP* CCSP
Figure 4. FOXF1/Gli1 expression and expansion of Gli1+ mesenchymal cells in an orthotopic mouse lung transplant model. (A) Generation of three-dimensional epithelial organoids in co-culture of Foxf1_tdTomato*Col1GFP+ MCs and bronchioalveolar stem cells (BASCs) from Rosa26mTmG mice. Scale bars: 500 µm. (B) Flow cytometry-sorted cell populations from organoids were analyzed by real-time PCR. (C) Foxf1+ MCs were co-cultured with BASCs±Shh signaling inhibitor (LDE225:0.75 µM) and compared to Foxf1_MC cultured in matrigel alone. (D) Mesenchymal(Lin−PDGFRα+) and epithelial(Lin−EpCAM+) cells sorted from isografts and allografts in the orthotopic mouse lung transplant model were analyzed for the indicated genes by real-time PCR. (E) Schemata for generation of Gli1CreERT2WT;Rosa26mTmG donor mice and lung transplant experiments. (F) Representative immunofluorescent images of lineage-traced Gli1_GFP+ MCs in two control and three transplanted lungs. Scale bar: 100 µm (zoomed cropped 50µm). Values=means±SD. *p<0.05; **p<0.01; ***p<0.001. Statistics: 2-tailed unpaired t test (B, D), one-way ANOVA; Bonferroni (C).