nature portfolio

Peer Review File

Emergence of fibrotic pericytes and their transcriptional regulation in pulmonary fibrosis.

Corresponding Author: Professor Tanya Kalin

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The submitted manuscript presents highly interesting data regarding the role of pericytes in pulmonary fibrosis. The authors identify the FOXF1 pathway as a central regulator in lung fibrosis. Overexpression of FOXF1 in pericytes leads to protection in the bleomycin-induced mouse model. Moreover, induction of FOXF1 expression in pericytes results in the attenuation of already established pulmonary fibrosis. While this part of the study is compelling, the analyses of the FOXF1-mediated protective functions are insufficiently addressed. The mechanisms that maintain lung homeostasis following injury would be of particular interest. Instead, the authors focus on a FOXF1 low-expressing profibrotic pericyte subpopulation. However, also this cell subset is not studied in detail.

1) The number of pericytes appears to be very small. Likewise, the two identified subpopulations also seem limited in number. It would be beneficial to include immunohistochemical staining of pericytes in healthy and IPF lungs, ideally accompanied by lower-magnification overview images. It is questionable whether such a small number of profibrotic pericytes can indeed exert a major influence on the development of pulmonary fibrosis.

2) In this context, it should be noted that the immunofluorescence data are not entirely convincing. In particular, the ACTA2 staining appears to be false positive. It seems that extracellular matrix (e.g., collagen), rather than ACTA2, has been labeled —possibly as an artifact.

3) It is more likely that the "normal" pericytes exert a regulatory function. The manuscript lacks sufficient focus on the FOXF1-mediated protective functions of pericytes. In this regard, co-culture experiments involving pericytes and fibroblasts would be of high interest.

4) Additionally, it would be important to clarify which of the FOXF1-dependent molecules are actually responsible for the regulatory/homeostatic functions of pericytes. What is the role of ID3? Which proteins are modulated by ID3?

Reviewer #2

(Remarks to the Author)
In this manuscript, Gao et al investigated the pathological reprogramming of pericytes during the progression of pulmonary fibrosis (PF), and demonstrated a significant downregulation of the transcription factor FOXF1. Using single-cell RNA-seq data from human and mouse lungs, they identified a PF-specific pericyte cluster (Peri2) where FOXF1 expression was markedly decreased. In mouse models, pericyte-specific Foxf1 knockout worsened PF, whereas Foxf1 overexpression exhibited both preventive and therapeutic effects. They further showed that FOXF1 regulates a gene network involved in fibrosis, including TGF-β signaling. This study highlights the critical role of Foxf1 in pericytes and offers novel insight into PF pathogenesis. These findings are very interesting.

Major comments
1. The manuscript implies that downregulation of FOXF1 is a causal factor in PF pathogenesis; however, the current data only demonstrate an association rather than definitive causality. All statements asserting causation should be toned down to indicate that FOXF1 involved in disease progression.

2. Pericytes represent only ~10% of mesenchymal cells (Supplementary Fig. S1B) and ~1.2% of all lung cells (Supplementary Fig. S3D). It remains unclear how much this small cell population actually contributes to the overall PF phenotype. In your Foxf1 KO and OE mouse models, please analyze how these manipulations affect other cell populations —such as alveolar epithelial cells or vascular endothelial cells—after bleomycin injury. Using your existing datasets, please examine potential intercellular interactions.
3. In your human mesenchymal cell scRNA-seq dataset, please report FOXF1 expression levels across all mesenchymal subtypes (e.g., alveolar fibroblast 1 and 2, airway vascular smooth muscle cells, etc.). How does FOXF1 expression in pericytes compare to these other cell types?
4. What mechanism underlies FOXF1 downregulation in pericytes from PF lungs? Can this mechanism be recapitulated and tested in vitro?
5. Please add Ashcroft scores for untreated controls, bleomycin-treated controls, and bleomycin-treated periFoxf1KO mice to panel 4D.
Minor comments:
1. Figures 1F, 3C, 3G, and 3H lack indicators pointing to representative cells. Please add arrows or annotations for clarity.
2. Several images in Figures 3C (Donor), 3G (Ctrl), 4E, 4F, 5F, and 6F lack scale bars within the figures. Please include them.
3. Please indicate the number of cells in each cluster directly on the UMAP plots in Figure 2A, 2B, 2C, and 7A.

Reviewer #3

(Remarks to the Author)
This manuscript identifies a previously uncharacterized sub-population of pericytes (Peri2) that emerges exclusively in fibrotic human and murine lungs and demonstrates—via combination of human scRNA-seq, two independent mouse scRNA datasets, lineage tracing, and inducible knockout/over-expression models. The authors also showed that the transcription factor FOXF1 is both necessary and sufficient to restrain the transition of pericytes into a pro-fibrotic, collagen-secreting state. The work is interesting and novel, will advance the conceptual framework of mesenchymal heterogeneity, and offer a plausible new treatment for lung fibrosis.
Major:
1. Reliance on the bleomycin model means relevance to human IPF remains inferential. Alternative IPF models (radiation, AdTGF-β, aged mice) for validation of pericytes (Peri2) and FOXF1 function would broaden impact.
2. Id3, Aplp2 and Acvrl1 are convincingly regulated by FOXF1, but their individual sufficiency or necessity has not been tested (e.g., CRISPR activation or knockout within FOXF1-null pericytes).
3. Whether continuous FOXF1 over-expression could perturb normal wound-repair or promote vascular malformations, long term observation is warranted.
4. The authors simply report that FOXF1 mRNA and protein are markedly lower in the newly defined “Peri2” pericyte cluster and in bleomycin-treated mouse lungs, but they do not propose or test a mechanism for why FOXF1 is suppressed. Recently, several studies indicated that low oxygen (hypoxia) is the primary trigger that suppress FOXF1 expression in lung pericytes and endothelial cells (Front Physiol. 2024 Jan 11:14:1309155.; Nat Commun. 2023 May 4;14(1):2560.). Does this indicate that FOXF1 is a passive factor, not a predisposing factor under hypoxic state due to pulmonary fibrosis?

Minor:
The paper uses “pericyte” and “perivascular mesenchymal cell” interchangeably; a stricter nomenclature would aid readers.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Ad 1) I requested immunohistochemistry data including H&E staining. Instead, the authors provided composite confocal microscopy images, which are not sufficient to assess lung architecture and cellular localization within the tissue context. In particular, distinguishing these cells from ACTA2-positive smooth muscle cells, which are also known to expand in IPF, appears essential.

Furthermore, it would be more informative to perform the CellChat analysis not only in periFOXF1KO mice, but also using the human datasets. Specifically, CellChat analyses for human pericyte type 1 and type 2 populations would be helpful. In addition, an analysis of receptor–ligand interactions with endothelial cells and human fibroblast populations would be of considerable interest.

Ad 2) The ACTA2 staining has undoubtedly improved; however, the images are still only marginally convincing. Additional images from other lesions and samples would be very helpful. A broader overview image using H&E or trichrome staining to better contextualize the region is still missing.

Moreover, how do the authors explain that the pericyte marker NG2, encoded by the gene CSPG4, does not appear to be expressed by the $ACTA2^{+}$ pericyte population according to Fig. 1C? This discrepancy requires clarification. In this context, it is not sufficiently demonstrated that pericyte type 2 cells truly arise from pericyte type 1 cells in IPF or in the mouse model.

Based on the marker profile, these cells could also represent smooth muscle actin–positive smooth muscle cells. This limitation needs to be addressed and discussed more thoroughly.

Ad 3) The authors have taken up my previous suggestions and included the corresponding experiments. In my view, these results should be integrated into the main manuscript rather than being presented only in the supplement.

Ad 4) The authors have made efforts to address my questions. However, the manuscript still largely focuses on describing what pericyte type 1 cells do not do compared to pericyte type 2 cells (which may potentially represent inflammatory smooth muscle cells). The authors should use the above-mentioned receptor–ligand analyses to clarify which signals pericyte type 1 cells send to endothelial cells that are no longer sent by pericyte type 2 cells. It would also be helpful to understand which signals produced by pericyte type 1 cells to fibroblasts are downregulated in pericyte type 2 cells.

Ad 5) The questions raised by the other reviewers were also only partially addressed.

Reviewer #2

(Remarks to the Author)
All of my comments have been adequately addressed. The revisions have significantly strengthened the manuscript, and I have no further comments.

Reviewer #3

(Remarks to the Author)
This manuscript identifies a previously uncharacterized sub-population of pericytes (Peri2) that emerges exclusively in fibrotic human and murine lungs and demonstrates—via combination of human scRNA-seq, two independent mouse scRNA datasets, lineage tracing, and inducible knockout/over-expression models. The authors also showed that the transcription factor FOXF1 is both necessary and sufficient to restrain the transition of pericytes into a pro-fibrotic, collagen-secreting state. The work is interesting and novel, will advance the conceptual framework of mesenchymal heterogeneity, and offer a plausible new treatment for lung fibrosis.
The authors have done a great job answering my question. There is just a minor error, "the sample names are missing in Figure 4G".

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Unfortunately, the authors did not mark their changes in the manuscript, which made the review process unnecessarily difficult. The authors have included the H&E stainings I requested. Based on these stainings and the morphology of the cell cluster in the IPF lung shown in Figure 1F, I consider the magnified cells depicted there to be smooth muscle cells and not pericytes. Peri2 and smoozh muscle cells are obviousely difficult to discriminate and may share similar funktions in fibrotic lungs. Although the authors claim to have added this major limitation as a discussion point, it cannot be found in the text or discussion section. I expect the authors to provide a photograph of a different region for Figure 1F and to include a paragraph in the discussion addressing the limitations of their data.

A point-by-point response to the Reviewers.

We would like to thank the Editor and the Reviewers for their time, efforts, and insightful comments. We greatly appreciate the constructive feedback and feel that the manuscript is significantly improved by addressing the Reviewers' comments.

Reviewer #1 (Remarks to the Author):

The submitted manuscript presents highly interesting data regarding the role of pericytes in pulmonary fibrosis. The authors identify the FOXF1 pathway as a central regulator in lung fibrosis. Overexpression of FOXF1 in pericytes leads to protection in the bleomycin-induced mouse model. Moreover, induction of FOXF1 expression in pericytes results in the attenuation of already established pulmonary fibrosis. While this part of the study is compelling, the analyses of the FOXF1-mediated protective functions are insufficiently addressed. The mechanisms that maintain lung homeostasis following injury would be of particular interest. Instead, the authors focus on a FOXF1 low-expressing profibrotic pericyte subpopulation. However, also this cell subset is not studied in detail.

We appreciate the reviewer's affirmative comments. We have performed additional experiments to improve the mechanistic part of our manuscript (see the answers on the specific questions below).

1) The number of pericytes appears to be very small. Likewise, the two identified subpopulations also seem limited in number. It would be beneficial to include immunohistochemical staining of pericytes in healthy and IPF lungs, ideally accompanied by lower-magnification overview images. It is questionable whether such a small number of profibrotic pericytes can indeed exert a major influence on the development of pulmonary fibrosis.

We agree and would like to thank the reviewer for the comment. As the reviewer requested, we have performed immunohistochemical staining of pericytes in human donor and IPF lungs and have added the new lower-magnification overview images (new Supplementary Fig. S1B in the revised manuscript). Low-magnification images show that pericytes are present throughout the donor lung and are enriched within fibrotic regions of IPF lungs.

Furthermore, using single-cell RNA sequencing data, we performed cell-cell communication network analysis to characterize interactions among lung cell populations (number of interactions and interaction strength). The analysis showed extensive crosstalk between pericytes and other lung cell types, especially alveolar fibroblasts and endothelial cells (new Supplementary Fig. S7C). These data suggest the important role of pericytes in mediating intercellular signaling within lung microenvironment and can explain why such a small number of pericytes exert a major influence on the development of pulmonary fibrosis. The data have been incorporated into the revised manuscript (new Supplementary Fig. S7C).

2) In this context, it should be noted that the immunofluorescence data are not entirely convincing. In particular, the ACTA2 staining appears to be false positive. It seems that extracellular matrix (e.g., collagen), rather than ACTA2, has been labeled—possibly as an artifact.

We agree with the reviewer. As requested, we have performed the new ACTA2 staining and replaced the old images in revised main Fig.1F. High magnification IPF lungs show the presence of aSMA protein in lung pericytes located in the fibrotic regions.

3) It is more likely that the "normal" pericytes exert a regulatory function. The manuscript lacks sufficient focus on the FOXF1-mediated protective functions of pericytes. In this regard, co-culture experiments involving pericytes and fibroblasts would be of high interest.

We agree with the reviewer's insightful comment regarding the regulatory role of pericytes, particularly concerning FOXF1-mediated protective functions. As the reviewer suggested, we have performed the co-culture experiments using primary human lung pericytes and human lung fibroblasts. Lung fibroblasts cultured in the presence of conditioned medium (CM) from FOXF1-deficient lung pericytes exhibited increased migration, proliferation and expression of pro-fibrotic *CTHRC1*, *COL1A1* and *ACTA2* mRNAs compared to fibroblasts cultured in the presence of CM from control pericytes. These new data support the FOXF1-mediated protective functions of pericytes. The data have been incorporated into the revised manuscript (new Supplementary Fig. S8A-F).

4) Additionally, it would be important to clarify which of the FOXF1-dependent molecules are actually responsible for the regulatory/homeostatic functions of pericytes. What is the role of ID3? Which proteins are modulated by ID3?

We agree. We have performed additional experiments and provided new data requested by the reviewer. To assess the FOXF1-dependent role of ID3 in pericytes, we have restored ID3 expression in FOXF1-deficient human lung pericytes *in vitro*. Restoration of ID3 decreased expression of pro-fibrotic *ACTA2, COL1A1*, and *COL3A1* mRNAs in FOXF1-deficient pericytes as shown by qRT-PCR (new Supplementary Fig. S13A-B), indicating that ID3 is important to maintain homeostatic phenotype of lung pericytes.

We next collected conditioned medium (CM) from FOXF1-deficient pericytes with or without ID3 restoration and used it to culture primary human lung fibroblasts (new Supplementary Fig. S13C-E). Restoration of ID3 decreased both migration and proliferation of lung fibroblasts (Supplementary Fig. S13C-E).

Finally, to assess the changes in pericytes' CM after ID3 restoration, we analyzed CM using human cytokine array. Restoration of ID3 in FOXF1-deficient human lung pericytes decreased the secretion of pro-fibrotic/pro-inflammatory IL-8 and CXCL1 by these cells (new Supplementary Fig. S13F-G). Altogether, our new findings identified ID3 as the FOXF1-dependent regulator of pericyte homeostatic functions, acting in part through regulation of

secreted profibrotic mediators, such as IL-8 and CXCL1. The new data have been incorporated into the revised manuscript (new Supplementary Fig. S13 and results section pages 16-18).

Reviewer #2 (Remarks to the Author):

In this manuscript, Gao et al investigated the pathological reprogramming of pericytes during the progression of pulmonary fibrosis (PF), and demonstrated a significant downregulation of the transcription factor FOXF1. Using single-cell RNA-seq data from human and mouse lungs, they identified a PF-specific pericyte cluster (Peri2) where FOXF1 expression was markedly decreased. In mouse models, pericyte-specific Foxf1 knockout worsened PF, whereas Foxf1 overexpression exhibited both preventive and therapeutic effects. They further showed that FOXF1 regulates a gene network involved in fibrosis, including TGF-β signaling. This study highlights the critical role of Foxf1 in pericytes and offers novel insight into PF pathogenesis. These findings are very interesting.

We would like to thank the reviewer for summarizing out work and for positive assessment of our manuscript.

Major comments
1. The manuscript implies that downregulation of FOXF1 is a causal factor in PF pathogenesis; however, the current data only demonstrate an association rather than definitive causality. All statements asserting causation should be toned down to indicate that FOXF1 involved in disease progression.

We agree. As the reviewer suggested, we have toned down the statements asserting causation throughout the manuscript. Statements have been adjusted to indicate that FOXF1-deficient pericytes are involved in pulmonary fibrosis and are promoting disease progression, rather than causing it. We have also changed the title. New title does not state the causative role of FOXF1 in pulmonary fibrosis but reflects the emergence of pro-fibrotic subset of lung pericytes.

2. Pericytes represent only ∼10% of mesenchymal cells (Supplementary Fig. S1B) and ∼1.2% of all lung cells (Supplementary Fig. S3D). It remains unclear how much this small cell population actually contributes to the overall PF phenotype. In your Foxf1 KO and OE mouse models, please analyze how these manipulations affect other cell populations—such as alveolar epithelial cells or vascular endothelial cells—after bleomycin injury. Using your existing datasets, please examine potential intercellular interactions.

We agree and want to thank the reviewer for the suggestion. As requested, we used our existing scRNAseq datasets to examine transcriptional changes in multiple cell types and determine how pericyte-specific deletion of FOXF1 influences intercellular communications following bleomycin injury. The CellChat analysis demonstrates the existence of the crosstalk between pericytes and other lung cell types, especially alveolar fibroblasts and endothelial cells (Endo)

(new Supplementary Fig. S7C), indicating the important role of pericytes in mediating intercellular signaling within lung microenvironment.

In addition, we assessed the contribution of FOXF1-deficient lung pericytes to fibrotic phenotype by performed new co-culture experiments using primary human lung pericytes and human lung fibroblasts. Specifically, we collected conditioned medium (CM) from control and FOXF1-deficient lung pericytes and added it to cultured lung fibroblasts (new Supplementary Fig. S8A-B). Lung fibroblasts cultured in the presence of CM from FOXF1-defecient lung pericytes exhibited increased migration, proliferation and expression of pro-fibrotic *CTHRC1*, *COL1A1* and *ACTA2* mRNAs compared to fibroblasts cultured in the presence of CM from control pericytes (new Supplementary Fig. S8C-F). Thus, FOXF1-deficient pericytes promoted pro-fibrotic phenotype in lung fibroblasts in co-culture conditions. The new data have been incorporated into the revised manuscript (new Supplementary Fig. S8 and results section page 17).

3. In your human mesenchymal cell scRNA-seq dataset, please report FOXF1 expression levels across all mesenchymal subtypes (e.g., alveolar fibroblast 1 and 2, airway vascular smooth muscle cells, etc.). How does FOXF1 expression in pericytes compare to these other cell types?

We agree. As requested, using human mesenchymal cell scRNA-seq dataset we have quantified FOXF1 expression levels across all mesenchymal subtypes, including alveolar fibroblast 1 and 2, airway vascular smooth muscle cells, etc. The new data is presented in the new Supplementary Fig. S4B, demonstrating the highest FOXF1 expression levels in Peri1 pericytes.

4. What mechanism underlies FOXF1 downregulation in pericytes from PF lungs? Can this mechanism be recapitulated and tested in vitro?

Based on our recent publications, pulmonary fibrosis is frequently associated with a hypoxic lung microenvironment, which leads to a reduction in FOXF1 expression in endothelial cells (Front Physiol. 2024 Jan 11:14:1309155.; Nat Commun. 2023 May 4;14(1):2560.). To test whether hypoxia decreases FOXF1 in pericytes, human lung pericytes were cultured in 1% oxygen using hypoxic chamber or were treated with a chemical hypoxia inducer (cobalt chloride, Cocl2). In both models of hypoxia, *FOXF1* mRNA in human lung pericytes was decreased, indicating that hypoxic environment in the fibrotic lungs could decrease FOXF1 in pericytes. We incorporated these new data in the new Supplementary Fig. S14A-B and results section (page 17).

5. Please add Ashcroft scores for untreated controls, bleomycin-treated controls, and bleomycin-treated periFoxf1KO mice to panel 4D.

We agree. As the reviewer requested, the Ashcroft scores were quantified and added to the revised Figure 4F.

Minor comments:
1. Figures 1F, 3C, 3G, and 3H lack indicators pointing to representative cells. Please add arrows or annotations for clarity.

We agree. As requested, arrows have been added to the revised figures.

2. Several images in Figures 3C (Donor), 3G (Ctrl), 4E, 4F, 5F, and 6F lack scale bars within the figures. Please include them.

We agree. Scale bars have been added to the revised figures.

3. Please indicate the number of cells in each cluster directly on the UMAP plots in Figure 2A, 2B, 2C, and 7A.

We agree. As requested, the number of cells in each cluster (previously listed in the table), have been added to the revised figures (Figure 2A, 2B, and 7A).

Reviewer #3 (Remarks to the Author):

This manuscript identifies a previously uncharacterized sub-population of pericytes (Peri2) that emerges exclusively in fibrotic human and murine lungs and demonstrates—via combination of human scRNA-seq, two independent mouse scRNA datasets, lineage tracing, and inducible knockout/over-expression models. The authors also showed that the transcription factor FOXF1 is both necessary and sufficient to restrain the transition of pericytes into a pro-fibrotic, collagen-secreting state. The work is interesting and novel, will advance the conceptual framework of mesenchymal heterogeneity, and offer a plausible new treatment for lung fibrosis.

We would like to thank the reviewer for the time invested to review out manuscript and for the positive comments.

Major:
1. Reliance on the bleomycin model means relevance to human IPF remains inferential. Alternative IPF models (radiation, AdTGF-β, aged mice) for validation of pericytes (Peri2) and FOXF1 function would broaden impact.

We agree with this insightful comment. As suggested, we have performed additional experiments to verify our conclusion using two additional models of pulmonary fibrosis: (1) silica-induced pulmonary fibrosis in mice; (2) mouse precision-cut lung slices (PCLS) treated with fibrosis-induced cocktail ex vivo. In both models, the deletion of FOXF1 from lung pericytes exacerbated lung fibrosis, supporting our finding from bleomycin model. These data have been added to the

revised manuscript in new Supplemental Figure S9A-E for the silica-induced pulmonary fibrosis in mice and in new Supplemental Figure S9F-J for PCLS model.

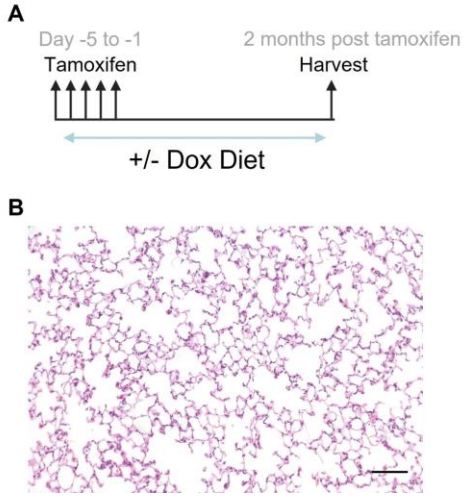
2. Id3, Aplp2 and Acvrl1 are convincingly regulated by FOXF1, but their individual sufficiency or necessity has not been tested (e.g., CRISPR activation or knockout within FOXF1-null pericytes).

We agree. To assess the FOXF1-dependent roles of ID3 or APLP2 in pericyte functions, we have restored ID3 or APLP2 expression in the FOXF1-deficient human lung pericytes using expression plasmids *in vitro*. We have also knockdown *ACVRL1* in pericytes using si*ACVRL1*. Restoration of either ID3 or APLP2, or depletion of ACVRL1 in FOXF1-deficient pericytes decreased fibrosis-associated *ACTA2, COL1A1,* and *COL3A1* mRNAs, indicating that each gene individually contributed to the fibrotic phenotype of FOXF1-deficient pericytes. The new data have been added to the revised manuscript in new Supplementary Figure S13A-B.

Additionally, human lung fibroblasts were cultured with conditioned medium from FOXF1-deficient lung pericytes with or without restored expression of ID3 or APLP2, or from FOXF1-deficient pericytes after ACVRL1 depletion. Migration and proliferation assays demonstrated that ID3, APLP2, ACVRL1 in pericytes contribute to the regulation of pro-fibroblast behavior in lung fibroblasts. The data have been added to the revised manuscript in new Supplementary Figure S13C-D and in the Results section (page 16-18).

3. Whether continuous FOXF1 over-expression could perturb normal wound-repair or promote vascular malformations, long term observation is warranted.

We agree. To address the review's comment, we have overexpressed FOXF1 in pericytes and harvested mouse lungs two months after overexpression. The two-month time point was chosen due to the limited resubmission deadline. The prolonged overexpression of *Foxf1* in pericytes did not alter lung histology.



4. The authors simply report that FOXF1 mRNA and protein are markedly lower in the newly defined "Peri2" pericyte cluster and in bleomycin-treated mouse lungs, but they do not propose or test a mechanism for why FOXF1 is suppressed. Recently, several studies indicated that low oxygen (hypoxia) is the primary trigger that suppress FOXF1 expression in lung pericytes and endothelial cells (Front Physiol. 2024 Jan 11:14:1309155.; Nat Commun. 2023 May 4;14(1):2560.). Does this indicate that FOXF1 is a passive factor, not a predisposing factor under hypoxic state due to pulmonary fibrosis?

We agree with the reviewer. To test whether hypoxia is the primary trigger that suppresses FOXF1 expression in lung pericytes, the primary human lung pericytes were exposed to 1% of oxygen using a hypoxic chamber or were treated with a chemical hypoxia inducer (cobalt chloride, Cocl2). In both models of hypoxia, *FOXF1* expression in pericytes was decreased. The data have been added to the revised manuscript in new Supplemental Figures S14A-B.

Minor:
The paper uses "pericyte" and "perivascular mesenchymal cell" interchangeably; a stricter nomenclature would aid readers.

We apologize for the confusion. We have revised our manuscript and corrected our statements to indicate that "perivascular mesenchymal cells" represent a broader category, which includes both pericytes and vascular smooth muscle cells. We now carefully distinguish between "pericytes" and "perivascular mesenchymal cells" to improve clarity of the manuscript for the readers.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Ad 1) I requested immunohistochemistry data including H&E staining. Instead, the authors provided composite confocal microscopy images, which are not sufficient to assess lung architecture and cellular localization within the tissue context. In particular, distinguishing these cells from ACTA2-positive smooth muscle cells, which are also known to expand in IPF, appears essential.

Furthermore, it would be more informative to perform the CellChat analysis not only in periFOXF1KO mice, but also using the human datasets. Specifically, CellChat analyses for human pericyte type 1 and type 2 populations would be helpful. In addition, an analysis of receptor–ligand interactions with endothelial cells and human fibroblast populations would be of considerable interest.

As the reviewer requested, we have performed additional immunohistochemistry as well as H&E staining to assess lung architecture and cellular localization within the tissue context. The new images have been added to the revised Fig. 1F and Suppl. Fig. S1B and S1C.

We appreciate the suggestion to expand our CellChat analysis to include human datasets. We agree that these additional analyses provide a more comprehensive understanding of the cellular interactions. As suggested by the Reviewer, the CellChat analysis of human datasets was provided in the revised manuscript (new Fig. 2A-D). Ligand-receptor interaction analysis between these pericyte subsets and the lung niche cells was also performed. Based on this analysis, Peri1 govern a "Homeostatic Support function". Signaling hierarchy analysis identifies Peri1 as the dominant source of Angiopoietin 1 (ANGPT1), a critical homeostatic growth factor that promotes lung vascular stability, reduces capillary leakage, and decreased inflammation after lung injury (Fig. 2B-D). In contrast, ANGPT1 is drastically decreased in Peri2 (Fig. 2C). Interestingly, Peri2 start to secrete Angiopoietin 2 (ANGPT2), which is an antagonist to ANGPT1-TIE2 signaling and is known to promote blood vessels destabilization, remodeling and vessel leakiness (Fig. 2C). The ligand-receptor analysis identified Peri2 population as governing a "Pathogenic Activation function", transitioning toward a reactive, pro-fibrotic,

pro-inflammatory state. This pathological reprogramming in Peri2 subset is evidenced by increased Fibronectin 1 (FN1), Thrombospondin (THBS), NOTCH and Collagen signaling networks, that collectively can promote myofibroblast differentiation, excessive collagen deposition and vessels remodeling (Fig. 2B-C). Moreover, by upregulating Macrophage Migration Inhibitory Factor (MIF), Peri2 can recruit and retain inflammatory cells, driving a persistent inflammatory environment (Fig. 2B). Together, these results identify Peri2 as the primary pericyte subset of the transition to progressive interstitial fibrosis, while Peri1 remains specialized for homeostatic maintenance. These new data have been incorporated into the revised manuscript (Figure 2A-D).

Ad 2) The ACTA2 staining has undoubtedly improved; however, the images are still only marginally convincing. Additional images from other lesions and samples would be very helpful. A broader overview image using H&E or trichrome staining to better contextualize the region is still missing.

Moreover, how do the authors explain that the pericyte marker NG2, encoded by the gene CSPG4, does not appear to be expressed by the ACTA2^{+} pericyte population according to Fig. 1C? This discrepancy requires clarification. In this context, it is not sufficiently demonstrated that pericyte type 2 cells truly arise from pericyte type 1 cells in IPF or in the mouse model. Based on the marker profile, these cells could also represent smooth muscle actin–positive smooth muscle cells. This limitation needs to be addressed and discussed more thoroughly.

As the Reviewer requested, we have included broader overview images to better contextualize the regions analyzed. Specifically, low-magnification overview images from multiple fibrotic lesions and independent mouse samples are now shown using H&E staining (new Suppl. Fig. S2F). These overview images highlight the fibrotic regions from which the higher-magnification ACTA2 images were acquired, thereby providing appropriate anatomical and pathological context. We believe these additions address the reviewer's concern regarding regional context and sample representation.

We would like to thank the Reviewer for the insightful comments and for raising the important question regarding the origin and marker expression of pericyte type 2 cells. We have performed pseudotime analysis using both human and mouse single-cell RNA sequencing datasets (Fig. 1E and Suppl. Fig. S2E) . This analysis strongly supports a lineage relationship between pericyte type 1 (Peri1) and pericyte type 2 (Peri2), indicating that Peri2 likely arises from Peri1. To further investigate the potential lineage of Peri2, we utilized a lineage tracing approach using *NG2-CreER; Rosa26-LSL-tdTomato* mice, which is a gold standard to identify the origin of the new cell types. Based on lineage-tracing, NG2-labeled pericytes, which do not express Acta2 in control mice, begin to express Acta2 following bleomycin treatment (Figure 3H-J). These data provide strong evidence that Peri2 cells differentiate from $NG2^{+}Acta2^{-}$ cells (Peri1). The lineage-tracing combined with the results of pseudotime analysis from mouse and human scRNAseq datasets, strongly suggest that Peri2 cells can derive from Peri1 cells. Of note, it is known that no single cell marker can distinguish pericytes from other cell types. In Fig. 1C, we have based our conclusion about existence of Peri2 subpopulation on expression of several well-accepted markers of pericytes, including Higd1b and Cox4I2. Even though, CSPG4 was almost undetectable in Peri2 based on scRNAseq (Fig. 1C), this cell population was sill expressing lower levels of two other pericyte markers Higd1b and Cox4I2 indicating that these cells are still pericytes. We agree with the Reviewer that Peri2 cells can potentially derive from smooth muscle cells, and this cannot be distinguished by immunostaining. We have acknowledged this possibility in the Discussion section (pages 19-20).

Ad 3) The authors have taken up my previous suggestions and included the corresponding experiments. In my view, these results should be integrated into the main manuscript rather than being presented only in the supplement.

We would like to thank the Reviewer for this suggestion. We agree that these results are important for the main conclusions of the study. Accordingly, we have now integrated these data into the main manuscript in the revised Fig. 1F, new Fig. 2A-D, new Fig. 4C, and new Fig. 9A-H.

Ad 4) The authors have made efforts to address my questions. However, the manuscript still largely focuses on describing what pericyte type 1 cells do not do compared to pericyte type 2 cells (which may potentially represent inflammatory smooth muscle cells). The authors should use the above-mentioned receptor–ligand analyses to clarify which signals pericyte type 1 cells send to endothelial cells that are no longer sent by pericyte type 2 cells. It would also be helpful to understand which signals produced by pericyte type 1 cells to fibroblasts are downregulated in pericyte type 2 cells.

We agree. We have provided the CellChat analysis in the new Figure 2A-D (human datasets) and new Suppl. Figure S3A-D (mouse datasets). The ligand-receptor interaction analysis clearly identifies two functionally distinct pericyte subtypes in pulmonary fibrosis (Peri1 and Peri2) and identifies their interactions with other cell types as Peri1 having “Homeostatic Support function” and Peri2 having "Pathogenic Activation function" (see response to question #1 above). Particularly, the receptor–ligand analysis identifies Peri1 as the dominant sender of Angiopoietin 1 (ANGPT1), a critical homeostatic growth factor that promotes lung vascular stability, reduces capillary leakage, and is anti-inflammatory (Fig. 2B). In contrast, Peri2 are no longer sending ANGPT1 (Fig. 2B), but start sending Angiopoietin 2 (ANGPT2), which is an antagonist to ANGPT1-TIE2 signaling and is known to promote blood vessels destabilization, remodeling and vessel leakiness. These new data have been incorporated in the revised manuscript.

Ad 5) The questions raised by the other reviewers were also only partially addressed.

We have improved our responses to the concerns raised by the other Reviewers. Based on our responses, the Reviewer 2 indicated that his/her concerns were satisfactorily resolved. The Reviewer 3 has requested a minor revision, which we have adressed by adding the sample names to the revised Figure 4G.

A point-by-point response to the Reviewer's comments.

Reviewer #1 (Remarks to the Author):

Unfortunately, the authors did not mark their changes in the manuscript, which made the review process unnecessarily difficult. The authors have included the H&E stainings I requested. Based on these stainings and the morphology of the cell cluster in the IPF lung shown in Figure 1F, I consider the magnified cells depicted there to be smooth muscle cells and not pericytes. Peri2 and smoozh muscle cells are obviousely difficult to discriminate and may share similar funktions in fibrotic lungs. Although the authors claim to have added this major limitation as a discussion point, it cannot be found in the text or discussion section. I expect the authors to provide a photograph of a different region for Figure 1F and to include a paragraph in the discussion addressing the limitations of their data.

To address the reviewer's comment, we have performed additional immunostaining using MYH11, a well-established smooth muscle cell marker which is not expressed by pericytes.

Our results demonstrated that, in IPF lungs, smooth muscle cells around vessels were highly positive for MYH11. However, while a subset of NG2-positive pericytes acquired a fibrotic phenotype characterized by α-SMA expression (NG2$^+$/α-SMA$^+$), they remained negative for MYH11, clearly distinguishing them from smooth muscle cells. Thus, these new findings helped to distinguish activated pericytes from vascular smooth muscle cells in the fibrotic area. The new data was included in the new Supplementary Fig. S1D.

In addition, as suggested by the reviewer, we have also modified the Discussion section addressing the limitations associated with distinguishing pericytes from smooth muscle cells in fibrotic lung tissue (page 20).

We hope that these additional changes have addressed the remaining concern.