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Targeting of the pulmonary capillary vascular niche promotes lung alveolar repair and ameliorates fibrosis

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Abstract

Although the lung can undergo self-repair after injury, fibrosis in chronically injured or diseased lungs can occur at the expense of regeneration. Here we study how a hematopoietic-vascular niche regulates alveolar repair and lung fibrosis. Using intratracheal injection of bleomycin or hydrochloric acid in mice, we show that repetitive lung injury activates pulmonary capillary endothelial cells (PCECs) and perivascular macrophages, impeding alveolar repair and promoting fibrosis. Whereas the chemokine receptor CXCR7, expressed on PCECs, acts to prevent epithelial damage and ameliorate fibrosis after a single round of treatment with bleomycin or hydrochloric acid, repeated injury leads to suppression of CXCR7 expression and recruitment of vascular

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Author Contributions

Z.C. designed the study, performed the experiments, interpreted the results, and wrote the paper. R.L. and M.G. performed the experiments and analysed the data. D.C. performed mouse experiments, collected and analyzed the data. K.S. and S.Y.R. helped to collect the data. G.-H.F. generated floxed *Vegfr1* mice and interpreted the results. T.P.S. analyzed the data and edited the manuscript. S.R. helped to formulate the hypothesis and edited the manuscript. B.-S.D. conceived the project, performed the experiments, analysed the data and wrote the paper.

endothelial growth factor receptor 1 (VEGFR1)-expressing perivascular macrophages. This recruitment stimulates Wnt/ β -catenin–dependent persistent upregulation of the Notch ligand Jagged1 (encoded by *Jag1*) in PCECs, which in turn stimulates exuberant Notch signaling in perivascular fibroblasts and enhances fibrosis. Administration of a CXCR7 agonist or PCEC-targeted *Jag1* shRNA after lung injury promotes alveolar repair and reduces fibrosis. Thus, targeting of a maladapted hematopoietic-vascular niche, in which macrophages, PCECs and perivascular fibroblasts interact, may help to develop therapy to spur lung regeneration and alleviate fibrosis.

Introduction

The lung, which facilitates oxygen exchange and defends against inhaled toxicants, is frequently exposed to infectious or noxious injury. Injured lungs can undergo facultative regeneration to restore alveolar architecture and cellular components^{1–8}. During lung repair, fibroblasts produce matrix to facilitate this process. However, uncontrolled matrix production by fibroblasts results in exuberant scar formation and fibrosis^{9–14}, perturbing pulmonary function. Thus, understanding the mechanisms that modulate fibroblast function in lung repair is crucial for designing strategies to promote lung regeneration and inhibit fibrosis.

Vascular endothelial cells regulate lung function in ways that extend beyond their role in delivering oxygen^{15–18}. Specialized pulmonary capillary ECs (PCECs) produce paracrine factors to stimulate the propagation of alveolar progenitor cells^{15,19}. The majority of alveolar fibroblasts are localized in the vicinity of PCECs, implicating a possible contribution of PCECs in regulating the properties of perivascular fibroblasts^{20–22}. Nevertheless, how aberrantly activated PCECs might stimulate perivascular fibroblasts to evoke fibrosis remains to be studied²³. To this end, cell type-specific gene engineering is needed to elucidate the interaction between PCECs and perivascular fibroblasts during lung repair.

The Notch pathway is pivotal in controlling the phenotype of lung cells, such as pulmonary artery and bronchial smooth muscle cells and fibroblasts^{6,7,12,24–26}, suggesting the possible contribution of this pathway in modulating perivascular fibroblasts. Moreover, Notch ligands are expressed by lung fibroblasts at low levels, suggesting non-cell autonomous regulation of Notch in fibroblasts²⁷. In contrast, endothelial cells express high amounts of Notch ligands with distinct functions, including Jagged1 (*Jag1*), Jagged2, and Delta-like ligand 1 and 4 (*Dll1* and *Dll4*)^{28–33}. As such, we hypothesized that PCECs express Notch ligands to modulate juxtacrine Notch signaling in perivascular fibroblasts, thereby orchestrating lung repair following injury. In this study, we reveal the contribution of PCEC-expressed *Jag1* in regulating lung repair and fibrosis, as well as its modulation by macrophages in a pulmonary hematopoietic-vascular niche.

Results

Repeated lung injury inhibits repair and stimulates fibrosis

To test the influence of PCECs on lung repair and fibrosis, we utilized a repetitive intratracheal bleomycin injection model³⁴ (Fig. 1a). One to six doses of bleomycin were injected into the trachea of mice, and lung gas exchange function was monitored by measuring the blood oxygen level after each injection. After each of the first three injections of bleomycin, the blood oxygen level decreased but then recovered; however, this alveolar functional recovery no longer occurred after the fourth injection (Fig. 1b). Since type 1 alveolar epithelial cells (AEC1s) are the main cell type that mediates lung gas exchange, we examined AEC1 distribution. Acute or chronic injury was induced by one or six injections of bleomycin, respectively. Epithelial damage and re-epithelialization after injury was tested by immunostaining of the AEC1 markers Aquaporin-5 and Podoplanin. AEC1 architecture was damaged by single bleomycin injection and subsequently restored in a time-dependent manner (Fig. 1b, Supplementary Fig. 1a). In contrast, this re-epithelialization was inhibited after six bleomycin injections, leading to a sustained disruption of alveolar epithelial morphology. Moreover, proliferation of surfactant protein C (SFTPC)⁺ type 2 alveolar epithelial cells (AEC2s) occurred after initial bleomycin injections, but this response no longer happened after the fifth injection (Supplementary Fig. 1b,c). Therefore, restoration of epithelial structure and of gas exchange function in injured alveoli are impeded by repetitive (chronic) lung injury.

In parallel, we examined fibrotic responses after each injection of bleomycin, as assessed by the protein levels of α -smooth muscle actin (SMA) and collagen I (Fig. 1d, e). SMA and collagen I protein levels were reduced at day 35 after one or two bleomycin injections, but this time-dependent resolution of fibrosis no longer occurred after the fourth injection (Supplementary Fig. 1d). Multiple bleomycin injections caused substantially higher levels of fibrosis than did a single bleomycin injection, as assessed by hydroxyproline levels, Sirius red staining to detect collagen I deposition, and hematoxylin & eosin (H&E) staining (Fig. 1f,g, Supplementary Fig. 1e–h). Thus, repetitive bleomycin injection causes sustained fibrosis in the injured lungs.

Notch signaling modulates the phenotype of lung fibroblasts^{26,27}. We therefore employed transgenic Notch reporter (TNR) mice, in which GFP expression is driven by the Notch effector RBP-J, to examine Notch activation in the injured lungs (Fig. 1h, Supplementary Fig. 2). Notch activation (GFP expression) was preferentially induced in desmin⁺ perivascular fibroblasts after six bleomycin injections. Based on this result, we postulated that exuberant Notch signaling in perivascular fibroblasts might contribute to impaired lung repair and fibrosis after chronic injury.

Jagged1 in aberrantly activated PCECs stimulates lung fibrosis

To study the mechanism whereby Notch is activated in perivascular fibroblasts after bleomycin injury, we examined the expression pattern of Notch ligands in injured mouse lungs. Among the tested Notch ligands, there was increased expression of Jag1 in PCECs after the fourth bleomycin injection, the time point at which sustained fibrosis became

evident (Fig. 2a, Supplementary Fig. 3a–c). Immunostaining of lung sections obtained from individuals with interstitial pulmonary fibrosis also suggested upregulation of Jag1 preferentially in PCECs, as compared to Jag1 expression in normal human lungs (Supplementary Fig. 3d). Hence, we hypothesized that aberrantly activated PCECs produce Jag1 to instigate Notch signal in perivascular fibroblasts, promoting fibrosis. To test this hypothesis, we used an endothelial cell-specific inducible gene deletion strategy. VE-cadherin-Cre^{ERT2} mice, in which a tamoxifen responsive-Cre is expressed under the control of the endothelial cell-specific VE-cadherin promoter, were crossed with mice harboring floxed *Jag1* (Fig. 2b). Treatment of the resulting mice (VE-cadherin-Cre^{ERT2} *Jag1*^{LoxP/LoxP}, referred to as *Jag1*^{EC/EC}) with tamoxifen selectively ablated *Jag1* in endothelial cells of adult mice. Mice with haplodeficiency of *Jag1* (VE-cadherin-Cre^{ERT2} *Jag1*^{LoxP/+}, referred to as *Jag1*^{EC/+}) were used as the control group. After repeated bleomycin injections, the extent of the fibrotic response in the lungs of *Jag1*^{EC/EC} mice was markedly lower than in control mice, as assessed by SMA and collagen I protein levels and hydroxyproline content (Fig. 2c–e, Supplementary Fig. 3e,f). These results indicate that chronic bleomycin injury upregulates Jag1 expression in PCECs to enhance fibrosis.

To monitor Notch activation, we also generated *TNR⁺Jag1*^{EC/EC} mice. Compared to *TNR⁺Jag1*^{EC/+} mice, Notch activation (GFP signal) was reduced in PDGFR-β⁺ fibroblasts of *TNR⁺Jag1*^{EC/EC} mice (Supplementary Fig. 3g). We then investigated the PCEC–lung fibroblast interaction in a co-culture system. Primary mouse PCECs were incubated with lung fibroblasts on Matrigel (Supplementary Fig. 4a). Among tested Notch downstream effectors, Hes1 was the most predominantly activated in the co-cultured fibroblasts (Supplementary Fig. 4b), and both Hes1 expression and activation of the co-cultured fibroblasts were reduced by genetic silencing of Jag1 in PCECs or of Notch1 in fibroblasts (Supplementary Fig. 4c–e). Notably, we also observed a time-dependent activation of Hes1 in lung fibroblasts of bleomycin-injured mice (Supplementary Fig. 4f,g). In addition, Notch signaling in co-cultured lung fibroblasts was associated with Smad3 activation³⁵ (Supplementary Fig. 5a–c). This result led us to examine Smad activation in lung fibroblasts after bleomycin injury. As assessed by electrophoretic mobility shift assay (EMSA), Smad3 activity in injured lung fibroblasts was attenuated in *Jag1*^{EC/EC} mice, as compared to control mice (Fig. 2f). Thus, Jag1 upregulation in PCECs induces Notch signaling in lung fibroblasts, which might result in fibrosis.

Effect of vascular-targeted Jag1 shRNA on lung fibrosis

The pro-fibrotic function of PCEC Jag1 in chronic lung injury suggests that targeting Jag1 in PCECs could prevent fibrosis. The large surface area of PCECs that is accessible to the blood circulation can be targeted by agents conjugated with antibodies that recognize endothelial cell antigens³⁶. We therefore generated an endothelial cell-specific gene transduction system, using a pseudotyped lentivirus³⁷ (Supplementary Fig. 6a). This packaging system incorporates an immunoglobulin G (IgG) recognizing motif in viral surface proteins, facilitating conjugation with IgG molecules. To achieve endothelial cell-specific targeting of Jag1, the rat monoclonal antibody (mAb) Mec13.3 recognizing the endothelial cell-enriched antigen CD31 was conjugated with pseudotyped lentivirus encoding Jag1 shRNA (*shJag1*^{EC} virus), a scrambled sequence control (*Srb*^{EC} virus) or *Gfp*

(*GFP^{EC}* virus). The *in vivo* effects of the conjugated lentiviruses were tested after jugular vein injection. Injection of *GFP^{EC}* virus resulted in GFP expression in VE-cadherin⁺ PCECs but not E-cadherin⁺ AECs (Fig. 2g, Supplementary Fig. 6b,c). Injection of *shJag1^{EC}* virus after the fourth bleomycin injection, and every 6 days thereafter, blocked Jag1 upregulation in PCECs and attenuated pulmonary SMA protein levels, as compared to injection of control *Srb^{EC}* virus (Fig. 2h). Moreover, these effects were associated with reduced levels of hydroxyproline, collagen I, and fibroblast Hes1 in the injured lungs (Fig. 2i,j, Supplementary Fig. 6c–h). Thus, targeting of induced Jag1 in PCECs during chronic lung injury could potentially abrogate fibrosis.

Contribution of CXCR7 in lung repair after bleomycin injury

Next, we sought to define the molecular mechanisms involved in the upregulation of Jag1 in PCECs. Induction of chemokine receptor CXCR7 expression, expressed predominantly in endothelial cells, promotes liver repair and limits atherosclerosis^{23,38}. Thus, we examined CXCR7 expression in PCECs. CXCR7 expression in PCECs was attenuated in repeatedly bleomycin-injected mice and in individuals with pulmonary fibrosis (Fig. 3a,b, Supplementary Fig. 7a–b), implicating a protective function of PCEC CXCR7. Compared to a low level of CXCR7 expression level in liver endothelial cells, CXCR7 protein was highly expressed by PCECs (Supplementary Fig. 7c–d). In parallel, CXCR7 ligand CXCL12/SDF-1 expression in the lungs was upregulated by bleomycin injection (Supplementary Fig. 7e). Thus, we tested the effect of CXCR7 agonist TC14012 on fibrotic responses. Indeed, local (intratracheal) infusion of the CXCR7 agonist TC14012 after the third bleomycin injection reduced collagen deposition and prevented alveolar epithelial damage (Fig. 3c–e, Supplementary Fig. 7g–i).

To test whether Jag1 induction and CXCR7 suppression are linked, we tested the effect of TC14012 in mice with endothelial cell-specific deletion of *Cxcr7* (*Cxcr7^{EC/i EC}*) and control mice with endothelial cell-specific *Cxcr7* haplodeficiency (*Cxcr7^{EC/+}*). Local instillation of TC14012 attenuated protein levels of Jag1, SMA and Hes1 in injured control but not *Cxcr7^{EC/i EC}* lungs (Fig. 3f–h, Supplementary Fig. 8a–b). Moreover, injection of *shJag1^{EC}* virus reduced protein levels of SMA and collagen I in both WT and *Cxcr7^{EC/i EC}* mice (Fig. 3i, Supplementary Fig. 8c–d). These data suggest that CXCR7 activation in PCECs protects against epithelial damage and prevents pro-fibrotic responses in PCECs, such as Jag1 upregulation (Supplementary Fig. 8e).

CXCR7 inhibits β -catenin-dependent Jag1 induction in injured PCEC

As the Wnt– β -catenin pathway can stimulate Notch ligand upregulation^{39,40}, we examined β -catenin pathway activation in bleomycin-injured mice using *Axin2–lacZ* reporter mice. We observed activation of β -catenin (β -galactosidase) in VE-cadherin⁺ PCEC at day 10 after four injections of bleomycin (Fig. 4a, Supplementary Fig. 9a), the time point at which endothelial Jag1 expression was persistently upregulated. In cultured mouse PCECs, addition of Wnt3A increased Jag1 expression, and this effect was dampened by TC14012 in wild type but not *Cxcr7*-deficient PCECs (Fig. 4b, Supplementary Fig. 9b). These data implicate Wnt– β -catenin pathway activation in PCECs in perpetuating Jag1 expression.

We next sought to discover the cellular mechanisms leading to β -catenin activation in PCECs after chronic injury. Macrophages and monocytes produce Wnt ligands to modulate the vascular phenotype^{13,41–46}. Thus, we tested the contribution of macrophages to pathological β -catenin activation in injured lungs⁴⁷. We generated an endothelial cell-specific β -catenin gain of function mouse line by crossing mice harboring a floxed *Ctnnb1* exon3 allele with VE-cadherin–Cre^{ERT2} mice, generating *Ctnnb1-Ex3^{fl}*^{EC/+} mice (Fig. 4c). The region of the β -catenin protein encoded by exon 3 mediates degradation of the protein; therefore, deletion of exon 3 generates a constitutively active form of β -catenin^{48,49}. We subjected control and *Ctnnb1-Ex3^{fl}*^{EC/+} mice to clodronate liposome-mediated macrophage depletion before the fourth bleomycin injection. As assessed by PCEC Jag1 expression lung SMA levels, macrophage and monocyte depletion prevented fibrotic responses in control but not *Ctnnb1-Ex3^{fl}*^{EC} mouse lungs (Fig. 4d, Supplementary Fig. 9c–d). In contrast, TC14012 attenuated Jag1 induction in both groups (Supplementary Fig. 9e–f). Thus, after repeated lung injury, macrophages appear to mediate over-activation of β -catenin in PCECs, leading to upregulation of pro-fibrotic Jag1.

VEGFR1⁺ macrophages modulate β -catenin pathway activation in PCECs

To unravel how macrophages mediate β -catenin activation in PCECs, we examined Wnt ligand production in F4/80⁺CD11b⁺ macrophages after repeated bleomycin injections. We observed preferential upregulation of Wnt3A in isolated macrophages (Fig. 4e). *In vivo*, Wnt3A expression in F4/80⁺CD11b⁺ macrophages was specifically enhanced by injection of placental growth factor (Plgf), a ligand of VEGFR1^{50–54} (Fig. 4f). Indeed, Plgf expression was upregulated in chronically injured lungs (Supplementary Fig. 10a). Based on these findings, we examined the recruitment of VEGFR1⁺ cells to bleomycin-injured lungs using *Vegfr1*–lacZ reporter mice⁵². Repeated bleomycin injections led to a significant increase in the numbers of CD11b⁺F4/80⁺VEGFR1⁺ macrophage (Fig. 4g, Supplementary Fig. 10b,c). Moreover, deletion of *Vegfr1* using LysM–Cre (*Vegfr1*^{LysM/LysM}) blocked expression of Wnt3A in F4/80⁺CD11b⁺ macrophages after bleomycin treatment (Fig. 4h, Supplementary Fig. 10d–f). Jag1 induction in PCECs was similarly abrogated in *Vegfr1*^{LysM/LysM} mice (Supplementary Fig. 10g,h). These findings suggest that, after chronic lung injury, VEGFR1-expressing perivascular macrophages evoke pathological activation of the β -catenin pathway in PCECs.

To formally test the effect of VEGFR1⁺ macrophages on the β -catenin–Jag1 axis in PCECs, we utilized an adoptive monocyte transfer strategy⁴⁷ in wild type and *Ctnnb1-Ex3^{fl}*^{EC/+} mice (Fig. 5a). Transplantation of *Vegfr1*^{–/–} monocytes (obtained from *Vegfr1*^{LysM/LysM} mice) into WT mice caused a significantly lower extent of fibrosis after bleomycin injection, compared to mice receiving *Vegfr1*^{+/+} monocytes (obtained from wild type mice) (Fig. 5b–d). In contrast, this differential effect of *Vegfr1*^{–/–} and *Vegfr1*^{+/+} monocytes was lost in *Ctnnb1-Ex3^{fl}*^{EC/+} mice. Notably, CXCR7 agonist TC14012 attenuated lung fibrosis in all of these groups (Fig. 5c,d). Moreover, decreased lung fibrosis, in both the setting of *Vegfr1*^{–/–} monocyte infusion and TC14012 treatment, was associated with lower Jag1 expression (Fig. 5e,f). These findings further implicate recruitment of VEGFR1-activated macrophages in stimulating a pro-fibrotic β -catenin–Jag1 axis in PCECs, which is tempered by CXCR7 signaling in PCECs (Supplementary Fig. 10i).

Role of CXCR7 and Jag1 after repeated acid aspiration injury

To examine whether the effects of CXCR7 and Jag1 in the bleomycin model can be generalized to another lung injury model, we used intratracheal instillation of 0.1 M hydrochloric acid (Fig. 6a). A single treatment with acid disrupted alveolar epithelial structure, followed by recovery of AEC1 numbers and restoration of lung respiratory capacity (Supplementary Fig. 11a–c). In contrast, repeated acid treatment blocked re-epithelialization and increased collagen I deposition in the injured lungs (Supplementary Fig. 11d,e). Next, we utilized this acid injury model to test the therapeutic effect of the CXCR7 agonist TC14012 and of *shJag1*^{EC} virus. TC14012 infusion attenuated alveolar epithelial injury and reduced fibrotic responses after repetitive acid treatment (Fig. 6b–e, Supplementary Fig. 12a–d). Similarly, injection of *shJag1*^{EC} virus maintained alveolar epithelial architecture, blocked lung fibrosis, and inhibited fibroblast Notch activation after the fifth round of acid aspiration (Fig. 6f–h, Supplementary Fig. 12e–f). Moreover, *shJag1*^{EC} virus injection preserved the gas exchange function in injured lungs (Fig. 6i). Thus, endothelial cell-specific activation of CXCR7 knockdown of Jag1 in chronically injured PCECs can stimulate functional lung repair and mitigate fibrosis.

Discussion

Fibrosis is involved in the progression of various lung diseases and can have fatal consequences. Modulation of lung regeneration and fibrosis could have substantial value in treating lung disease^{9–11,34}. After surgical removal of the left lung lobe in rodents, the remaining lobes regrow without fibrosis, and, in many patients with acute lung injury, scar deposition in the lungs after injury resolves over time. By contrast, chronic insult to the lungs (for example, due to asbestos or silica deposition) can lead to exuberant scar formation, resulting in fibrosis that perturbs lung function. Here we show that iterative lung injury with bleomycin affects CXCR7 expression in mouse PCECs, leading to upregulation of pro-fibrotic Jag1. In parallel, Jag1 induction in PCECs is promoted via an interaction of PCECs with VEGFR1⁺ perivascular macrophages, leading to β -catenin activation in PCECs. We also showed that the effects of endothelial CXCR7 and Jag1 could be generalized to a second lung injury model, acid aspiration. Our findings indicate that after sustained lung injury, VEGFR1⁺ perivascular macrophages interact with PCECs to form a maladapted “hematopoietic–vascular niche” evoking fibrosis (Fig. 6j).

Spatial and temporal regulation of Notch signaling intricately balances lung regeneration and fibrosis after injury. For example, a recent study suggested that the Notch signaling is essential for activation of lineage-negative epithelial stem/progenitor cells (LNEPs) in the lungs after influenza infection⁶. In this setting, Notch-Hes1 signaling in LNEPs stimulates their proliferation and migration, but a subsequent decrease in Notch signaling in LNEPs is necessary for these lung progenitors to differentiate into alveolar epithelial cells. Aberrantly sustained Notch activity in the injured lungs led to alveolar cyst architecture that is indicative of fibrotic phenotype⁶. Here we found that persistent upregulation of Notch ligand, Jag1, in chronically injured PCECs causes sustained Notch activation in perivascular fibroblasts. Notably, both Notch activation and fibrotic injury were attenuated in the lungs of *Jag1*^{EC/EC} mice, implicating endothelial Jag1 in the induction of pro-fibrotic Notch

signaling in perivascular fibroblasts. How other Notch ligands expressed in different cell types modulate Notch activity in injured lungs remains to be clearly defined^{31,33}.

Suppression of the “built in” protective CXCR7 pathway in PCECs by repeated injury prevents alveolar repair and promotes fibrosis. The anti-fibrotic role of CXCR7 was at least partially due to inhibition of β -catenin-dependent induction of Jag1^{39,40}. Future studies should investigate the role of CXCR7 in modulating the physiological function of the β -catenin pathway in endothelial cells of specific vascular beds^{48,49}, as well as the potential involvement of the CXCL12 receptor CXCR4^{23,55}. The anti-fibrotic function of CXCR7 in PCECs extends the previously discovered role of PCECs in enabling lung alveolar regeneration^{15,18}, and future study is also needed to identify CXCR7-triggered endothelial paracrine molecules¹⁸ that evoke alveolar epithelial repair.

Macrophages regulate vascular remodeling and patterning^{13,41–44}. Here, we showed that VEGFR1⁺ perivascular macrophages stimulate β -catenin dependent Jag1 expression in PCECs. The contribution of VEGFR1 in macrophages and/or monocytes^{44,53} was evidenced by the reduced level of lung injury in *Vegfr1*^{LysM/ LysM} mice and by the beneficial effects of adoptive transfer of *Vegfr1*-deficient monocytes. LysM-Cre can also be expressed by AEC2s; however, VEGFR1 expression in AEC2s was negligible compared to that in macrophages and monocytes in both control and injured lungs (Fig. 4g and Supplementary Fig. 10b–c), such that the effects of LysM-Cre-mediated *Vegfr1* deletion were likely due to effects on monocytes and/or macrophages, rather than on AEC2s. Other Wnt ligands and macrophage-derived factors might also modulate PCEC function in lung repair, and future studies using cell type-specific knockouts will be needed to explore crosstalk between perivascular macrophages and PCECs.

Macrophages are important in both the promotion and resolution of fibrosis during organ repair^{41–47,56–59}. The pro-fibrotic VEGFR1⁺ perivascular macrophages that we have identified are reminiscent of a previously described pro-fibrotic monocyte and macrophage population⁶⁰. The influence of the maladapted hematopoietic-vascular niche containing VEGFR1⁺ perivascular macrophages and PCECs might be context- and stage-specific^{50–54}. For example, it is conceivable that increased levels of the VEGFR1-specific ligand Plgf in chronically injured lungs specifically triggers the pathological function of VEGFR1 in macrophages, and future study is needed to identify the cellular source of Plgf, as well as how VEGFR1 is induced in the pro-fibrotic subset of macrophages. The contribution of dysfunctional epithelium in subverting the repair function of the hematopoietic-vascular niche also should be studied^{1,9,11}.

Taken together, our results indicate that chronic lung injury recruits pro-fibrotic macrophages and suppresses a protective CXCR7 mechanism in PCECs, perpetuating the production of a pro-fibrotic endothelial signal—Jag1—that prohibits epithelial repair. Targeting of the hematopoietic-vascular niche¹⁸, which is readily accessible to the circulation, could enable regenerative therapy for lung disorders. Our findings might also help to develop treatments for various fibrosis-related diseases that are major causes of death in developed countries^{1,9,11}.

Online Methods

Animals

Cxcr7^{loxP/loxP} mice²³, *Jag1*^{loxP/loxP} mice⁶¹, and mice harboring a floxed exon3 *ctnnb1* allele^{48,49,62} were crossed with VE-cadherin–Cre^{ERT2} (cdh5–PAC–Cre^{ERT2}) transgenic mice³³. These crosses generated VE-cadherin–Cre^{ERT2} *Cxcr7*^{loxP/loxP}, VE-cadherin–Cre^{ERT2} *Jag1*^{loxP/loxP}, and VE-cadherin–Cre^{ERT2} *Ctnnb1-Ex3*^{loxP/+} mice. Four week old male mice were then treated with tamoxifen at a dose of 200 mg/kg intraperitoneally (i.p.) for 6 days and rested for at least ten days after last injection, resulting in endothelial-specific deletion of *Cxcr7*, *Jag1*, and *ctnnb1* gain of function in adult mice (*Cxcr7*^{EC/i EC}, *Jag1*^{EC/i EC}, *Ctnnb1-ex3*^{EC/+}).

Six to ten week old male mice with endothelial cell-specific haplodeficiency of *Jag1* or *Cxcr7* (*Jag1*^{EC/+} or *Cxcr7*^{EC/+}) were used as control groups for male *Jag1*^{EC/i EC} and *Cxcr7*^{EC/i EC} mice at the same age. Six to eight week old male wild type mice were utilized for mouse lung injury models and related treatments. Six to eight week old male transgenic Notch Reporter (TNR)⁶³ mice were used to track Notch pathway activity in the injured lungs. TNR mice were also bred with VE-cadherin–Cre^{ERT2} *Jag1*^{loxP/loxP} to generate six to ten week old male TNR⁺ *Jag1*^{EC/i EC} mice after tamoxifen injection. *Vegfr1*–driven lacZ reporter mice and floxed *Vegfr1* mice were generated by Dr. Guo-Hua Fong (University of Connecticut)⁵². LysM–Cre (Stock No. 004781) and male β -catenin reporter Axin2–lacZ reporter (Stock No. 009120) mice were from the Jackson Lab. Six to eight week old *Vegfr1*–driven lacZ and *Axin2*–lacZ mice were used to measure *Vegfr1* expression and β -catenin pathway activation after bleomycin injection.

Investigators who performed mouse lung injury experiments and who analyzed the pattern and extent of cell activation/proliferation were randomly assigned with animals or samples from different experimental groups and were blinded to the genotype of the animals or samples. All animal experiments were carried out under the guidelines set by the Institutional Animal Care and Use Committee at Weill Cornell Medical College.

Mouse lung injury models

A repetitive intratracheal bleomycin injection model was used to induce lung fibrosis³⁴. Bleomycin sulfate powder (EMD) was suspended and dissolved in sterile PBS and injected intratracheally into six-eight week old male wild type mice or six to ten week old mice of the indicated genotypes at a dose of 1 unit/kg in a total volume of 50 μ l PBS. During the injection process, mice were anesthetized with a cocktail of Ketamine and Xylazine. Mice were suspended vertically on a stand for orotracheal instillation, and 50 μ l of bleomycin solution was administered through a 27-gauge angiocatheter. Intratracheal injection of hydrochloric acid (acid) was similarly performed as previously described⁶⁴. Orotracheal instillation was performed in anesthetized mice, and 20 μ l of an iso-osmolar solution of 0.1 M hydrochloric acid was instilled. After each injection of bleomycin or hydrochloric acid, mice were observed to ensure full recovery from anesthesia, and body temperature was maintained using an external heat source. After recovery, mice were transferred to ventilated cages with access to food and water. To examine the proliferation of type 2 alveolar

epithelial cells (AEC2s) after bleomycin treatment, mice were sacrificed 1 hour after i.p. injection of 5-bromo-2'-deoxyuridine (BrdU). At the indicated time points after bleomycin or acid treatment, the oxygen tension in arterial blood of treated mice was measured as previously described using I-Stat (Abbott Laboratories, Abbott Park, IL)³⁶.

Macrophage and monocyte depletion by clodronate liposome injection

We adopted a clodronate liposome administration method to selectively and effectively deplete macrophages and monocytes, including pulmonary macrophages⁶⁰. The procedure and schedule of clodronate liposome injection was based on previously described kinetics of macrophage and monocyte depletion in both control and bleomycin-treated mice⁶⁰.

To perform clodronate liposome administration, negatively-charged clodronate encapsulated in liposomes (Clodosome-A, Cat. No. F70101C-A) was obtained from Formu Max (Palo Alto, CA). Clodosome-A was prepared following the vendor's guidance. 0.3 ml of Clodosome-A or empty control liposomes (Formu Max, Palo Alto, CA, USA) was intravenously (i.v.) injected into mice one day before the 4th injection of bleomycin and every ten days thereafter to deplete macrophages/monocytes during chronic lung injury. The Clodosome-A method depletes more than 90% of macrophages in the lungs 24 hours after injection, an effect which persists for up to 5 days⁶⁰.

Macrophage and monocyte isolation and adoptive transfer

In order to interrogate the contribution of VEGFR1⁺ macrophages and monocytes during lung repair, six to eight week old male wild type mice were injected with 50 µg of Plgf or equal amounts of CXCL12/SDF-1, GM-CSF, and VEGF-A (BioVision, Milpitas, CA) a day before the 4th injection of bleomycin or before PBS injection and every three days thereafter. Macrophages and monocytes were isolated from mouse lungs using the Monocyte Isolation Kit (Miltenyi Biotec) at day 10 after the 4th bleomycin injection or after PBS injection. The expression of cytokines and Wnt3A was examined by quantitative PCR. To examine the contribution of VEGFR1-expressing macrophages and monocytes in lung repair, monocytes were isolated from the bone marrow of mice with selective *Vegfr1* deletion, using LysM-Cre (*Vegfr1*^{LysM/LysM}) or control (*Vegfr1*^{+/+}) mice. 3 x 10⁶ *Vegfr1*^{-/-} monocytes (from *Vegfr1*^{LysM/LysM} mice) or *Vegfr1*^{+/+} monocytes were intravenously infused into macrophage-depleted control and *Ctnnb1-ex3*^{EC} mice after the 4th injection of bleomycin (the schedule is shown in Figure 5a). This adoptive transfer of monocytes was repeated after the 5th and 6th injections of bleomycin. Lung fibrotic responses were compared between control and *Ctnnb1-ex3*^{EC} mice receiving *Vegfr1*^{+/+} and *Vegfr1*^{-/-} monocytes. To determine the effect of CXCR7 activation, 10 mg/kg TC14012 or vehicle was infused into the recipient mice through the trachea after monocyte transfer and repeated every six days. Jag1 and SMA protein levels in the lungs were detected by immunoblot. Used antibody information is described in supplementary Table 1. Collagen deposition and morphology were tested by Sirius red and H&E staining.

Tissue harvesting and histology

For lung tissue collection, both lungs were thoroughly perfused with PBS via the pulmonary artery to remove residual blood in the vasculature. The right lung was removed from the

thoracic cavity after the right hilum was tied. Lung lobes were separated, collected, and processed for subsequent experiments such as protein isolation and detection. The left lung was inflated from the identified and isolated trachea with a 21-gauge needle and syringe. The trachea was then tied under pressure. Each parameter from each individual animal was measured at least twice and averaged.

Fixed lungs were either embedded in paraffin or snap-frozen in OCT compound (Miles, Elkhart, IN). 10 μ m thick lung cryosections were made for immunofluorescent staining. Sirius red and H&E stainings were performed on paraffin embedded lung sections to determine the lung morphology and distribution of collagen, using the procedure of Histoserv (Germantown, MD).

Immunofluorescence, β -galactosidase (LacZ) staining, and morphometric analysis

Lung frozen sections were blocked (5% donkey serum from Jackson ImmunoResearch (West Grove, PA, USA) (Cat. No. 017-000-121) & 0.3% Triton X-100) and incubated with primary antibodies (described in Supplementary Table 1) at 4 °C overnight¹⁵. Lung sections from six to eight week old male TNR mice, in which GFP fluorescence indicates Notch activation, were co-stained with the PCEC marker VE-cadherin and the fibroblast antigen desmin. After the sections were washed with PBS and incubated with fluorescent dye-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), nuclear staining was carried out with DAPI using Prolong Gold mounting medium (Invitrogen). Cell proliferation (BrdU incorporation) was measured by immunostaining for BrdU. Fluorescent images were captured on an AxioVert LSM710 microscope (Zeiss). To determine the fluorescent signal in tissue sections, fluorescent cells in five different high-power fields from each slide were quantified. Image analysis was performed in a blinded fashion. Investigators were not aware of the genotype of animals or the identity of samples during scoring.

β -galactosidase (LacZ) activity in mouse lungs was determined from the cryopreserved lung slides after fixation with 0.1% glutaraldehyde as previously described⁵². Six to eight week male Vegfr1-LacZ and Axin2-LacZ mice were utilized. Alveolar morphology was independently assessed by two investigators using five randomly selected fields in each H&E slide.

Lung fibrosis determination

Right top lung lobes were homogenized in tissue lysis buffer. Immunoblotting of SMA and collagen I was performed using the lung tissue lysates. PFA-fixed lung sections were stained with Sirius Red to assess collagen deposition and distribution, following the protocol of Histoserv (Germantown, MD). Lung fibrotic parenchyma was assessed using five random fields in each section and quantified as previously described²³.

Hydroxyproline content was quantified in the lungs to determine the extent of fibrosis^{10, 65}. Right lower lobes were weighed, homogenized, and baked in 12 N hydrochloric acid overnight at 120 °C. Then, the samples were aliquoted and added to 1.4% chloramine T in 0.5 M sodium acetate/10% isopropanol (Sigma). Following incubation for 20 minutes at room temperature, the samples were incubated with Erlich's solution at 65 °C for 15 minutes. Absorbance at the wavelength of 540 nm was determined, and the content of

hydroxyproline was calculated by comparing the absorbance to a hydroxyproline standard curve. Hydroxyproline level in the lung was determined based on the weight of the right lower lung lobe and of the total lung.

Cells

Mouse PCECs and lung fibroblasts were isolated from lungs by flow sorting or sheep anti-rat Dyna beads (Invitrogen) as previously described^{15, 66}. PCECs and lung fibroblasts were identified by rat anti-mouse CD31 (clone Mec13.3) and VE-cadherin (clone Bv13) antibodies (for PCECs) and anti-mouse PDGFR β clone APB5 antibody (for fibroblasts). For Dyna bead-based isolation, Dyna beads were prepared one day before isolation. Beads were washed three times and suspended in 200 μ l PBS containing 0.1% bovine serum albumin (BSA), 2 mM EDTA, and 1% penicillin/streptomycin/fungizone (P/S/F). 2.5 μ g of each type of rat antibody was added to 10 μ l of beads and rotated for 1 hour at room temperature and then 4°C overnight. Then, beads were washed three times and suspended in the original buffer with same volume.

For lung digestion, we utilized a digestion cocktail solution containing 2 mg/ml collagenase A and 1 mg/ml Dispase (Roche Life Science) in Hanks' Balanced Salt Solution (HBSS). 1 ml digestion solution was directly instilled via the trachea and used to perfuse via pulmonary artery to accelerate the digestion process. Perfused mouse lung tissues were then removed from the chest cavity to RPMI1640 medium (Gibco), gently minced, and disrupted by passing through a 18 G syringe. Lung tissues were then suspended in digestion cocktail (100 mg in 2.5 ml) for 15 minutes at 37 °C. Digested tissue was filtered through a sterile 40 μ m nylon mesh (cell strainer), centrifuged, and suspended in the original volume. After filtration, released lung cells were incubated with 1 μ g/ml fluorescently labeled rat-anti mouse VE-cadherin and CD31 antibodies for flow sorting or 10 μ l of conjugated beads for Dyna bead isolation. After the supernatant was carefully aspirated and washed 5 times in PBS + 0.1% BSA + 2 mM EDTA + 1% P/S/F, fluorescent antibody-stained or bead-bound cells were collected using a flow sorter or magnet. Isolated PCECs or fibroblasts were directly subjected to subsequent analyses, unless specified as cultivated cells.

Cell cultivation and shRNA transduction

Cultivation of mouse PCECs was performed on 25 μ g/ml fibronectin-coated plates as previously described⁶⁷. Isolated mouse PCECs were transduced with the E4ORF1 gene and with a gene encoding hyperactive c-Raf to maintain endothelial cell survival in serum-free conditions⁶⁷. Five shRNA clones (TRCN0000028933, TRCN0000028887, TRCN0000028869, TRCN0000028860 and TRCN0000028850) were obtained from Open Biosystems and used to perform gene knockdown of Jag1 in cultivated PCECs. Clone TRCN0000028887 (indicated as clone 2) showed the highest efficiency in silencing Jag1 in cultivated PCECs. A negative control vector was constructed using a scrambled (Srb) sequence designed by GeneCopoeia that does not match any genomic sequence (Cat. No. CSHCTR001). Lentiviral particles were generated by co-transfecting 15 μ g of shuttle lentiviral vector containing *Jag1* shRNA or Srb sequence, 3 μ g of pENV/VSV-G, 5 μ g of pRRE, and 2.5 μ g of pRSV-REV in 293T cells by the Fugene (Roche) method. Viral supernatants were concentrated by Lenti-X concentrator (Clontech, Cat. No. 631232). After

titration with Lenti-X p24 rapid titer kit (Clontech, Cat. No. 632200), 25,000 pg of virus was used to transduce 1,000,000 PCECs.

Human pulmonary fibrosis samples

Human pulmonary fibrosis and normal tissues were purchased from Origene (Rockville, MD). The characteristics of the pulmonary fibrosis samples were as follows: Patient 1 (Cat. No. CS502727): H&E staining shows 45% alveoli, 0% bronchioles, 30% fibrovascular tissue, 25% diffuse interstitial fibrosis. Patient 2: (Cat. No. CS504978): H&E staining shows 65% alveoli, 10% bronchioles, 25% fibrovascular septae; contains interstitial fibrosis and chronic inflammation. Patient 3 (Cat. No. CS504492): H&E staining shows 65% alveoli, 15% bronchioles, 20% fibrovascular septae, and interstitial fibrosis. Two individual normal lung tissues exhibiting regular alveolar architecture and morphology in H&E staining were similarly analyzed and compared with patient samples.

In vivo modulation of gene expression in PCECs by pseudotyped lentiviral particles

To couple lentiviral particles with an antibody recognizing endothelial surface antigen, we adopted a packaging system to generate pseudotyped lentivirus³⁷. Viral particles were produced by transfection of 293T cells with lentiviral constructs of Jag1 shRNA, scrambled sequence (Srb), or GFP and packaging vector pMDL/pPRE, and pRSV-REV, as well as pseudotyped vector 2.2 that replaced pVSV-G³⁷. Inclusion of pseudotyped vector 2.2 in the packaging system enables the generation of lentiviral particles bearing an immunoglobulin G (IgG) binding motif on the particle surface. Lentiviral particles were concentrated using the Lenti-X concentrator kit (Clontech). Virus titer was normalized to lentiviral core/capsid protein p24 using the Lenti-X p24 Rapid Titer Kit (Clontech). For conjugation of Mec13.3 antibody (BD Biosciences) with lentiviral particles, concentrated virus was suspended in PBS at a concentration of 30 µg/ml p24 capsid protein and incubated with a 2-fold excess of Mec13.3 antibody or rat IgG (Jackson ImmunoResearch, Cat. No. 012-000-002). Conjugated lentiviral particles were purified again and resuspended in sterile PBS.

To test the efficiency of virus delivery, two groups of six to eight week old male wild type mice were intravenously injected with GFP-expressing lentivirus conjugated to Mec13.3 or rat IgG, with the titer normalized to 10 µg p24 capsid protein. GFP expression in VE-cadherin⁺ PCECs and E-cadherin⁺ AECs was determined by co-staining of GFP with antibodies to VE-cadherin and E-cadherin (BD Biosciences).

Effects of CXCR7 agonist and endothelial cell-specific delivery of Jag1 shRNA on lung fibrosis

Pseudotyped lentiviral particles containing shJag1 were conjugated with anti-mouse CD31 antibody Mec13.3 to induce shJag1 expression in endothelial cells (*shJag1*^{EC}). Five individual viral particles containing different mouse Jag1 shRNA clones were conjugated with anti-CD31 mAb Mec13.3 separately, resulting in five types of *shJag1*^{EC} viruses (*shJag1*^{EC} C1-C5). Lentiviral particles containing the Srb construct was similarly processed with Mec13.3 as a control group (*Srb*^{EC}). After the fourth bleomycin injection, six to eight week old male wild type mice were subjected to Mec13.3-conjugated *shJag1*^{EC} or *Srb*^{EC} every six days at a dose of 10 µg p24 capsid protein. To test the ability of individual

Mec13.3-coupled virus in attenuating Notch activation in the lungs of bleomycin-injected mice, lung fibroblasts were isolated at day 35 after sixth bleomycin injection as aforementioned, and Hes1 protein expression in the isolated mouse lung fibroblasts was analyzed by immunoblotting (Abcam, Cat. No. ab71559). Knockdown of Jag1 in PCECs after injection of *shJag1^{EC}* was tested by immunostaining and immunoblot. Since Jag1 shRNA clone 2 conjugated to Mec13.3 exhibited the highest efficiency in abrogating Hes1 activation in mouse lung fibroblasts after repeated bleomycin injection, results obtained from this *shJag1^{EC}* experimental group are presented throughout, unless otherwise specified. *shJag1^{EC}* and *Srb^{EC}* were similarly administered into six to eight week old male wild type mice after the second administration of acid with the same dose and schedule. Six to ten week old male *Cxcr7^{EC/i EC}* and randomly distributed male and female *Cxcr7^{+/+}* mice were also treated with *shJag1^{EC}*, *Srb^{EC}*, equal amounts of Mec13.3, or rat IgG, respectively. Same injection dose and schedule were used for *Cxcr7^{+/+}* and *Cxcr7^{EC/i EC}* mice.

The CXCR7-selective agonist TC14012 (R&D Systems) and vehicle were intratracheally administered into mice at 10 mg/kg immediately after the third injection of bleomycin or second administration of acid and every four days thereafter. After injection of CXCR7 agonist or *shJag1^{EC}*, alveolar epithelial structure was examined by co-staining for aquaporin-5 (Abcam, Cat. No. ab78486) and podoplanin (R&D, Cat. No. AF3244). Lung fibrotic responses were determined at the indicated time points after bleomycin or acid administration, including immunoblot analysis of SMA and collagen I, Sirius red staining, and determination of lung hydroxyproline content.

PCEC–lung fibroblast co-culture

For PCEC–lung fibroblast co-culture experiments, lung fibroblasts were transduced with lentiviral vector encoding Gfp and cultivated in DMEM supplemented with recombinant platelet-derived growth factor (PDGF)- β (5 ng/ml), recombinant epidermal growth factor (EGF) (10 ng/ml) and antibiotics. PDGF- β and EGF were obtained from BioVision (Milpitas, CA). Co-culture was performed on Matrigel (BD Biosciences) under serum-free conditions. 250 μ l Matrigel was seeded in a 24 well plate, and 100,000 mCherry fluorescent protein-transduced PCECs and 25,000 lung fibroblasts were seeded on solidified Matrigel in Ex vivo medium (Invitrogen). Cells were retrieved 8 days after co-culture; lung fibroblasts were purified as described above for subsequent analyses.

Detection of Notch and Smad activation in lung fibroblasts

Activation/phosphorylation of Smad3 (p-Smad3) in lung fibroblasts was detected using antibodies from Cell Signaling (Cat. No. 9520). Notch 1 (Clone TRCN0000025902) and 3 (clone TRCN0000075570) were silenced with shRNA (Open Biosystems) in lung fibroblasts to test the contribution of each of these Notch receptors on Notch-mediated activation in lung fibroblasts. An electrophoretic mobility shift assay (EMSA) kit (Panomics, Fremont, CA) was used to detect Smad protein DNA binding activity in lung fibroblasts. 10 μ g nuclear extract was mixed with a labeled Smad 3/4 binding element probe (Panomics, Fremont, CA, Cat. No. AY1042P) and analyzed. Attenuation of Smad activation was assessed by comparing the optical density of shifted bands among groups.

Flow cytometry

Stained lung fibroblasts and PCECs were measured using an LSRII flow cytometer (Becton Dickinson). Compensation for multivariate experiments was carried out with FACS Diva software (Becton Dickinson Immunocytometry Systems). Flow cytometry analysis was performed using various controls, including isotype antibodies and unstained PCECs and lung fibroblasts, for determining gates and compensations.

Statistical analysis and reproducibility of experiments

The number of animals in each group is listed in the figure legends. Differences among groups were assessed using one-way ANOVA. All presented representative images were obtained from independently repeated experiments. Representative images from each animal group are presented in the figures. Image analyses were performed in a blinded fashion. Investigators were unaware of the genotype of animals or the identity of samples during assessment. Results are presented as means $\pm$ standard error of the mean (s.e.m.). Each point in dot plots represents an individual animal or cell sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Repetitive lung injury causes sustained alveolar epithelial cell damage, irreversible pulmonary fibrosis, and persistent Notch signaling in perivascular fibroblasts. **(a)** The experimental scheme for inducing chronic lung injury in mice using repeated intratracheal injections of bleomycin (Bleo). **(b)** Blood oxygen levels in mice after bleomycin treatment. Each dot represents data from one animal; $n = 10$ animals in the PBS-treated groups and the 1 Bleo and 2 Bleo groups, and $n = 9$ mice for the other groups; * $P < 0.05$. One-way ANOVA was used to compare the statistical difference between groups throughout the study. **(c)** Immunostaining for the AEC1 markers aquaporin-5 and podoplanin, and for the endothelial cell-specific antigen VE-cadherin. The percentage of AEC1s in lung cryosections is quantified in Supplementary Fig. 1a. Scale bar, 50 μ m. **(d)** Immunoblotting of α -smooth muscle actin (SMA) and collagen I in lungs following bleomycin injection. Here and in all figures, each lane contains a sample from an individual mouse. **(e)** Quantification of the immunoblotting data for collagen I; quantitation of the immunoblotting data for SMA is shown in Supplementary Fig. 1d. $n = 8$ mice in each Bleo group and $n = 9$ mice in the PBS group. **(f)** Collagen deposition in mouse lungs at days 20 or 35 after single or six bleomycin injections was examined by Sirius red staining. Sirius red area is quantified in Supplementary Fig. 1e, f. Scale bar, 350 μ m. **(g)** Lung hydroxyproline levels after single or

six bleomycin injections. $n=9$ per group. $*P<0.05$. Error bars indicate standard error of the mean (s.e.m.). **(h)** Analysis of Notch activation in mouse lungs in Transgenic Notch reporter (TNR) mice. For each of the two conditions shown, the micrograph on the left shows GFP fluorescence (reporting Notch activation), and the other micrographs show immunostaining for GFP, the fibroblast marker desmin and endothelial cell-specific VE-cadherin. Note the Notch activation (GFP) signal in desmin-positive perivascular fibroblasts (inset). Scale bars, 50 μm .

Figure 2.

Pro-fibrotic role of the Notch ligand Jag1 in mouse PCECs after bleomycin injury. **(a)** Jag1 expression in lung cryosections of mice at day 10 after the fourth injection of bleomycin (Bleo) or PBS. Other Notch ligand expression is shown in Supplementary Fig. 3b–c; Scale bar, 50 μm in Figure 2. **(b)** Inducible endothelial cell-specific deletion of *Jag1* in adult mice (*Jag1*^{EC/EC}) was generated by breeding mice expressing VE-cadherin (Cdh5)-driven tamoxifen-responsive Cre (VE-Cad-Cre^{ERT2}) with *Jag1*^{loxP/loxP} mice. Mice with endothelial cell-specific haploinsufficiency of *Jag1* (*Jag1*^{EC/+}) served as a control. **(c)** Sirius red staining of control and *Jag1*^{EC/EC} mouse lungs at day 35 after the sixth bleomycin injection. **(d,e)** Levels of collagen I, SMA, and hydroxyproline in injured mouse lungs at day 35 after the sixth bleomycin injection; **P* < 0.05; *n* = 8 mice in the *Jag1*^{EC/EC} group and *n* = 9 animals in the control group. Error bars denote s.e.m. in Figure 2. Quantification of protein levels is shown in Supplementary Fig. 3f,g. **(f)** Smad3 activity in lung fibroblasts of the indicated groups of mice was analyzed at day 35 after the sixth bleomycin injection by electrophoretic mobility shift assay (EMSA). Quantification is shown in Supplementary Fig. 5c. **(g)** GFP expression in WT mouse lungs after intravenous injection of endothelial cell-targeted virus encoding GFP (*Gfp*^{EC}). GFP localization was examined in VE-cadherin⁺ PCECs and E-cadherin⁺ AECs. The generation and characterization of endothelial cell-

targeted virus are shown in Supplementary Fig. 6a–f. **(h–j)** After injection of EC–targeted Jag1 shRNA (*shJag1^{EC}*), levels of Jag1, SMA, and hydroxyproline were examined in mouse lungs, and Hes1 protein was detected by immunoblotting in isolated lung fibroblasts. Antibody Mec13.3 recognizing endothelial-enriched antigen CD31 was coupled with pseudotyped virus expressing scrambled sequence (*Srb^{EC}*) or different Jag1 shRNA clones (*shJag1^{EC}*, C1–C5). Anti-CD31 mAb and Rat IgG lanes indicate mouse groups that received equal amounts of uncoupled mAb Mec13.3 or rat IgG isotype control. Assays were performed at day 35 after the sixth bleomycin injection. **P* < 0.05; *n* = 8 mice per group. Immunoblot quantification is shown in Supplementary Fig. 6h.

Figure 3.

The CXCL12 receptor CXCR7 promotes lung repair and suppresses fibrosis after bleomycin injury. **(a)** Immunostaining for CXCR7, SMA and VE-cadherin in lung cryosections in mice at day 10 after the fourth injection of bleomycin (Bleo) or PBS. Scale bar, 50 μm in Figure 3. **(b)** Immunoblotting for CXCR7 in mouse PCECs from mice of the indicated groups. Quantification is shown in Supplementary Fig. 7b. **(c)** Collagen deposition and tissue morphology of mouse lungs 35 days after the sixth bleomycin injection, in mice treated with local infusion of the CXCR7 agonist TC14012 or vehicle. H&E, Hematoxylin & Eosin staining. Quantification of the Sirius red-positive area and lung hydroxyproline levels is shown in Supplementary Fig. 7f, g. **(d,e)** Alveolar epithelial structure and function were determined in mice at day 35 after the sixth bleomycin injection. The percentage of

aquaporin-5⁺Podoplanin⁺ AEC1 area **(d)** and blood oxygenation **(e)** were analyzed after treatment with vehicle (Veh) or TC14012. $n = 8$ mice per group. Representative staining is shown in Supplementary Fig. 7h. $*P < 0.05$. **(f,g)** Immunoblotting for Jag1 and SMA in mouse lungs **(f)** and Hes1 in lung fibroblasts **(g)** in $Cxcr7^{fl/EC}$ and control $Cxcr7^{fl/EC/+}$ mice treated with TC14012 or vehicle. **(h)** Quantification of the immunoblotting data in **f** and **g**. Error bars depict s.e.m. in Figure 3. $*P < 0.05$; $n = 8$ mice per group. **(i)** Immunoblotting for SMA and collagen I in the indicated groups of mice at day 35 after the sixth bleomycin injection. Quantification is shown in Supplementary Fig. 8c,d.

Figure 4.

VEGFR1⁺ perivascular macrophages induce β-catenin-dependent Jag1 upregulation in PCECs. **(a)** Immunostaining for β-galactosidase (LacZ), SMA and VE-cadherin in β-catenin reporter (Axin2-lacZ) mice that were subjected to PBS or four bleomycin injections. Scale bars, 50 μm in Fig. 4. **(b)** Immunoblotting for Jag1 in wild type and *Cxcr7*-deficient mouse PCECs treated with Wnt3A and TC14012 as indicated. **(c)** Experimental scheme for interrogating the influence of macrophages on endothelial β-catenin activation. Mice with endothelial (EC)-specific β-catenin activation (*Ctnnb1-Ex3^{ΔEC/+}*) or control mice (*Ctnnb1-Ex3^{+/+}*) were treated with clodronate liposomes to deplete macrophages and monocytes before the fourth bleomycin injection, or were treated with the CXCR7 agonist TC14012. **(d)** Immunoblotting for Jag1, SMA, and collagen I in *Ctnnb1-Ex3^{ΔEC/+}* mice after

clodronate liposome injection (day 35 after the sixth bleomycin injection). N/A indicates mouse group without treatment of clodronate liposome or vehicle. **(e)** Levels of endothelial cell-activating factors in mouse lung macrophages at day 10 after the fourth bleomycin injection. $n = 10$ mice in the Wnt3A group and $n = 9$ mice in the other groups. Each dot indicates an individual mouse. SDF-1, stromal-derived factor 1; VEGF-A, vascular endothelial growth factor-A; FGF-2, fibroblast growth factor-2. **(f)** Wnt3A expression in mouse macrophages after injection of the indicated cytokines. Plgf, placental growth factor; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor. $n = 10$ mice in the Plgf group and $n = 9$ animals in the other groups. **(g)** VEGFR1⁺F4/80⁺CD11b⁺ macrophages in WT mouse lungs at day 10 after the fourth injection of bleomycin or PBS. **(h)** Immunostaining for Wnt3A, CD11b, F4/80, and VE-cadherin in injured mouse lungs (day 10 after the fourth bleomycin injection) in WT (*Vegfr1*^{+/+}) or *Vegfr1*^{LysM}/^{LysM} mice.

Figure 5.

In chronically injured lungs, stimulation of β -catenin–Jag1 pathway in PCECs by VEGFR1⁺ macrophages is suppressed by CXCR7 agonist. (a) Experimental scheme for studying the effect of VEGFR1⁺ macrophages and monocytes on lung fibrosis. Monocytes from *Vegfr1*^{+/+} or *Vegfr1*^{LysM^{-/-}} mice were intravenously transplanted to macrophage-depleted wild type or *Ctnnb1-Ex3*^{EC/+} mice after the fourth bleomycin injection and analyzed for fibrotic responses. To examine the influence of CXCR7, mice were also treated with vehicle or the CXCR7 agonist TC14012 after monocyte transfer. (b–f) Fibrosis in injured wild type (b,d, top graphs) and *Ctnnb1-Ex3*^{EC/+} (b,d, bottom graphs) mice after receiving *Vegfr1*^{+/+} or *Vegfr1*^{-/-} monocytes and vehicle or TC14012. Determination of pulmonary hydroxyproline levels (b), Sirius red staining (c,d), H&E staining (c), and

immunoblotting of Jag1 and SMA (**e,f**) were performed in the indicated groups of mice. Representative images are shown in **c**. In **b, d** and **f**, * indicates $P < 0.05$ between vehicle and TC14012 treatment (in both WT and *Ctnnb1-Ex3^{fl}*^{EC/+} mice). # indicates $P < 0.05$ between mice infused with *Vegfr1*^{+/+} and *Vegfr1*-deficient monocytes. Error bars denote s.e.m.; $n = 7$ mice in the TC14012 groups and $n = 8$ mice in the vehicle groups. Scale bars, 50 μm .

Figure 6.

Lung repair and fibrosis in mice after repeated hydrochloric acid aspiration. **(a)** The experimental scheme for inducing lung injury by single or multiple intratracheal instillations of hydrochloric acid (acid). **(b–e)** AEC1 morphology (as assessed by immunostaining for the AEC1 markers aquaporin-5 and podoplanin **(b)**), blood oxygenation levels **(c)**, lung Jag1 and SMA protein expression and pulmonary hydroxyproline levels **(d)** were determined 25 days after the 5th acid treatment in mice treated with vehicle or TC14012. Representative Sirius red and H&E staining images of the injured lungs are shown in **e**. $n = 6$ mice per group. Error bars depict s.e.m. Scale bars, 50 μm in Figure 6. **(f–i)** In mice intravenously injected *shJag1*^{EC} or control *Srb*^{EC} virus, effects on lung repair were examined at day 25

after the fifth acid treatment, as assessed by AEC1 morphology (**f**), protein levels of lung fibroblast Hes1 and of pulmonary SMA and collagen I (**g,h**), and blood oxygenation (**i**). * $P < 0.05$ between *shJag1*^{EC} and *Srtb*^{EC} with groups. Quantification of protein levels is shown in Supplementary Fig. 12. $n = 9$ animals per group. (**j**) The lung hematopoietic–vascular niche modulates alveolar repair and fibrosis. After acute injury, CXCR7 in PCECs promotes an epithelial response (AEC proliferation) and prevents fibrosis. After chronic injury, recruitment of VEGFR1⁺ perivascular macrophages instigates pathological Jag1 upregulation in PCECs, dependent on Wnt– β –catenin signaling. This sustained upregulation of Jag1 elicits juxtacrine Notch activation in perivascular fibroblasts, promoting pulmonary fibrosis. Targeting of this niche could enhance alveolar epithelial repair and ameliorate lung fibrosis.