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Fibrocytes Are Not an Essential Source of Type I Collagen during Lung Fibrosis

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Progressive fibrosis involves accumulation of activated collagen-producing mesenchymal cells. Fibrocytes are hematopoietic-derived cells with mesenchymal features that potentially have a unique and critical function during fibrosis. Fibrocytes have been proposed as an important direct contributor of type I collagen deposition during fibrosis based largely on fate-mapping studies. To determine the functional contribution of hematopoietic cell-derived type I collagen to fibrogenesis, we use a double-transgenic system to specifically delete the type I collagen gene across a broad population of hematopoietic cells. These mice develop a robust fibrotic response similar to littermate genotype control mice injured with bleomycin indicating that fibrocytes are not a necessary source of type I collagen. Using collagen–promoter GFP mice, we find that fibrocytes express type I collagen. However, fibrocytes with confirmed deletion of the type I collagen gene have readily detectable intracellular type I collagen indicating that uptake of collagen from neighboring cells account for much of the fibrocyte collagen. Collectively, these results clarify several seemingly conflicting reports regarding the direct contribution of fibrocytes to collagen deposition. The Journal of Immunology, 2014, 193: 000–000.

Fibrosis is a common feature of many systemic inflammatory conditions and can also occur as a primary progressive disease. Fibrosis can lead to organ dysfunction and significant morbidity. Collectively, fibrosis is the leading cause of death in developed countries. Idiopathic pulmonary fibrosis is the most common primary fibrotic lung disorder, affecting >5 million people worldwide. The median survival of patients with this condition is 3–5 y from the time of diagnosis and current medical therapy is largely ineffective (1–5). Unfortunately, despite intense investigation, we still have a poor understanding of the pathogenesis of tissue fibrosis and fibrotic diseases are difficult to treat. Recent evidence suggests that fibrogenesis is more complex than originally thought and involves activation of and coordination of many cells types that contribute directly and indirectly to fibrogenesis. A more thorough delineation of the processes that underlie fibrogenesis requires the investigation of novel pathways and cell types.

Fibrosis is characterized by accumulation of activated fibroblasts and excessive deposition of fibrotic extracellular matrix proteins, especially type I collagen. Extensive research has focused on mechanisms of type I collagen synthesis by fibroblasts. However, the origin of type I collagen secreting cells remains unclear and controversial (6). Injury leads to sequential remodeling of the extracellular matrix with rapid replacement by plasma derived matrix proteins and eventual replacement with fibrillar collagens derived from activated fibrogenic cells. Initiation of these events can occur through recruitment of circulating cells with fibrogenic potential into the microenvironment, proliferation and activation of quiescent fibroblasts, and transdifferentiation of structural cells into fibroblast-like cells. The original paradigm assumed that the accumulated activated fibroblasts were derived from proliferation and activation of quiescent resident tissue fibroblasts. However, recently other possibilities for the origin of collagen producing cells have been proposed including epithelial cells, endothelial cells, pericytes, mesenchymal stem cells, and fibrocytes (7–14). Differentiating among these possibilities is important because pathways leading to their activation may be distinct. There is significant overlap in mechanisms of activation of fibroblasts, epithelial cells, endothelial cells, and pericytes. For example, TGF-β is the most well established cytokine leading to fibroblast to myofibroblast transition and epithelial to mesenchymal transition (15, 16). Most, if not all, factors promoting mesenchymal transition of epithelial and endothelial cells have also been studied in models of fibroblast activation. Fibrocyte recruitment may be unique in their ability to respond to a number of cytokines and chemokines typically associated with activation of inflammatory cells (17). Fibrocytes are hematopoietic bone marrow–derived cells that express both fibroblast and leukocyte markers. They circulate in peripheral blood and can be isolated from many tissues including the lung. Cultured fibrocytes have been shown to express a number of fibrotic extracellular matrix proteins including collagen I, collagen III, and fibronectin (17–21). In addition, fibrocytes maintain expression of common leukocyte markers including CD45, CD13, and CD34. Fibrocytes have been shown to secrete a number of profibrotic cytokines that could potentially help orchestrate fibrogenesis. Importantly, fibrocytes express a number of chemokine receptors, including CXCR4, CCR7, and CCR2, which likely mediate recruitment and activation of fibrocytes to areas of tissue damage (22–24). Thus, recruitment of fibrocytes to sites of injury suggests an important transition from inflammation to fibrogenesis with the ability of fibrocytes to respond to inflammatory cytokines,
produce fibrotic extracellular matrix proteins, and foster further activation of fibroblasts and other cell types (17). Although several reports indicate that fibrocytes express type I collagen, others have suggested that uptake of secreted type I collagen by hematopoietic cells accounts for the fibrocyte population (25, 26). Thus, whether fibrocytes contribute to matrix accumulation via direct or indirect mechanisms remains unclear.

Prior studies on the origin of fibrogenic effector cells have relied primarily on generation of fate-mapping chimeric mice by either transplanting bone marrow from a transgenic reporter mouse into a wild-type mice or by crossing cell type–specific Cre mice with transgenic lox-stop-lox reporter mice. Following induction of fibrosis, the genetically labeled cells are then costained for markers of activated fibroblasts, such as α-smooth muscle actin. However, fate-mapping studies have only led to further controversy. For example, there are contrasting reports on occurrence of the fibrocyte–myofibroblast transition, epithelial–mesenchymal and pericyte–myofibroblast transition in models of fibrosis (12–14, 27–35). Furthermore, myofibroblasts are unlikely to be the only collagen-producing cells during fibrogenesis (36). Finally although fate-mapping and costaining for extracellular matrix protein markers demonstrate some capacity of the labeled cell type to express fibrotic matrix proteins, fate-mapping studies have a limited ability to determine the quantitative and functional contribution of different cell types to accumulation of fibrotic matrix proteins. An alternative approach is to delete genes important for the function of fibrogenic cells within specific cell populations.

In this study, we investigate the possibility that hematopoietic cells are a major contributor to type I collagen deposition during lung fibrogenesis using mice in which the Col1a1 gene has been specifically deleted within hematopoietic cells.

Materials and Methods

Mice

The floxed Col1a1 mice in a C57BL/6 background are described previously (37). Vav-iCre mice are from The Jackson Laboratory (38). Type I collagen-GFP (Col-GFP) reporter mice are described previously (39). These mice were genotyped by PCR. Six- to 8-week-old mice were given 50 μl intratracheal injections of saline or bleomycin (2 U/kg) dissolved in saline via surgical tracheotomy (37). At various time points after injection, mice were euthanized, and samples were collected for analysis. All mice were bred and maintained in a specific pathogen-free environment, and all animal experiments were approved by the University Animal Care and Use Committee at University of Michigan.

Reagents

Bleomycin was purchased from Millipore (Billerica, MA). Biotin-conjugated rat anti–mouse CD16/32 and CD45 Abs, PE- and Cy7-conjugated anti–mouse CD11b Ab, and Cytofix/Cytperm and Perm/wash were from BD Biosciences (San Jose, CA). Anti–mouse CD45 magnetic beads and MACS Cell Separation columns were obtained from Miltenyi Biotec (San Diego, CA). Collagen I Ab was purchased from Rockland (Gilbertsville, PA); HRP-conjugated secondary Abs were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Collagenase A was purchased from Roche (Indianapolis, IN). Alexa Fluor–conjugated secondary Abs and fluorescein-conjugated type I collagen were obtained from Life Technologies (Grand Island, NY). Rabbit IgG isotype control Ab was purchased from Southern Biotechnology Associates (Birmingham, AL). Collagen III Ab was from Abcam (Cambridge, MA). LAMP1 Ab was from the University of Iowa Developmental Studies Hybridoma Bank. Environodes expressing Cre was from the University of Iowa Gene Transfer Vector Core Facility. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation of primary mouse lung mesenchymal cells

Primary lung mesenchymal cells were isolated as described previously (22). Briefly, 6- to 10-week-old uninjured or bleomycin-injured mice were sacrificed, and lungs were perfused with PBS. Lungs were removed and minced to ~1-mm cubed pieces. Mined lungs were maintained in DMEM supplemented with 10% FBS in a 37°C, 5% CO2 incubator allowing primary lung mesenchymal cell outgrowth over 2–4 wk. Primary mesenchymal cells were analyzed between passages 2–4 and within 4 wk of isolation. In some experiments, primary lung mesenchymal cells from Col1a1−/− mice were treated with adenovirus encoding Cre (50 PFU/cell) (37).

Isolation of whole lung single cell suspension

Whole-lung single-cell suspensions were prepared as described previously (22, 40, 41). In brief, mice were euthanized at the time points indicated. Lungs were perfused with PBS, excised, and minced as above. Mined lung fragments were incubated in digestion solutions containing collagenase (1 mg/ml) and DNase (25 U/ml) in RPMI 1640 medium at 37°C for 30 min. Samples were dispersed by passing up and down 20 times with a 10-ml syringe. After pelleting, supernatants were decanted, and hypotonic shock was performed to remove RBCs. Samples were washed once with RPMI 1640 medium. Cells were isolated from the crude digest by passing through a 40-μm cell strainer. The cells were washed in 40% percoll and then resuspended in RPMI 1640 medium. Immediately after isolation, purified whole lung single cell suspensions were used for further analysis.

Flow cytometry and cell sorting

Primary lung mesenchymal cells and whole-lung single-cell suspensions were analyzed by flow cytometry as described previously (22, 23). Resuspended cells were incubated in blocking buffer containing 1% BSA and anti–CD16/32 Ab in PBS for 30 min. Cells were then incubated with fluorescent-conjugated CD45 and CD11b Abs in blocking buffer for 30 min. In some experiments, cells were fixed and permeabilized with Cytofix/Cytperm, according to the manufacturer’s protocol. Cells were then stained with rabbit anti–collagen I Ab or rabbit IgG isotype control in Perm/wash buffer for at least one hour. After washing cells were stained with fluorescent-conjugated anti–rabbit secondary Ab and analyzed by flow cytometry using an Attune Flow Cytometer (Life Technologies). Cells were sorted using a MoFlo Astrios (Beckman Coulter) or FACSAria III (BD Biosciences). Cell suspensions and cell surface collagen-FITC were quenched with trypan blue (200 μg/ml) prior to flow cytometry analysis as described (42–44).

H&E staining and Masson’s trichrome assay

Lungs from sacrificed mice were inflated to 25 cm H2O pressure and fixed with formaldehyde. Lungs were embedded in paraffin, sectioned and stained with H&E and Masson’s trichrome by the McClinchey Histology Lab (Stockbridge, MA). Lungs sections were visualized on an Olympus BX-51 microscope, and images were captured with an Olympus DP-70 camera (37).

Immunofluorescence staining

Primary lung mesenchymal cells from wild-type mice were seeded on chamber slides (27, 37, 45, 46). After 24 h, cells were fixed, permeabilized, and stained for the proteins indicated as described previously. Stained slides were visualized as above.

Hydroxyproline assay and bronchoalveolar lavage

Bronchoalveolar lavage samples were obtained and quantified on a hemocytometer as described previously. Hydroxyproline was measured by methods described previously (37, 46, 47). Briefly, homogenates from both lungs were incubated in 12 N HCl for at least 16 h at 120°C. After addition of citrate buffer and chloramine T solutions, samples were incubated for 20 min. Then, Griess’s solution was added to the mixture and incubated for an additional 15 min at 65°C. The absorbance at 540 nm was measured, and the hydroxyproline concentration was determined against a hydroxyproline standard curve.

Gene expression analysis

mRNA was isolated from cells and tissue with TRizol (Invitrogen), according to the manufacturer’s protocol. Reverse transcription was performed with SuperScript III First-strand synthesis kit (Invitrogen), and RT-PCR was performed to using the POWER SYBR Green PCR MasterMix.
Kit (Applied Biosystems) and Applied Biosystems 7000 sequence detection system. The relative expression levels of col1a1 in fold changes were calculated against β-actin or GAPDH as described previously (37, 46).

**DNA analysis**

DNA was isolated from cells by proteinase K digestion. PCR primers within the floxed portion of the floxed col1a1 gene (exon2 2-5) were as follows: forward, 5'-AGTCAGCTGCATACAAATGCCCT-3', and reverse, 5'-ATACGATGCTTACCCTTGGGCTT-3'. Equal loading of DNA for PCR was confirmed using primers from an adjacent region outside the floxed portions (exon 6): forward, 5'-AGAGACCGAGGAGCAAGAATA-3'; and reverse, 5'-CAGTCGAGTTTCTCCCTCTAA-3'. PCR products were separated on agarose gels and visualized using a UVP transilluminator (UVP). Quantitative PCR analysis of the floxed col1a1 exons 2–5 was determined using the POWER SYBR Green PCR MasterMix Kit (Applied Biosystems) and Applied Biosystems 7000 sequence detection system. Relative amount of col1a1 exons 2–5 were calculated against col1a1 exon 6.

**Immunoblot**

Immunoblot of protein lysate was performed as described previously (27, 37, 45, 46). Scanned immunoblots are representative of at least three separate experiments. In some experiments, densitometry was quantified by ImageJ as described previously (37, 46).

**Statistical analysis**

Data are expressed as mean ± SEM. The two-tailed Student t test was used to determine differences between groups. A p value < 0.05 was accepted as significant.

**Results**

**Primary lung mesenchymal cells contain CD45-positive, collagen I expressing cells**

We and others have shown that primary fibroblast-like mesenchymal cells derived from outgrowth of minced lung tissue contain a subpopulation of fibrocytes that have a mixed hematopoietic and mesenchymal phenotype and protein expression (17, 22). To verify these prior observations, primary murine lung mesenchymal cells were isolated and cultured for 2 wk. Cells were stained for CD45, a leukocyte common Ag present on hematopoietic cells, and analyzed by flow cytometry. The cultured primary lung mesenchymal cells were found to contain ∼75% (50–90%) CD45-negative cells (fibroblasts) and ∼25% (10–50%) CD45-positive fibrocytes. We confirmed expression of type I collagen, a mesenchymal marker, within both populations by several techniques. The CD45-positive and -negative populations were sorted and analyzed by immunoblot and compared with alveolar macrophages as a negative control. As expected, both CD45-positive and -negative cells had type I collagen protein compared with undetectable levels of collagen I in alveolar macrophages (Fig. 1). There were greater levels of type I collagen in the CD45-negative population compared with the CD45-positive population consistent with prior observations (22). Type I collagen protein in fibroblasts and fibrocytes was further verified by coimmunostaining for CD45 and type I collagen. Again, both populations stained for type I collagen but there was greater type I collagen within CD45-negative fibroblasts. Finally, to ensure expression of type I collagen within CD45-positive fibrocytes, we used a previously described type I collagen promoter-GFP (Col-GFP) reporter mouse (39). Activation of the type I collagen promoter within fibroblasts and fibrocytes was confirmed by isolating primary lung mesenchymal cells from wild-type and Col-GFP mice. After 2 wk in culture cells were stained for CD45-expression and analyzed by flow cytometry. CD45-positive and CD45-negative lung mesenchymal cells from Col-GFP mice had significantly greater FL1 (GFP) signal compared with wild-type cells indicating type I collagen promoter activity within cultured fibroblasts and fibrocytes (Fig. 2). Consistent with the above results, CD45-negative cells had greater FL1 signal compared with CD45-positive population indicating greater activation of the collagen I promoter within fibroblasts. Collectively, these results indicate that primary lung mesenchymal cells contain a mixture of CD45-negative/collagen I-positive fibroblasts and CD45-positive/collagen I-positive fibrocytes.

**Generation of mice with hematopoietic deletion of type I collagen**

To determine the direct contribution of hematopoietic cells to collagen production during fibrogenesis, we used the previously described floxed col1a1 (col1a1fl/fl) mouse in which loxP sites flank exons 2–5 enabling permanent deletion of type I collagen.
within cells expressing Cre recombinase (37). Primary lung mesenchymal cells from col1a1fl/fl mice were treated in vitro with an adenovirus expressing Cre. After 2 wk, there was near complete deletion of type I collagen protein within CD45-positive and -negative populations. Floxed collagen mice were then crossed with transgenic Vav-cre mice, which have been previously reported to delete floxed genes specifically within all hematopoietic cells (38). Double-transgenic Vav-cre/col1a1fl/fl (Vav-Col) mice have normal lung histology at baseline compared with littermate control mice lacking either the Vav-cre or the col1a1fl genes (Fig. 3). Primary lung mesenchymal cells were isolated from Vav-Col and littermate control mice. After 2 wk in culture, cells were analyzed by flow cytometry. Vav-Col lung mesenchymal cells had similar proportions of CD45-positive and -negative cells compared with lung mesenchymal cells from littermate control mice or wild-type mice. Cells were then FACS sorted for CD45-positive and CD45-negative populations and lysed for DNA and protein isolation. DNA was analyzed by PCR using primers within the floxed portion of the floxed col1a1 allele. CD45-positive cells from the Vav-Col mice demonstrated significant loss of the floxed col1a1 exons and loss of col1a1 mRNA expression indicating robust recombination/deletion of type I collagen within fibrocytes. In contrast, CD45-negative cells from Vav-Col mice demonstrated persistent levels of the floxed col1a1 exons compared with CD45-positive and -negative cells from control mice (Fig. 4). Thus, Vav-Col mice have deletion of the col1a1 gene specifically within fibrocytes. Next, protein isolated from the CD45-sorted cells were analyzed by immunoblot for type I collagen. As before, CD45-negative fibroblasts had more type I collagen than CD45-positive fibrocytes. Surprisingly, fibrocytes from Vav-Col mice had similar amounts of type I collagen compared with fibrocytes from littermate control mice (Fig. 4), suggesting that much of the type I collagen protein within these cells is not through transcription/translation of type I collagen but perhaps through uptake of type I collagen secreted by the cocultured fibroblasts.

Primary mesenchymal cells derived from outgrowth of minced lung tissue 2 wk after intratracheal bleomycin administration demonstrate a similar pattern. CD45-positive fibrocytes from bleomycin-injured Col-GFP mice demonstrate expression of GFP that is significantly less than GFP expression by CD45-negative fibroblasts (Fig. 5A, 5B). CD45-positive fibrocytes from Vav-Col mice contain type I collagen protein despite absence of col1a1 exons 2–5 DNA and col1a1 mRNA (Fig. 5C–E).

**Hematopoietic-deletion of collagen does not attenuate fibrosis**

Next, Vav-Col mice and littermate genotype control mice were injured with intratracheal bleomycin or saline. After 2 wk, single-
cell preparations from the injured lungs were analyzed by flow cytometry for CD45 and intracellular type I collagen. As expected, bleomycin leads to increased numbers of collagen positive cells. Surprisingly, Vav-Col mice and genotype control mice injured with bleomycin exhibit similar numbers of CD45/collagen I double-positive cells (Fig. 6). There was a trend toward more CD45-negative/collagen I–positive cells in Vav-Col mice, suggesting that other cell types might be compensating for loss of functional fibrocytes, but these differences were not statistically significant ($p = 0.3$). To ensure deletion of col1a1 within the CD45/collagen double-positive population, single-cell suspensions of bleomycin-injured mice were FACS sorted. DNA was immediately isolated from the sorted cells and analyzed for recombination using primers within the floxed region. As with the in vitro experiments, CD45-positive/collagen I–positive cells from Vav-Col mice had robust recombination/deletion of the col1a1 gene compared with CD45-negative/collagen I–positive cells from Vav-Col mice and cells from genotype control mice. Furthermore, protein and mRNA from CD45-positive and CD45-negative populations demonstrate loss of col1a1 mRNA within Vav-Col fibrocytes but similar levels of collagen I and collagen III protein by immunoblot (Fig. 7). Sorted cells were further characterized for CD11b, another common fibrocyte marker, indicating high expression of CD11b within CD45-positive/collagen I–positive fibrocytes from Vav-Col and littermate control mice but not within CD45-negative/collagen I–positive fibroblasts (Fig. 7F, 7G). Three weeks after bleomycin injury, the extent of fibrosis was determined by hydroxyproline assay and trichrome staining, and the extent of injury was determined by bronchoalveolar lavage cell count. Bleomycin injury led to increased total collagen content in both Vav-Col mice and littermate genotype control mice. The extent of fibrosis and injury was similar in Vav-Col and genotype control mice after bleomycin injury (Fig. 8, Supplemental Fig. 1).

Fibrocyte endocytosis of type I collagen

Our studies suggest that although hematopoietic cells have some capacity for activation of collagen genes, much of the type I collagen within these cells is not through production of type I collagen but rather through uptake of collagen produced by neighboring cells. Many hematopoietic cells have a significant capacity for uptake of particles and proteins through phagocytosis and pinocytosis (25, 26). To determine the capacity of primary
lung mesenchymal cells to endocytose type I collagen in the culture media, we used a purified type I collagen directly conjugated with FITC (Col-FITC) (42). Primary lung mesenchymal cells were isolated from wild-type mice. After 2 wk in culture, cells were incubated with Col-FITC for 0, 10, 30, and 90 min. After the indicated times, cells were thoroughly washed to remove free Col-FITC, and then, cells were stained for CD45 and analyzed by flow cytometry. Both the CD45-positive and -negative populations demonstrated increased FL1 (FITC) signaling that increased over the 90 min indicating some capacity for Col-FITC uptake, consistent with prior reports of collagen uptake by different cell types (42, 48–51). There was significantly greater Col-FITC within the CD45-positive fibrocyte population compared with the CD45-negative fibroblast population. Internalization of Col-FITC was confirmed by colocalization with LAMP1, a lysosomal marker, and by cell surface fluorescence quenching with trypan blue. This indicates that fibrocytes have a robust ability to endocytose extracellular collagen (Fig. 9, Supplemental Fig. 2).

Discussion

These results demonstrate that cells of hematopoietic origin produce type I collagen but are not a necessary source of type I collagen during experimental lung fibrosis. Furthermore, using Vav-Cre transgenic mice to specifically delete type I collagen within hematopoietic cells, we surprisingly find substantial intracellular type I collagen in fibrocytes despite confirming absence of the col1a1 gene. As our studies clarify, identification of intracellular type I collagen does not necessarily indicate collagen I production by that cell. Furthermore, because procollagen I is secreted and matured into collagen I by proteolytic cleavage in the extracellular space, identification of intracellular procollagen I also does not necessarily indicate collagen I production by that cell (52). Notably, we find activation of the type I collagen promoter by fibrocytes using GFP-reporter mice. Collectively, our results suggest that fibrocytes express some type I collagen, but a large portion of its intracellular collagen is through uptake from neighboring collagen-secreting cells.

We confirm prior observations that a commonly used minced lung cell outgrowth protocol to generate primary lung fibroblasts produces a mixed population of collagen-positive lung mesenchymal cells made up of CD45-negative fibroblasts and CD45-positive fibrocytes (22). The resulting coculture system may crudely model the close approximation of activated fibroblasts and fibrocytes that occurs in vivo during fibrogenesis. Both in vivo and in vitro we readily identify intracellular collagen within CD45-positive cells despite deletion of the collagen I gene indicating uptake of fibroblast-derived type I collagen.

The contribution of fibrocytes to fibrogenesis remains controversial (17). Studies from fibrotic human tissue indicate that fibrocytes express some type I collagen protein and mRNA and that fibrocytes likely contribute to fibrogenesis (34, 53–56) Given the importance of type I collagen to fibrosis and the evidence that fibrocytes express type I collagen, it is compelling to infer that collagen deposition is a major function of fibrocytes. However, the importance of fibrocyte-derived type I collagen to fibrogenesis has not been directly investigated. Several prior studies investigating the contribution of fibrocytes to fibrogenesis have used reporter gene chimera mice to quantify the fraction of collagen-positive cells of hematopoietic origin (14, 34, 35, 57). These prior studies are limited in their ability to assess the functional contribution of...
hematopoietic cells to collagen deposition, which involves multiple regulated steps after collagen promoter activation (translation, secretion, maturation, fibrillogenesis, and so on) (52). Although our studies suggest that fibrocytes (and other hematopoietic cells) are not a necessary source of type I collagen during fibrosis, it remains possible that hematopoietic cells do contribute to some of the collagen accumulation. Profibrotic cytokines released during injury may lead to recruitment of diverse populations of potential fibrotic effector cells. In this model, loss of one source of type I collagen may lead to augmented compensatory contribution of other collagen-producing cells. Indeed, there is a suggestion of increased numbers of collagen-positive/CD45-negative cells in

FIGURE 5. In vitro-derived fibroblasts from bleomycin-injured mice express type I collagen. (A and B) Two weeks after intratracheal bleomycin injury, minced lung outgrowth mesenchymal cells from wild-type and Col-GFP mice were isolated and analyzed by flow cytometry. (A) Histogram overlay of GFP expression by CD45-positive (CD45+) and CD45-negative (CD45-) cells. (B) Quantification of GFP expression demonstrating greater GFP expression in CD45+ fibroblasts ($p < 0.01$), but significant GFP within CD45+ fibrocytes from Col-GFP mice compared with wild-type cells ($p < 0.05$); $n = 4$. (C–E) Two weeks after bleomycin, minced lung outgrowth mesenchymal cells from control and Vav-Col mice were sorted for CD45 and compared with alveolar macrophage (AM) control. (C) Immunoblot for type I collagen demonstrating less type I collagen within CD45+ cells but similar amounts of type I collagen between CD45+ cells from control mice and Vav-Col mice. (D) DNA quantitative PCR for floxed col1a1 exons 2–5 DNA demonstrates CD45+ cells from Vav-Col mice have robust recombination compared with other samples ($p < 0.01$). $n = 4$. (E) Vav-Col mice have loss of col1a1 mRNA compared with CD45+ cells from control mice by qPCR ($p < 0.05$). $n = 4$.

FIGURE 6. Vav-Col mice have similar numbers of fibrocytes compared with genotype control mice after bleomycin. (A and B) Single-cell whole-lung preparation from control and Vav-Col mice were analyzed by flow cytometry for CD45 and type I collagen (B) or isotype control for collagen staining (A). (C) Bleomycin injury leads to increased numbers of collagen-positive cells. Bleomycin-injured Vav-Col mice have similar numbers of collagen-positive fibrocytes compared with littermate control mice ($n = 4$).
bleomycin-injured Vav-Col mice compared with genotype control mice (Fig. 6).

The cellular origin of type I collagen during fibrosis remains controversial. We recently reported that mice with deletion of type I collagen within alveolar epithelial cells developed less fibrosis but have more lung injury and death compared with genotype control mice, implying type I collagen from different cellular sources could have unique functions during fibrogenesis perhaps because of the unique spatial niche or timing of the collagen deposition (37). Indeed, we found that epithelial cell–derived type I collagen activated collagen receptor–mediated signaling on neighbor fibroblasts, potentially leading to augmented fibroblast activation. Type I collagen itself has been well studied as an initiator of cell signaling through a number of cell surface receptors and could coordinate mesenchymal cross-talk. The importance of type I collagen from other cell types such as pericytes and mesenchymal stem cells is currently being pursued, but we believe that the contribution of type I collagen by these different cell types will be more complicated than simply quantifying the fractional contribution to the overall collagen accumulation during fibrosis given the likely importance of collagen signaling and collagen-mediated cell migration.

Fibrocytes do not encompass all bone marrow–derived mesenchymal cells, which include mesenchymal stem/stromal cells (MSCs), which are defined, in part, as CD45 negative (58). There has been controversy on whether bone marrow–derived MSCs are derived from the hematopoietic lineage or from a separate lineage. Thus, the direct contribution of bone marrow–derived MSCs to fibrosis remains unclear. However, one recent report using Vav-Cre mice in a fate-mapping strategy found evidence of labeled CD45-negative cells during fibrogenesis suggesting a common origin with hematopoietic cells (59).

We found that fibrocytes have a robust capacity for type I collagen uptake. Whether uptake of collagen occurs via collagen receptor-mediated process or by a less specific pinocytosis mechanism is unclear. Inhibition of several type I collagen receptors did not affect type I collagen uptake in our system (data not shown). Uptake of collagen by fibrocytes may result in transcriptional/phenotypic changes within fibrocytes with potential consequences for fibrosis. Finally, fibrocyte uptake of type I collagen may be an

**FIGURE 7.** Hematopoietic cell deletion of col1a1 does not affect fibrocyte type I collagen protein levels in vivo. (A) DNA was isolated from FACS-sorted CD45-positive (CD45+)/collagen I–positive and CD45-negative (CD45−)/collagen I–positive cells from Vav-Col and control mice 2 wk after bleomycin injury. PCR demonstrates loss of col1a1 exons 2–5 in CD45+/collagen I–positive fibrocytes from Vav-Col mice compared with intact col1a1 in cells from genotype control mice and CD45− cells from Vav-Col mice. Primers outside the floxed region (exon 6) are used as a loading control. (B) Quantitative PCR DNA analysis for col1a1 exons 2–5 demonstrate significant recombination within CD45− cells from Vav-Col mice; n = 4. (C) Quantitative PCR analysis for col1a1 mRNA. CD45− cells have less col1a1 mRNA than CD45− cells. CD45− cells from Vav-Col mice have near complete absence of col1a1 mRNA compared with CD45− cells from genotype control mice (p < 0.05; n = 4). (D and E) Immunoblot (D) demonstrates similar levels of type I and third type III collagen within CD45− cells from littermate control and Vav-Col mice quantified by densitometry (E), n = 4. (F and G) CD45+/collagen I–positive (CD45+/Col+) fibrocytes from littermate control (F) and Vav-Col (G) mice also coexpress CD11b compared with CD45−/collagen I–positive (CD45−/Col−) fibroblasts.
important mechanism for turnover of degraded collagen I or for clearance of abundantly secreted procollagen I (26).

Although deletion of type I collagen within fibrocytes did not attenuate fibrosis, we did find expression of type I collagen within cultured fibrocytes. Several groups have found coexpression of traditional hematopoietic and mesenchymal proteins within fibrocytes including cell surface receptors and secreted proteins. These studies suggest that fibrocytes may have unique and necessary functions during fibrogenesis through paracrine activation of other collagen-producing cells. Indeed, adoptive transfer of

**FIGURE 8.** Mice with hematopoietic cell deletion of coll1a1 are not protected from bleomycin-induced lung fibrosis. (A and B) Trichrome-stained lung sections from littermate control (A) and Vav-Col (B) mice 3 wk after bleomycin injury demonstrate robust fibrosis in both (original magnification ×400). (C) Hydroxyproline assay from lungs of control and Vav-Col mice 3 wk after intratracheal saline or bleomycin demonstrate similar induction of fibrosis (n = 4–10).

**FIGURE 9.** Lung fibrocytes readily take up FITC-conjugated collagen in vitro. (A and B) Flow cytometry of primary mesenchymal cells for CD45 and FITC without addition of FITC-conjugated collagen I (t = 0) (A) or 90 min after addition of FITC-conjugated (50 μg/ml) collagen I to the culture media (t = 90) (B). (C) Overlay of t = 0 and t = 90 plots demonstrating greater uptake of FITC-conjugated collagen I in CD45-positive cells. (D) Time course of FITC-conjugated collagen I uptake demonstrating greater uptake by CD45-positive cells compared with CD45-negative cells. *p < 0.01.
fibrocytes leads to augmented fibrosis in mice and several studies have found a correlation between increased numbers of fibrocytes and worse disease progression (23). Future studies into the function of fibrocytes during fibrogenesis could apply a similar strategy to the one used in this report with deletion of mesenchymal genes within a hematopoietic population.

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Disclosures
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