Contribution of Epithelial-derived Fibroblasts to Bleomycin-induced Lung Fibrosis

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Rationale: Lung fibroblasts are key mediators of fibrosis resulting in accumulation of excessive interstitial collagen and extracellular matrix, but their origins are not well defined.

Objectives: We aimed to elucidate the contribution of lung epithelium-derived fibroblasts via epithelial-mesenchymal transition (EMT) in the intratracheal bleomycin model.

Methods: Primary type II alveolar epithelial cells were cultured from Immortomice and exposed to transforming growth factor- β_1 and epidermal growth factor. Cell fate reporter mice that permanently mark cells of lung epithelial lineage with β -galactosidase were developed to study EMT, and bone marrow chimeras expressing green fluorescent protein under the control of the fibroblast-associated S100A4 promoter were generated to examine bone marrow-derived fibroblasts. Mice were given intratracheal bleomycin (0.08 unit). Immunostaining was performed for S100A4, β -galactosidase, green fluorescent protein, and α -smooth muscle actin.

Measurements and Main Results: *In vitro*, primary type II alveolar epithelial cells undergo phenotypic changes of EMT when exposed to transforming growth factor- β_1 and epidermal growth factor with loss of prosurfactant protein C and E-cadherin and gain of S100A4 and type I procollagen. *In vivo*, using cell fate reporter mice, approximately one-third of S100A4-positive fibroblasts were derived from lung epithelium 2 weeks after bleomycin administration. From bone marrow chimera studies, one-fifth of S100A4-positive fibroblasts were derived from bone marrow at this same time point. Myofibroblasts rarely derived from EMT or bone marrow progenitors.

Conclusions: Both EMT and bone marrow progenitors contribute to S100A4-positive fibroblasts in bleomycin-induced lung fibrosis. However, neither origin is a principal contributor to lung myofibroblasts.

Keywords: epithelial-mesenchymal transition; fibroblasts; S100A4; lung epithelium

Fibrotic lung diseases, including the clinically distinct disorder of idiopathic pulmonary fibrosis (IPF), represent a heterogeneous group of diseases in which progressive parenchymal

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Fibroblasts are the effector cells in pulmonary fibrosis, responsible for the deposition of collagen and other extracellular matrix. Multiple origins are implicated for the lung fibroblast population, including the possibility that these cells arise through epithelial-mesenchymal transition (EMT).

What This Study Adds to the Field

Both EMT and bone marrow progenitors contribute to S100A4-positive fibroblasts in bleomycin-induced lung fibrosis. However, neither origin is a principal contributor to lung myofibroblasts.

fibrosis disrupts the structure and gas-exchanging functions of the lungs (1). Fibroblasts are largely responsible for the augmented collagen and matrix synthesis and deposition that occur in pulmonary fibrosis (2). The origin of lung fibroblasts during pulmonary fibrosis has not been well defined, but potential sources include proliferation of resident lung interstitial fibroblasts, differentiation of progenitor cells from the bone marrow, and transition of epithelial cells to a fibroblast phenotype, a process termed epithelial-mesenchymal transition (EMT) (3). Prior animal studies have demonstrated that *in situ* proliferation contributes to the lung fibroblast population (4, 5). In addition, animal studies have demonstrated that during experimentally induced pulmonary fibrosis, a subset of lung fibroblasts arise from bone marrow progenitor cells (6) and that fibrocytes contribute to the fibrogenic process (7, 8). The idea that EMT can contribute to organ fibrosis has received considerable attention, but studies to evaluate this process, particularly in the lungs, are limited.

EMT is observed both in embryogenesis and organogenesis, allowing cells to transition between epithelial and mesenchymal phenotypes (3). In addition to its roles in tissue and organ development, EMT has been shown to play a major role in the metastatic potential of epithelial-based malignancies (9). Until more recently, it was assumed that in mature tissues the epithelial phenotype was a terminal event; however, studies demonstrating the occurrence of EMT in mature epithelial cells have challenged this long-held idea (3, 10), including evidence of EMT in several organs (3, 10–12). Two studies have identified EMT-derived cells in lungs undergoing parenchymal fibrosis (13, 14). However, the extent to which EMT contributes to fibroblast and myofibroblast populations in the lungs requires further investigation.

In these studies, we asked whether EMT is an important contributor to the population of effector fibroblasts during bleomycin-induced lung fibrosis, the most commonly employed

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animal model of lung fibrosis, and how EMT-derived fibroblasts compare with fibroblasts derived from bone marrow and other local sources. We used *in vitro* studies of murine type II alveolar epithelial cells (AECs), as well as the *in vivo* experimental mouse model of pulmonary fibrosis induced by intratracheal bleomycin (15, 16) with a cell fate reporter system to determine the contribution of EMT to the lung fibroblast population. Some of the results of this study have been presented previously in abstract form (17, 18).

METHODS

For detailed methods, see the online supplement.

Transgenic Mice

Immortomice, with temperature-sensitive expression of simian virus 40 large T antigen (19), from R. Whitehead (Vanderbilt University, Nashville, TN), were used to obtain type II AECs. Cells from these mice proliferate when cultured with IFN- γ at 33°C; however, when cultured without IFN- γ at 37°C, cells lose expression of the simian virus 40 antigen and revert to the primary cell phenotype (see additional details in the online supplement).

Mice expressing Cre recombinase under the control of the surfactant protein C promoter (SPC.Cre) were obtained from B. Hogan (Duke University, Durham, NC). R26Rosa.Stop.LacZ reporter mice, with R26Rosa promoter-driven expression of a *loxP*-flanked STOP cassette upstream of *lacZ*, were obtained from Jackson Laboratory (Bar Harbor, ME). Mice expressing enhanced green fluorescent protein (eGFP) under the control of the fibroblast-associated S100A4 promoter (S100A4.GFP) and mice expressing Cre under the control of the S100A4 promoter (S100A4.Cre) have been described previously (16, 20). Mice expressing eGFP under the control of the chicken actin promoter (ACTB.GFP) were obtained from Jackson Laboratory. All mice were C57BL/6J background. All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Models

Bleomycin (0.08 unit) was injected intratracheally and lungs were harvested as previously described (15, 16). Fetal liver transplantation (FLT) was performed as previously described (21), generating bone marrow chimeras with S100A4.GFP or ACTB.GFP mice as donors and wild-type mice as recipients.

Cell Culture/Isolation

Type II AECs were isolated from Immortomice as previously described (16) and placed in Dulbecco's modified Eagle's medium (DMEM) with IFN- γ (0.2 ng/ml) (Sigma, St. Louis, MO) at 33°C. After two passages, AECs were placed in DMEM without IFN- γ at 37°C. After 48 hours, transforming growth factor (TGF)- β_1 (10 ng/ml) (R&D Systems, Minneapolis, MN) and epidermal growth factor (EGF, 100 ng/ml) (R&D Systems) were added for 72 hours. AECs were collected as previously described (16). Fibroblasts were cultured as previously described (16).

Histology/Microscopy

Lungs were formalin-fixed and paraffin-embedded as previously described (16). For frozen sections, lungs were processed in 20% sucrose with freezing in liquid nitrogen. Microscopy was performed with an Olympus IX81 microscope with IX2 biological disk scanning unit (Olympus, Tokyo, Japan).

Immunostaining Studies

Immunocytochemistry was performed on formalin-fixed preparations and immunofluorescence on frozen sections as previously described (16) and detailed in the online supplement. Antibodies included S100A4: rabbit polyclonal (from E.G.N.), prosurfactant protein C (pro-SP-C): goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), GFP: AV peptide antibody (Clontech, Mountain View, CA), β -galactosidase: chicken polyclonal (Abcam, Cambridge, MA), α -smooth muscle actin (α -SMA): Cy3 conjugated (Sigma), and fluorescent secondary

(Jackson ImmunoResearch, West Grove, PA). Nuclear staining was done with 4',6-diamidino-2-phenylindole (DAPI) VECTASHIELD mount (Vector Laboratories, Burlingame, CA). 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) staining was performed on frozen sections as previously described (11). Cells were counted on nonoverlapping high-power fields at $\times 600$ magnification.

Western/Northern Blots

Western and Northern blots were performed as previously described (16). Antibodies included the following: S100A4, E-cadherin (rabbit polyclonal; Santa Cruz Biotechnology), and p44 and p42 mitogen-activated protein kinase (MAPK) (rabbit polyclonal; Invitrogen, Carlsbad, CA). Northern blots were performed for type 1 procollagen, S100A4, and 18S ribosomal RNA (16).

Statistics

Statistical analyses were performed with GraphPad InStat (GraphPad Software, San Diego, CA). Differences among groups were assessed by one-way analysis of variance. Results are presented as means $\pm$ standard error of the mean (SEM). *P* values less than 0.05 were considered significant.

RESULTS

TGF- β_1 and EGF Induce EMT in Primary Murine Type II AECs

To determine whether primary murine type II AECs are capable of undergoing EMT *in vitro*, we isolated these cells from Immortomice. On the basis of previously published work (22) and preliminary studies, we used a combination of TGF- β_1 (10 ng/ml) and EGF (100 ng/ml) to induce EMT in type II AECs. As shown in Figure 1, this treatment resulted in cell dispersion with loss of cell-cell contact, development of an elongated fibroblast-like appearance, loss of pro-SP-C production, and induction of S100A4 expression. S100A4, also identified in some literature as fibroblast-specific protein-1, is an 11-kD protein that belongs to the calmodulin-S100-troponin C superfamily of binding proteins that has been used in a number of studies to identify cells with a fibroblast phenotype (15, 16, 23–26). Type II AECs exposed to this combination of TGF- β_1 (10 ng/ml) and EGF (100 ng/ml) also lose expression of the epithelial cell protein E-cadherin and begin expressing S100A4 and type 1 procollagen, indicating a mesenchymal cell phenotype (Figure 2). Taken together, these studies demonstrate that type II AECs undergo EMT *in vitro* when exposed to appropriate stimuli.

Interstitial Fibroblasts in Areas of Bleomycin-induced Lung Fibrosis Are of Epithelial Lineage

To evaluate EMT *in vivo* in the bleomycin model, we used a cell fate mapping strategy based on lineage-specific expression of the *lacZ* gene product β -galactosidase (β -Gal) (Figure 3). In this model, we crossed SPC.Cre mice with R26Rosa.Stop.LacZ reporter mice. When Cre recombinase is activated under the control of the SP-C promoter, the STOP cassette between the two *loxP* sites is removed, which irreversibly activates β -Gal expression in SP-C⁺ lung epithelial cells. As a result of recombination, the vast majority of lung epithelial cells from bronchi to alveoli are permanently genetically marked with β -Gal expression because of SP-C expression in early lung development. Whereas only type II AECs express SP-C in developed lungs, progenitor cells for proximal and distal lung epithelium are SP-C positive during early lung development (27). Cell fate mapping becomes possible because the recombined cell and all daughter cells constitutively express β -Gal (marking their epithelial origin) (Figure 3). Ongoing reporter gene expression is driven by the constitutively active ROSA promoter. We tested the efficiency of this strategy by enzymatic

Figure 1. Identification of epithelial-mesenchymal transition (EMT) by primary type II alveolar epithelial cells (AECs) obtained from Immortomice. Cells were cultured in media with or without transforming growth factor (TGF)- β_1 (10 ng/ml) and epidermal growth factor (EGF, 100 ng/ml) for 72 hours. With TGF- β_1 and EGF treatment, type II AECs (A and B) demonstrated a change to a morphologic appearance consistent with that of fibroblasts on phase-contrast imaging, (C and D) showed reduced expression of the type II AEC protein prosurfactant protein C (pro-SP-C), and (E and F) gained expression of the mesenchymal protein S100A4 as demonstrated by immunocytochemistry.

substrate staining for β -Gal, using X-Gal. X-Gal⁺ precipitate (blue) revealed efficient Cre recombination in the lung epithelium in R26Rosa.Stop.LacZ.SPC.Cre mice (Figure 3). Lungs from single transgenic SPC.Cre or R26Rosa.Stop.LacZ mice did not have X-Gal⁺ precipitate. Subsequently, we treated R26Rosa.Stop.LacZ.SPC.Cre mice and controls with intratracheal bleomycin (0.08 unit) and harvested lungs 1, 2, and 3 weeks later, with frozen lung sections stained for X-Gal. Lung tissue sections demonstrated blue (X-Gal⁺) cells in fibrotic areas, raising the question of how many of these cells were simply epithelial cells or possibly epithelial cell-derived fibroblasts (Figure 3). Thus, this transgenic system provides a means to follow lung epithelial cell fate under experimental conditions, including a means to evaluate for EMT.

To quantify the extent to which EMT contributes to the lung fibroblast population, we performed confocal double immunofluorescence staining of frozen lung sections from cell fate reporter mice treated with bleomycin and untreated control mice for S100A4 and β -Gal expression. Lung sections from untreated reporter mice showed rare S100A4⁺ fibroblasts in the interstitium and peribronchial areas, whereas β -galactosidase antibody staining was widely detected in alveolar and airway epithelial cells (Figure 4). After intratracheal bleomycin treatment, lung tissue fibrosis was prominent as detected by Trichrome staining (*see* Figure E1 in the online supplement) and S100A4⁺ cells increased substantially at 2 weeks, consistent with our prior observations (16). On merged images, expression of β -Gal and S100A4 colocalized in some interstitial cells, indicating that these S100A4⁺ fibroblasts were derived from

epithelium via EMT (Figure 4). No β -Gal staining was detected in lung sections from control R26Rosa.Stop.LacZ mice (*see* Figure E2 in the online supplement). On these lung sections, we quantified the total number of S100A4⁺ cells and the number of S100A4⁺ cells that also expressed β -Gal. Quantification demonstrated rare S100A4⁺ β -Gal⁺ cells in untreated reporter mice whereas the number of S100A4⁺ β -Gal⁺ cells increased after bleomycin treatment, peaking at the 2-week time point. Approximately one-third of the S100A4⁺ fibroblasts were derived from lung epithelium 2 weeks after bleomycin administration (Figure 4). This estimate is made under the assumption that the cell fate mapping system marks all the lung epithelium with β -Gal; if not, then the number of epithelial-derived fibroblasts could be underestimated. Subsequently, outgrowth fibroblast culture studies were performed from R26Rosa.Stop.LacZ.SPC.Cre reporter mouse lungs 2 weeks after intratracheal bleomycin. Outgrowth fibroblasts at passages 1 and 2 from R26Rosa.Stop.LacZ.SPC.Cre demonstrated rare X-Gal-positive cells, whereas positive controls (R26Rosa.Stop.LacZ.S100A4.Cre lung fibroblasts) were uniformly X-Gal positive (*see* Figure E3 in the online supplement). On the basis of these results, it appears that EMT-derived lung fibroblasts may be phenotypically different from fibroblasts of other origin. Specifically, we speculate that EMT-derived fibroblasts may have a decreased ability to proliferate in outgrowth culture. Taken together, these studies demonstrate that EMT results in a substantial subset of S100A4⁺ lung fibroblasts in bleomycin-induced lung fibrosis. In addition, EMT-derived fibroblasts may be phenotypically different compared with non-EMT-derived fibroblasts.

Figure 2. Time course for loss of epithelial markers and gain of mesenchymal markers after treatment with transforming growth factor (TGF)- β_1 (10 ng/ml) and epidermal growth factor (EGF, 100 ng/ml). (A and B) Western blots for (A) E-cadherin and (B) S100A4 in Immortomouse type II alveolar epithelial cells (AECs) after treatment with TGF- β_1 and EGF. Results were normalized for p44/p42 mitogen-activated protein kinase (MAPK). (C) Northern blot showing increased expression of type I procollagen and S100A4 expression after treatment with TGF- β_1 and EGF. Results are normalized for 18S.

Myofibroblasts Represent a Cell Population Distinct from EMT-derived S100A4⁺ Fibroblasts

Myofibroblasts have long been recognized as key cells in the deposition of extracellular matrix in lung fibrosis (28). To determine the extent to which EMT contributes to the lung

myofibroblast population, we performed coexpression analysis using anti- α -SMA antibodies conjugated with Cy3 (Sigma) and β -Gal in the cell fate mapping model described previously. In untreated lungs, α -SMA expression is not observed in the interstitium and is restricted to vascular smooth muscle cells with no colocalization in β -Gal⁺ cells. In reporter mice treated with bleomycin, a small number of cells in the interstitium (relative to S100A4) are α -SMA⁺. Only rare α -SMA⁺ cells were also positive for β -Gal, even 2 weeks after bleomycin administration (Figure 5). Next, we performed coexpression analysis using anti- α -SMA antibodies and anti-S100A4 antibodies. Quantification of S100A4⁺ α -SMA⁺ cells per high-power field showed that 3.5% of S100A4⁺ cells have colocalized expression of α -SMA (Figure 6). Interestingly, although these *in vivo* studies showed rare S100A4/ α -SMA colocalization, *in vitro* studies reveal that S100A4⁺ fibroblasts have the capacity to transition to a myofibroblast phenotype. In these studies, lung fibroblasts cultured from non-bleomycin-treated S100A4.GFP mice did not express α -SMA at baseline, but after treatment with TGF- β_1 (10 ng/ml) for 24 hours, α -SMA expression was induced (see Figure E4 in the online supplement). Therefore, it appears that, in contrast to S100A4⁺ fibroblasts, myofibroblasts in the lung are rarely derived from EMT.

Some S100A4⁺ Fibroblasts Derive from Bone Marrow in Bleomycin-induced Lung Fibrosis

Our *in vivo* results described previously (Figure 4) suggest that approximately two thirds of S100A4⁺ lung fibroblasts at the 2-week time point after bleomycin treatment are derived from origins other than EMT. To determine whether S100A4⁺ lung fibroblasts derive from bone marrow precursors, we created bone marrow chimeras with S100A4.GFP donors, using a method of fetal liver transplantation (FLT) that results in more than 85% bone marrow reconstitution by 4 weeks (21). We previously used these S100A4.GFP mice in the bleomycin model to demonstrate that S100A4⁺ cells in the lung express α_1 type 1 procollagen (16). Four weeks after FLT, we treated mice with bleomycin (0.08 unit) and harvested the mice 2 weeks later. This time point was selected to compare with the time

Figure 3. Cell fate mapping strategy to identify epithelial-mesenchymal transition (EMT) *in vivo*. (A) Schematic for construction of cell fate reporter mice. (B) 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) staining (blue) in lung sections from untreated R26Rosa.Stop.LacZ.SPC.Cre mice detects reporter gene expression in epithelial cells. (C) X-Gal staining is absent in control (single transgenic) mouse lungs. (D) Epithelial and interstitial cells (arrows) are X-Gal positive 2 weeks after bleomycin administration in lungs of cell fate reporter mice. (B–D) Original magnification, $\times 600$.

Figure 4. Detection of epithelial–mesenchymal transition (EMT)–derived fibroblasts in lungs after bleomycin treatment. Confocal images to detect fluorescence immunostaining for β -galactosidase (β -Gal) (green), S100A4 (red), and 4',6-diamidino-2-phenylindole (DAPI) (blue) in lungs from (A–C) untreated R26Rosa.Stop.LacZ.SPC.Cre reporter mice and (D–L) cell fate reporter mice 1, 2, and 3 weeks after intra-tracheal bleomycin. Cells that coexpress β -Gal and S100A4 are yellow (arrows point to representative cells). Fluorescence image capture was blanked to sections stained with secondary antibodies only. No green fluorescence was detected in single transgenic mice. (M) Graph representing the number of S100A4⁺ β -Gal⁺ cells compared with the total S100A4⁺ cell population in lung parenchyma per high-power field (HPF) from control (untreated) mice and mice 1, 2, and 3 weeks after bleomycin treatment. Mean of 10 HPFs per section $\pm$ SEM, n = 3 mice per time point. *P < 0.01 compared with untreated control. (A–L) Original magnification, $\times 600$. Control staining images are shown in Figure E2.

point of peak EMT-derived fibroblasts. In this model, bone marrow–derived fibroblasts express GFP but resident lung fibroblasts and other bone marrow–derived cells are GFP negative. After bleomycin, GFP⁺ fibroblasts were found in areas of lung fibrosis by microscopy (Figure 7). Serial lung sections were immunostained for S100A4 and GFP (Figure 7) and numbers of positive cells were counted in a blinded fashion on 10 sequential, nonoverlapping fields to determine total

S100A4⁺ fibroblasts and S100A4⁺ fibroblasts derived from bone marrow, respectively. As shown in Figure 7, rare GFP⁺ cells were identified in the absence of bleomycin treatment (3.3% of S100A4⁺ cells were GFP⁺). At 2 weeks, however, the percentage of GFP⁺ cells increased significantly (19.8% of S100A4⁺ cells were GFP⁺). This study indicates that bone marrow–derived cells migrate to the lungs and contribute to the fibroblast pool, accounting for approximately one-fifth of

Figure 5. α -Smooth muscle actin (α -SMA)⁺ myofibroblasts are rarely derived from epithelial–mesenchymal transition (EMT) after bleomycin treatment. Confocal images to detect fluorescence immunostaining of β -galactosidase (β -Gal)⁺ cells (green) and α -SMA⁺ cells (red) and 4',6-diamidino-2-phenylindole (DAPI) (blue). (A–C) Lungs from untreated R26Rosa.Stop.LacZ.SPC.Cre reporter mouse and (D–F) reporter mouse 2 weeks after bleomycin treatment. Cells that coexpress β -Gal and α -SMA are yellow (arrow points to representative cell). Dual staining β -Gal⁺ α -SMA⁺ cells were present but rare in the lung tissue after bleomycin treatment. Weeks 1 and 3 images are not shown but were similar to the Week 2 images. Original magnification, $\times 600$.

Figure 6. Few S100A4⁺ lung fibroblasts coexpress α-smooth muscle actin (α-SMA). Confocal images to detect fluorescence immunostaining for S100A4 (green), α-SMA (red), and 4',6-diamidino-2-phenylindole (DAPI) (blue) in lungs from (A–C) untreated R26Rosa.Stop.LacZ.SPC.Cre reporter mice and (D–F) reporter mice 2 weeks after bleomycin treatment. Cells that coexpress S100A4 and α-SMA are yellow (arrow points to representative cell). (G) Bar graph representing the number of S100A4⁺α-SMA⁺ cells compared with the total S100A4⁺ cell population in lung parenchyma per high-power field (HPF) from control (untreated) mice and mice 1, 2, and 3 weeks after bleomycin treatment. Mean of 5 HPF ± SEM, n = 3 mice per time point. *P < 0.01 compared with baseline. (A–F) Original magnification, ×600.

S100A4⁺ lung fibroblasts 2 weeks after bleomycin administration. As with the EMT studies described previously, outgrowth fibroblast culture studies were performed from chimeric mice with bone marrow reconstitution (via FLT) from two different GFP-expressing models (S100A4.GFP and ACTB.GFP). In contrast to the EMT studies, in both chimeric models, bone marrow-derived fibroblasts (as evidenced by GFP fluorescence) were easily cultured from the lung (data not shown). To identify bone marrow-derived myofibroblasts, immunocytochemistry was performed for α-SMA, but dual-positive cells (GFP⁺α-SMA⁺) were rarely encountered (data not shown) with 2.3% of outgrowth myofibroblasts also GFP⁺, suggesting that bone

marrow-derived cells are not a significant contributor to the myofibroblast population. Taken together, these studies demonstrate that (1) bone marrow-derived progenitor cells supply a subset of the S100A4⁺ lung fibroblast population in bleomycin-induced lung fibrosis, (2) bone marrow-derived fibroblasts can be passaged in culture, and (3) myofibroblasts are rarely of bone marrow origin.

DISCUSSION

We found that lung epithelial cells undergo EMT and contribute substantially to the lung fibroblast population during

Figure 7. Bone marrow-derived fibroblasts identified in S100A4.GFP bone marrow chimeras. Top: Immunostaining for (A) S100A4 and (B) green fluorescent protein (GFP; brown staining) in a lung section from a mouse 2 weeks after intratracheal bleomycin administration. (C) Graph showing the number of GFP⁺ and S100A4⁺ cells in lung parenchyma per high-power field (HPF) at baseline and 2 weeks after bleomycin treatment. Mean of 10 HPF ± SEM, n = 3 mice per time point. *P < 0.05 compared with untreated baseline. (A and B) Original magnification, ×400.

experimentally induced lung fibrosis. *In vivo*, we estimate that approximately one third of the S100A4⁺ lung fibroblasts derive from the lung epithelium 2 weeks after bleomycin administration. We also found that the bone marrow is a substantial contributor to the lung fibroblast population, accounting for approximately one fifth of the S100A4⁺ lung fibroblasts 2 weeks after bleomycin administration. However, these two origins together account for just over one half of the S100A4⁺ lung fibroblast population 2 weeks after bleomycin administration, suggesting that resident interstitial cells (or cells from another as yet unidentified origin) play a major role. Only a minority of S100A4⁺ fibroblasts are α -SMA⁺ myofibroblasts, and few of these cells appear to derive from EMT, suggesting that fibroblast origin impacts phenotype. Consistent with this idea, EMT-derived lung fibroblasts are difficult to outgrow in culture, whereas bone marrow-derived lung fibroblasts are easily grown in culture.

The concept that fibroblasts can derive directly from epithelial cells via EMT has important implications for lung fibrosis. Epithelium and mesenchyme (of which fibroblasts are the prototypic cell type) are found in all organs. In embryogenesis, a plasticity exists between these cell types that is required for the complex development of organ structures (29). The idea that switching between epithelial and mesenchymal phenotypes can occur in mature tissues under specific circumstances has been investigated. Interestingly, evidence indicates that EMT has been shown to play an important role in fibrotic conditions in a number of other organs, including kidney, liver, and gastrointestinal tract (3, 10–12, 22).

Multiple cytokines have been shown to be important in EMT including TGF- β , EGF, insulin-like growth factor-II, and fibroblast growth factor-2 (3, 22, 30, 31). TGF- β is the prototypical cytokine in the induction of EMT (3, 22) and has been shown to induce EMT in the human AEC line A549 (32) and in rat type II AECs (33, 34). Phenotypic characteristics of TGF- β -treated cells include increased expression of collagen-1 and α -SMA and loss of E-cadherin. TGF- β has been shown to be a key profibrotic cytokine promoting fibroblast proliferation and is one of the most important stimulators of extracellular matrix production (35). In murine models, overexpression of TGF- β in the lung has resulted in pulmonary fibrosis (36, 37). TGF- β has the potential to affect EMT through multiple pathways, including MAPK activation (38), G protein-mediated signaling (39), Smad-2/3-dependent transcription (40), and effects on β -catenin (41). In the microenvironment associated with EMT, there is an increase in EGF receptors on epithelial cells (42). EGF has been shown to induce EMT in renal tubular epithelial cells (22) when used in conjunction with TGF- β . EGF results in the down-regulation of E-cadherin (43), a mechanism that may in part be responsible for the effects of EGF on EMT. Furthermore, signaling through the EGF receptor has been implicated in the pathogenesis of lung fibrosis in multiple animal studies (44, 45). In our studies, we found that the combination of TGF- β ₁ and EGF was effective in inducing EMT in type II AECs *in vitro*, similar to the effect seen on renal tubular epithelial cells (22).

In 2005, Willis and colleagues (34) identified in lung sections from patients with IPF cells that coexpressed epithelial markers and α -SMA, indicating that cells undergoing phenotype transition may be present in IPF lungs. In this study, the first to describe the possibility of EMT in human lung fibrosis, the authors reported that more than 80% of lung epithelial cells expressing prosurfactant protein B or thyroid transcription factor-1 in IPF lung biopsies also expressed α -SMA as detected by dual-immunostaining techniques. Although it is not clear from this study if all of these α -SMA-positive cells are truly myofibroblasts, these studies do argue that epithelial cells in

human forms of lung fibrosis are capable of phenotypic transition to a mesenchymal phenotype. In 2006, Kim and colleagues (13) also evaluated human IPF lung biopsy samples, demonstrating that cells positive for pro-SP-C also expressed the mesenchymal protein N-cadherin. More convincing evidence of *in vivo* EMT in the lung comes from this same study by Kim and colleagues (13), in which they demonstrated EMT in the lung by overexpression of active TGF- β ₁ by adenoviral vector, using genetic lineage tracing with α -SMA and vimentin as mesenchymal markers. In this study, the investigators noted that approximately one third of vimentin-expressing cells also expressed β -Gal, using a cell fate mapping strategy similar to ours 3 weeks after TGF- β ₁ adenovirus administration. These investigators reported that less than 5% of β -Gal⁺ cells express α -SMA. In this study, the percentage of α -SMA⁺ cells that expressed β -Gal was not reported. More recently, this group has shown evidence of EMT in the bleomycin model when using a similar cell fate mapping model, this time with a GFP reporter (14). In this study, they demonstrated that 8.9% of GFP⁺ cells (indicating all cells of epithelial origin) expressed vimentin, 5.3% expressed α -SMA, and 4.8% expressed collagen-1 at 17 days after bleomycin administration. These investigators also implicated extracellular matrix components in regulating EMT in the lungs. In their studies, epithelial-specific deletion of α ₃ integrin reduced EMT and protected from lung fibrosis, apparently by inhibiting tyrosine phosphorylation of β -catenin and generation of β -catenin/Smad2 complexes (14). Our results regarding the proportion of EMT-derived fibroblasts are complementary to these other investigations. Specifically, our finding that approximately one third of S100A4⁺ fibroblasts in the lung after bleomycin treatment are of epithelial lineage is consistent with estimates of EMT-derived fibroblasts by Kim and colleagues after treatment with TGF- β ₁ adenovirus (13). In contrast to other reports, our studies indicate that EMT-derived α -SMA⁺ myofibroblasts are rare in the lungs after bleomycin treatment. Although the reasons for the discrepancy are not clear, studies by Kim and colleagues (13, 14) suggest a higher frequency of EMT-derived α -SMA⁺ myofibroblasts in the lungs during lung fibrosis.

On the basis of reports that circulating fibrocytes contribute to wound healing (46), several studies have been published showing that these bone marrow-derived cells home to the lungs after injury and contribute to fibrosis (6, 7, 47). One of the most convincing studies of the role of bone marrow-derived progenitor cells in lung fibrosis was published in 2004 when Hashimoto and colleagues created bone marrow chimeras with donor cells that constitutively express green fluorescent protein (GFP) and found increased numbers of GFP⁺ cells in the lungs after bleomycin (Day 21), and that many of these cells were positive for collagen-1 by flow cytometry (6). Interestingly, less than 1% of these cells were α -SMA⁺. In our study, we created bone marrow chimeras with an FLT model using GFP expression and found that approximately one fifth of the S100A4⁺ lung fibroblasts arise from bone marrow progenitors at the 2-week time point in the intratracheal bleomycin model. Myofibroblasts, as detected by α -SMA expression, were rarely of bone marrow origin in our study, corroborating the results observed earlier by Hashimoto and colleagues (6). Thus, our studies demonstrate that a subset of S100A4⁺ lung fibroblasts arises from bone marrow progenitors, but that the bone marrow is not a principal source of myofibroblasts. Although our studies were designed to estimate the contribution the bone marrow makes to the lung S100A4⁺ fibroblast population, these studies do not determine how these cells impact the lung fibrosis phenotype.

A limitation of this study is the fact that the bleomycin model leads to a transient lung fibrosis and does not recapitulate the human disease IPF (48). In addition, all cell fate mapping

studies rely on the ability to label the cell lineage of interest and to specifically identify the target cell population. In these studies, we chose R26Rosa.Stop.LacZ reporter mice to permanently mark cells of epithelial origin. Initially, we performed a number of experiments using Z/EG reporter mice (obtained from A. Nagy, University of Toronto, Toronto, ON, Canada), which express GFP after removal of a stop codon under the control of the chicken actin promoter. These experiments proved unsuccessful because of the unreliable activity of the actin promoter in the lungs, leading us to shift to the R26Rosa.Stop.LacZ reporter mice. To identify fibroblasts, we chose S100A4 and α -SMA as phenotype markers. Although no perfectly sensitive and specific fibroblast markers exist, we have observed good utility for S100A4 as a marker for lung fibroblasts (16), and α -SMA is the classical marker for myofibroblasts. Nevertheless, our data and other studies investigating the origins of fibroblasts must be interpreted with these caveats in mind.

Although our studies demonstrate that the lung fibroblast population receives contributions from both the bone marrow and through EMT, these studies also argue that resident interstitial cells are likely key contributors to the lung fibroblast population. With the models we employed, just over 50% of the lung fibroblast population 2 weeks after bleomycin administration can be accounted for by EMT and bone marrow-derived progenitors. It is possible that resident interstitial cells contribute significantly to the remainder, and other origins such as endothelial mesenchymal transition must also be considered.

Each source for effector fibroblasts (*in situ* proliferation, derivation from bone marrow precursors or fibrocytes, and EMT) likely has its own implications for understanding the pathogenesis of pulmonary fibrosis. Future studies investigating the functional heterogeneity of various fibroblast populations will likely contribute significantly to our understanding of fibrosing lung diseases. In addition, an improved understanding of the pathways governing the recruitment of lung fibroblasts by individual origin may potentially identify targets for new therapies designed to attenuate progression of disease.

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References

1. American Thoracic Society, European Respiratory Society. Idiopathic pulmonary fibrosis: diagnosis and treatment [international consensus statement]. *Am J Respir Crit Care Med* 2000;161:646–664.
2. Phan SH. The myofibroblast in pulmonary fibrosis. *Chest* 2002;122:286S–289S.
3. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003;112:1776–1784.
4. Zhang K, Rekhter MD, Gordon D, Phan SH. Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis: a combined immunohistochemical and *in situ* hybridization study. *Am J Pathol* 1994;145:114–125.
5. Vyalov SL, Gabbiani G, Kapanci Y. Rat alveolar myofibroblasts acquire α -smooth muscle actin expression during bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1993;143:1754–1765.
6. Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH. Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest* 2004;113:243–252.
7. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* 2004;114:438–446.
8. Moore BB, Kolodick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB. CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. *Am J Pathol* 2005;166:675–684.
9. Thiery JP. Epithelial-mesenchymal transitions in tumor progression. *Nat Rev Cancer* 2002;2:442–454.
10. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002;110:341–350.
11. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J Biol Chem* 2007;282:23337–23347.
12. Bataille F, Rohrermeier C, Bates R, Weber A, Rieder F, Brenmoehl J, Strauch U, Farkas S, Furst A, Hofstadter F, et al. Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn's disease. *Inflamm Bowel Dis* 2008;14:1514–1527.
13. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA. Alveolar epithelial cell mesenchymal transition develops *in vivo* during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci USA* 2006;103:13180–13185.
14. Kim KK, Wei Y, Szekeres C, Kugler MC, Wolters PJ, Hill ML, Frank JA, Brumwell AN, Wheeler SE, Kreidberg JA, et al. Epithelial cell $\alpha_3\beta_1$ integrin links β -catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. *J Clin Invest* 2009;119:213–224.
15. Lawson WE, Polosukhin VV, Stathopoulos GT, Zoia O, Han W, Lane KB, Li B, Donnelly EF, Holburn GE, Lewis KG, et al. Increased and prolonged pulmonary fibrosis in surfactant protein C-deficient mice following intratracheal bleomycin. *Am J Pathol* 2005;167:1267–1277.
16. Lawson WE, Polosukhin VV, Zoia O, Stathopoulos GT, Han W, Plieth D, Loyd JE, Neilson EG, Blackwell TS. Characterization of fibroblast-specific protein 1 in pulmonary fibrosis. *Am J Respir Crit Care Med* 2005;171:899–907.
17. Lawson WE, Zoia O, Polosukhin VV, Blackwell TS. Evidence of epithelial mesenchymal transition in primary type II alveolar epithelial cells and mesothelial cells [abstract]. *Proc Am Thorac Soc* 2005;2:A853.
18. Lawson WE, Polosukhin VV, Li B, Sherrill TP, Blackwell TS. Fibroblasts derive from epithelial cells and bone marrow progenitors in experimental lung fibrosis [abstract]. *Proc Am Thorac Soc* 2006;3:A559.
19. Whitehead RH, Joseph JL. Derivation of conditionally immortalized cell lines containing the *Min* mutation from the normal colonic mucosa and other tissues of an "Immortomouse"/Min hybrid. *Epithelial Cell Biol* 1994;3:119–125.
20. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG, Moses HL. TGF- β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004;303:848–851.
21. Everhart MB, Han W, Parman KS, Polosukhin VV, Zeng H, Sadikot RT, Li B, Yull FE, Christman JW, Blackwell TS. Intratracheal administration of liposomal clodronate accelerates alveolar macrophage reconstitution following fetal liver transplantation. *J Leukoc Biol* 2005;77:173–180.
22. Okada H, Danoff TM, Kalluri R, Neilson EG. Early role of Fsp1 in epithelial-mesenchymal transformation. *Am J Physiol* 1997;273:F563–F574.
23. Tager AM, Kradin RL, LaCamera P, Bercury SD, Campanella GS, Leary CP, Polosukhin V, Zhao LH, Sakamoto H, Blackwell TS, et al. Inhibition of pulmonary fibrosis by the chemokine IP-10/CXCL10. *Am J Respir Cell Mol Biol* 2004;31:395–404.
24. Fano G, Biocca S, Fulle S, Mariggio MA, Belia S, Calissano P. The S-100: a protein family in search of a function. *Prog Neurobiol* 1995;46:71–82.
25. Iwano M, Fischer A, Okada H, Plieth D, Xue C, Danoff TM, Neilson EG. Conditional abatement of tissue fibrosis using nucleoside analogs

- to selectively corrupt DNA replication in transgenic fibroblasts. *Mol Ther* 2001;3:149–159.
26. Okada H, Inoue T, Kanno Y, Kobayashi T, Watanabe Y, Ban S, Neilson EG, Suzuki H. Selective depletion of fibroblasts preserves morphology and the functional integrity of peritoneum in transgenic mice with peritoneal fibrosing syndrome. *Kidney Int* 2003;64:1722–1732.
 27. Perl AK, Wert SE, Nagy A, Lobe CG, Whitsett JA. Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc Natl Acad Sci USA* 2002;99:10482–10487.
 28. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 1999;277:C1–C9.
 29. Hay ED, Zuk A. Transformations between epithelium and mesenchyme: normal, pathological, and experimentally induced. *Am J Kidney Dis* 1995;26:678–690.
 30. Morali OG, Delmas V, Moore R, Jeanney C, Thiery JP, Larue L. IGF-II induces rapid β -catenin relocation to the nucleus during epithelium to mesenchyme transition. *Oncogene* 2001;20:4942–4950.
 31. Strutz F, Zeisberg M, Ziyadeh FN, Yang CQ, Kalluri R, Muller GA, Neilson EG. Role of basic fibroblast growth factor-2 in epithelial-mesenchymal transformation. *Kidney Int* 2002;61:1714–1728.
 32. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z. TGF- β_1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res* 2005;6:56.
 33. Yao HW, Xie QM, Chen JQ, Deng YM, Tang HF. TGF- β_1 induces alveolar epithelial to mesenchymal transition *in vitro*. *Life Sci* 2004;76:29–37.
 34. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor- β_1 : potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 2005;166:1321–1332.
 35. Sime PJ, O'Reilly KM. Fibrosis of the lung and other tissues: new concepts in pathogenesis and treatment. *Clin Immunol* 2001;99:308–319.
 36. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor- β_1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997;100:768–776.
 37. Lee CG, Cho SJ, Kang MJ, Chapoval SP, Lee PJ, Noble PW, Yehualaesht T, Lu B, Flavell RA, Milbrandt J, et al. Early growth response gene 1-mediated apoptosis is essential for transforming growth factor β_1 -induced pulmonary fibrosis. *J Exp Med* 2004;200:377–389.
 38. Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL. Integrin β_1 signaling is necessary for transforming growth factor- β activation of p38MAPK and epithelial plasticity. *J Biol Chem* 2001;276:46707–46713.
 39. Boyer AS, Erickson CP, Runyan RB. Epithelial-mesenchymal transformation in the embryonic heart is mediated through distinct pertussis toxin-sensitive and TGF- β signal transduction mechanisms. *Dev Dyn* 1999;214:81–91.
 40. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R. BMP-7 counteracts TGF- β_1 -induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003;9:964–968.
 41. Masszi A, Fan L, Rosivall L, McCulloch CA, Rotstein OD, Mucsi I, Kapus A. Integrity of cell-cell contacts is a critical regulator of TGF- β_1 -induced epithelial-to-myofibroblast transition: role for β -catenin. *Am J Pathol* 2004;165:1955–1967.
 42. Citterio HL, Gaillard DA. Expression of transforming growth factor α (TGF α), epidermal growth factor receptor (EGF-R) and cell proliferation during human palatogenesis: an immunohistochemical study. *Int J Dev Biol* 1994;38:499–505.
 43. Lu Z, Ghosh S, Wang Z, Hunter T. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of β -catenin, and enhanced tumor cell invasion. *Cancer Cell* 2003;4:499–515.
 44. Hardie WD, Le Cras TD, Jiang K, Tichelaar JW, Azhar M, Korfhagen TR. Conditional expression of transforming growth factor- α in adult mouse lung causes pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L741–L749.
 45. Korfhagen TR, Swantz RJ, Wert SE, McCarty JM, Kerlakian CB, Glasser SW, Whitsett JA. Respiratory epithelial cell expression of human transforming growth factor- α induces lung fibrosis in transgenic mice. *J Clin Invest* 1994;93:1691–1699.
 46. Metz CN. Fibrocytes: a unique cell population implicated in wound healing. *Cell Mol Life Sci* 2003;60:1342–1350.
 47. Moore BB, Murray L, Das A, Wilke CA, Herrygers AB, Toews GB. The role of CCL12 in the recruitment of fibrocytes and lung fibrosis. *Am J Respir Cell Mol Biol* 2006;35:175–181.
 48. Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L152–L160.