Emergence of Fibroblasts with a Proinflammatory Epigenetically Altered Phenotype in Severe Hypoxic Pulmonary Hypertension

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Emergence of Fibroblasts with a Proinflammatory Epigenetically Altered Phenotype in Severe Hypoxic Pulmonary Hypertension

Min Li,*,#1 Suzette R. Riddle,*,#1 Maria G. Frid,* Karim C. El Kasmi,‡ Timothy A. McKinsey,* Ronald J. Sokol,‡ Derek Strassheim,§ Barbara Meyrick,§ Michael E. Yeager,* Amanda R. Flockton,* B. Alexandre McKeon,* Douglas D. Lemon,‡ Todd R. Horn,‡ Adil Anwar,* Carlos Barajas,* and Kurt R. Stenmark*

Persistent accumulation of monocytes/macrophages in the pulmonary artery adventitial/perivascular areas of animals and humans with pulmonary hypertension has been documented. The cellular mechanisms contributing to chronic inflammatory responses remain unclear. We hypothesized that perivascular inflammation is perpetuated by activated adventitial fibroblasts, which, through sustained production of proinflammatory cytokines/chemokines and adhesion molecules, induce accumulation, retention, and activation of monocytes/macrophages. We further hypothesized that this proinflammatory phenotype is the result of the abnormal activity of histone-modifying enzymes, specifically, class I histone deacetylases (HDACs). Pulmonary adventitial fibroblasts from chronically hypoxic hypertensive calves (termed PH-Fibs) expressed a constitutive and persistent proinflammatory phenotype defined by high expression of IL-1β, IL-6, CCL2(MCP-1), CXCL12(SDF-1), CCL5(RANTES), CCR7, CXCR4, GM-CSF, CD40, CD40L, and VCAM-1. The proinflammatory phenotype of PH-Fibs was associated with epigenetic alterations as demonstrated by increased activity of HDACs and the findings that class I HDAC inhibitors markedly decreased cytokine/chemokine mRNA expression levels in these cells. PH-Fibs induced increased adhesion of THP-1 monocytes and produced soluble factors that induced increased migration of THP-1 and murine bone marrow-derived macrophages as well as activated monocytes/macrophages to express proinflammatory cytokines and profibrogenic mediators (TIMP1 and type I collagen) at the transcriptional level. Class I HDAC inhibitors markedly reduced the ability of PH-Fibs to induce monocyte migration and proinflammatory activation. The emergence of a distinct adventitial fibroblast population with an epigenetically altered proinflammatory phenotype capable of recruiting, retaining, and activating monocytes/macrophages characterizes pulmonary hypertension-associated vascular remodeling and thus could contribute significantly to chronic inflammatory processes in the pulmonary artery wall. The Journal of Immunology, 2011, 187: 000–000.

Several studies have documented that pulmonary hypertension (PH)-associated vascular remodeling is characterized by the early and persistent accumulation of mononuclear cells in the perivascular adventitia in animal models of PH, including chronically hypoxic calves, rats, mice, and monocrotaline-treated rodents (1–3). Further, in most chronic forms of human PH, perivascular accumulation of monocytes/macrophages is a common feature (4–6), and elevated levels of inflammatory cytokines, including IL-1β and IL-6, have been shown to predict survival in idiopathic and familial pulmonary arterial hypertension (7). Thus, pulmonary vascular remodeling is potentially perpetuated by a local/adventitial chronic inflammatory response.

Traditionally, vascular inflammation has been considered an “inside-out” response centered on monocyte/macrophage recruitment to the intima of blood vessels. However, growing evidence supports a new paradigm of an “outside-in” hypothesis, in which vascular inflammation is initiated and perpetuated by adventitial fibroblasts (8–11). The participation of fibroblasts in the regulation/orchestration of immune responses has been regarded traditionally as insignificant, whereas monocytes, dendritic cells, and T lymphocytes are the established key players. However, recent experimental evidence suggests that mesenchymal cells, specifically fibroblasts, are responsive to non-Ag-specific danger signals, which activate TLR/NF-κB signaling pathways. Thus, monocytes/macrophages are capable of recruiting, retaining, and activating monocytes/macrophages characterizes pulmonary hypertension-associated vascular remodeling and thus could contribute significantly to chronic inflammatory processes in the pulmonary artery wall. The Journal of Immunology, 2011, 187: 000–000.
innate activation of fibroblasts may play a central role in the initiation and perpetuation of inflammatory tissue responses (8, 11–13). Whether pulmonary adventitial fibroblasts, in the setting of PH, exhibit features of a phenotype compatible with innate immune activation characterized by the generation of canonical proinflammatory cytokines/chemokines as well as adhesion molecules is unknown. Moreover, in the pulmonary circulation, it is currently unknown if adventitial fibroblasts are capable of recruiting and retaining monocytes/macrophages to the pulmonary artery wall with subsequent activation of monocytes/macrophages toward a proinflammatory phenotype.

Acquired and stable changes in the phenotype of mesenchymal cells may require epigenetic processes such as might occur in response to altered histone acetylation (14). Histone-dependent packaging of genomic DNA into chromatin is a central mechanism for gene regulation. Expression of inflammatory genes, DNA repair, and proliferation have been shown to be controlled by the degree of acetylation of histone and nonhistone proteins produced by histone acetyltransferases and histone deacetylases (HDACs) (15–18). Several reports have documented changes in HDAC activity in fibroblasts in rheumatoid arthritis and juvenile idiopathic arthritis, with recent reports demonstrating specific increases in HDAC1 activity (19, 20). Additional reports have demonstrated anti-inflammatory effects of small-molecule HDAC inhibitors in animal models of inflammatory diseases, in fibrotic vascular disease, and in cancer (16, 21, 22). However, to our knowledge, no previous studies have addressed the possible role of HDACs in the control of inflammatory gene expression in the setting of PH.

The goal of this study was to test the hypothesis that, in the setting of chronic hypoxic PH, adventitial fibroblasts exhibit a “persistently activated” proinflammatory phenotype capable of inducing recruitment, retention, and proinflammatory activation of monocytes/macrophages. Our approach was to determine, in the setting of chronic hypoxic PH, whether: 1) pulmonary adventitial fibroblasts exhibit an activated proinflammatory phenotype (expressing elevated levels of cytokines, chemokines, and adhesion molecules); 2) pulmonary adventitial fibroblasts promote migration, adhesion, and proinflammatory activation of monocytes/macrophages; and 3) epigenetic modifications due to abnormal activity of histone-modifying enzymes contribute to this activated phenotype.

### Materials and Methods

#### Animals

The neonatal calf and rat models of severe hypoxia-induced PH have been described previously (1). Briefly, 1-day-old male Holstein calves were exposed to hypobaric hypoxia (\(P_F = 445 \text{ mm Hg}\)) for 2 wk (n = 7), whereas age-matched controls (n = 7) were kept at ambient altitude (\(P_F = 640 \text{ mm Hg}\)). Wistar–Kyoto rats were exposed to hypobaric hypoxia (\(P_F = 380 \text{ mm Hg}\)) for 4 wk (n = 9). Age-matched controls (n = 9) were kept at ambient altitude.

Monocrotaline treatment of rats (experimental group, n = 12; control group, n = 12) was performed as described previously (23).

Standard veterinary care was used following institutional guidelines for rats at the University of Colorado at Denver Center for Laboratory Animal Care in compliance with Institutional Animal Care and Use Committee-approved protocols and for calves at the Department of Physiology, School of Veterinary Medicine, Colorado State University (Fort Collins, CO). Animals of both species were euthanized by an overdose of sodium pentobarbital (160 mg/kg body weight).

#### Human specimens

Frozen sections of lung tissue from human subjects with idiopathic pulmonary arterial hypertension (iPAH) (n = 5) and controls (n = 4) were used for immunocytochemical analyses. Lung tissues from iPAH patients and control subjects were provided by the Pulmonary Hypertension Breakthrough Initiative. The tissues were procured at the Transplant Procurement Centers at Stanford University, University of California at San Diego, Vanderbilt University, and Allegheny General Hospital.

#### Cell culture

Isolation of adventitial fibroblasts from distal pulmonary arteries was performed as described previously (24). Experiments were performed on cells at passages 4–10. Conditioned media was collected from confluent fibroblast cultures and used in migration and/or activation studies with monocytes (24 h, serum-free media) or murine macrophages (72 h, complete media). THP-1 monocytes (a human leukemic mononuclear cell line routinely used in monocyte assays) were purchased from the American Type Culture Collection (Manassas, VA). Murine bone marrow-derived macrophages (BMDMs) were isolated from C57BL/6 mice as described previously (25).

#### Immunofluorescent staining

Mouse mAb or rabbit polyclonal Ab against the following Ags were used: bovine CD14 (mAb, 15 μg/ml; VMDR, Pullman, WA), human CD68 (cross-reacts with bovine and rat Ags) (mAb, 1:100 dilution; Dako, Carpinteria, CA), rat CD11b (mAb, 1:50 dilution; Chemicon International, Temecula, CA), human VCAM-1 (cross-reacts with bovine) (goat Ab, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), bovine IL-1β (mAb, 15 mg/ml; VMRD), vimentin (chicken Ab, 1:500 dilution; Millipore, Temecula, CA), heat shock protein 47 (Hsp47) (mAb, 1:400 dilution; Calbiochem, San Diego, CA), α-smooth muscle (SM)-actin (mAb, 1:200 dilution; Sigma-Aldrich, St. Louis, MO), SM-myosin heavy chains (polyclonal Ab, 1:2000, generously

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### Table I. Bovine and human primer sequences for real-time RT-PCR

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<td>COL1A1</td>
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Results

PH-associated vascular remodeling is associated with perivascular/adventitial accumulation of monocytes/macrophages

Marked accumulation of monocytes/macrophages (defined by expression of CD14, CD11b, and CD68 Ags) was observed in the pulmonary perivascular adventitia of chronically hypoxic calves and rats, monocrotaline-treated rats with PH, and in human subjects with iPAH (Fig. 1). These observations emphasize the adventitial/perivascular accumulation of monocytes/macrophages in several forms of PH.

Characterization of ex vivo fibroblast populations

Consistent with the previous data on fibroblasts from the large elastic pulmonary arteries (29), adventitial fibroblasts isolated...
from distal pulmonary arteries of chronically hypoxic hypertensive calves (hereafter termed “PH-Fibs”) were significantly smaller in size than those of control calves (termed “CO-Fibs”) and proliferated at markedly higher rates (data not shown). The phenotypes of PH-Fibs and CO-Fibs were characterized by immunofluorescence analysis using mesenchymal, smooth muscle, hematopoietic/progenitor, and monocyte/macrophage markers. Both PH-Fibs and CO-Fibs expressed mesenchymal Ags: a myofibroblast marker α-SM-actin (Fig. 2A, 2B) as well as Hsp47 (a molecular chaperone for type I procollagen) and vimentin (Fig. 2E, 2F, only PH-Fibs shown). Both PH-Fibs and CO-Fibs populations lacked the expression of the SM-specific marker SM-myosin H chain (Fig. 2C, only PH-Fibs shown) and lacked the expression of CD34, CD14, and CD68 (data not shown). However, a marked difference in the phenotypes of PH-Fibs and CO-Fibs was observed in the fact that PH-Fibs were almost completely deficient in THY1 mRNA expression, whereas CO-Fibs expressed THY1 at high levels (Fig. 2G).

**PH-Fibs express a proinflammatory stable phenotype**

Under serum-free conditions, PH-Fibs, as compared with CO-Fibs, exhibited constitutively augmented mRNA expression of cano-

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**FIGURE 2.** Phenotypic characterization of ex vivo pulmonary adventitial fibroblast populations. A and B, Both PH-Fibs (A) and CO-Fibs (B) populations expressed the myofibroblast marker α-SM-actin. The subcellular pattern of expression, though, differed between PH-Fibs and CO-Fibs. In PH-Fibs (A), α-SM-actin expression was predominantly cytoplasmic, as opposed to the mostly microfibrillar pattern in CO-Fibs (B). Note that PH-Fibs (A) are significantly smaller in size than CO-Fibs (B). C and D, Both PH-Fibs and CO-Fibs (only PH-Fibs are shown, C) lacked the expression of a SMC differentiation marker, SM myosin heavy chains, as compared with its positive expression in pulmonary artery SMCs (D). E and F, Both PH-Fibs (shown here) and CO-Fibs (data not shown) expressed mesenchymal cell markers, type 1 procollagen chaperone Hsp47 (E) and vimentin (F). G, PH-Fibs were almost completely deficient of THY1 mRNA expression, whereas CO-Fibs expressed THY1 message at high levels. Number of cell populations analyzed: PH-Fibs (n = 7), CO-Fibs (n = 5). Scale bars, 50 μm. *p < 0.01.
ical proinflammatory cytokines (IL-1β, IL-6, and GM-CSF), chemokines/cognate receptors [CCL2(MCP-1), CXCL12(SDF-1), CCL5 (RANTES), CCR7, and CXCR4], costimulatory molecules (CD40L and CD40), and VCAM-1 (Fig. 3, Table II). These phenotypical differences between PH-Fibs and CO-Fibs were maintained in culture through multiple passages (tested up to passage 10), thus representing a stable proinflammatory phenotype of PH-Fibs. Notably, the proinflammatory phenotype of PH-Fibs was characterized by the lack of expression of proinflammatory TNF-α, IL-12A (P35), IL-12B (P40), Th2 cytokines IL-4 and IL-13, or Th17 cytokine IL-17A. Little to no expression of IL-10, a canonical anti-inflammatory cytokine, was noted in both CO-Fibs and PH-Fibs.

RT-PCR results were confirmed at the protein level by immunofluorescent cytochemistry, ELISA, and Western blot analyses. As shown in Fig. 4, in vivo immunocytochemical analysis of lung tissues demonstrated augmented expression of IL-1β, CCL2(MCP-1), CXCL12(SDF-1), and VCAM-1 in the pulmonary adventitia of chronically hypertensive calves. In vitro analysis of cultured cells confirmed these observations by demonstrating augmented expression of these proteins by PH-Fibs compared with that by CO-Fibs. A 5.4-fold increase in CCL2(MCP-1) production in PH-Fibs (58.5 ± 8.1 versus 10.8 ± 7.1 pg/ml in CO-Fibs) was shown by ELISA analysis, and a 1.8-fold increase in VCAM-1 expression by PH-Fibs compared with that by CO-Fibs was shown by Western blot analysis. Limitations on the availability of bovine-specific Abs prevented the evaluation, at the protein level, of other molecules that were increased significantly at the mRNA level.

In contrast, little expression of a proinflammatory phenotype was observed in smooth muscle cells (SMCs) isolated from the

**FIGURE 3.** PH-Fibs express a proinflammatory phenotype (mRNA expression). A–C, PH-Fibs, as compared with CO-Fibs, exhibited constitutively augmented mRNA expression of a number of cytokines (A), chemokines and costimulatory molecules (B), and VCAM-1 (C). The observed differences between PH-Fibs and CO-Fibs were maintained in culture through multiple passages (tested up to passage 9). As shown, differences in TNF-α and CCR2 messages were not significant. Number of cell populations analyzed: PH-Fibs (n = 6), CO-Fibs (n = 5). *p < 0.05, **p < 0.001, ***p < 0.0001.
pulmonary arteries of hypertensive animals (Fig. 5A). In fact, PH-Fibs exhibited markedly higher mRNA expression levels than those of SMCs isolated from the same pulmonary arteries of hypertensive animals (PH-SMCs) for IL-1β, IL-6, CCL2(MCP-1), CXCL12(SDF-1), CCL5(RANTES), CXCR4, CD40, and VCAM-1 (Fig. 5B, Table III). No significant difference was observed between PH-Fibs and PH-SMCs for mRNA expression levels of TNF-α, GM-CSF, CCR7, and CD40L (Fig. 5B).

**Alterations in HDACs contribute to a proinflammatory phenotype of PH-Fibs**

To address the possible role of HDACs in the distinct phenotype of PH-Fibs, HDAC catalytic activity was quantified. As shown in Fig. 6Aa, class I HDAC catalytic activity was increased significantly in PH-Fibs compared with that in CO-Fibs. The activity was blocked completely by the selective class I HDAC inhibitor apicidin, confirming that deacetylation of the substrate was mediated by members of HDAC class I. Elevated class I HDAC catalytic activity correlated with increased abundance of HDAC1, HDAC2, and HDAC3 as determined by Western blot analysis (Fig. 6Ab, 6Ac).

To determine whether HDAC activity contributed to the proinflammatory phenotype of PH-Fibs, pharmacological HDAC inhibitors were used. Incubation of PH-Fibs with the pan-HDAC inhibitor SAHA or with the selective class I HDAC inhibitor apicidin resulted in the attenuation of a proinflammatory phenotype as defined by dramatically reduced mRNA expression levels of IL-6, CCL2(MCP-1), CXCL12(SDF-1), GM-CSF, and VCAM-1 (Fig. 6B). Notably, changes in mRNA expression levels of CCL5 (RANTES) (Fig. 6B), CXCR4, and CCR2 (data not shown) were statistically insignificant, indicating specific targeting of HDAC inhibitors. HDAC inhibition did not result in increased mRNA expression of the anti-inflammatory cytokine IL-10. HDAC inhibitors did not affect, at the concentrations tested, cell viability as tested by the trypan blue assay.

### Table II. Fold change in mRNA expression levels between PH-Fibs and CO-Fibs

<table>
<thead>
<tr>
<th>Gene</th>
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<td>IL-1β</td>
<td>2.41 ± 0.25</td>
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<td>IL-6</td>
<td>8.74 ± 1.35</td>
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<td>TNF-α</td>
<td>1.07 ± 0.35</td>
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<td>CCL2/MCP-1</td>
<td>2.66 ± 0.52</td>
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<tr>
<td>CXCL12/SDF-1</td>
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<tr>
<td>CCL5/RANTES</td>
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<td>CCR7</td>
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<td>CCR2</td>
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<td>CXCR4</td>
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<td>GM-CSF</td>
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<td>CD40L</td>
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<td>CD40</td>
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<tr>
<td>VCAM-1</td>
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</table>

### Figure 4. PH-Fibs express a proinflammatory phenotype (protein expression). A–F. At the protein level, PH-Fibs expressed higher levels of IL-1β (A), CCL2/MCP-1 (B, C), CXCL12(SDF-1) (D), and VCAM-1 (E, F). Number of cell populations analyzed: PH-Fibs (n = 5), CO-Fibs (n = 5). Scale bars, 50μm. *p < 0.02 (C), *p < 0.05 (F). adv., adventitia; M, media; PA, pulmonary artery.
**PH-Fibs induce increased migration, adhesion, and activation of monocytes**

We next tested whether PH-Fibs induced increased migration of THP-1 monocytes and primary murine BMDMs. Serum-free culture medium conditioned by PH-Fibs (PH-Fib-CM), as compared with that in CO-SMCs. Number of cell populations analyzed: PH-SMCs (n = 7), CO-SMCs (n = 9). PH-Fibs consistently exhibited markedly higher mRNA expression levels of a number of cytokines, chemokines, and VCAM-1, than those of PH-SMCs. No significant difference was observed for mRNA expression levels of TNF-α, GM-CSF, CCR7, and CD40L. Number of cell populations analyzed: PH-Fibs (n = 5), PH-SMCs (n = 7). *p < 0.05, **p < 0.001, ***p < 0.0001.

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**FIGURE 5.** PH-SMCs of hypertensive animals do not express a proinflammatory phenotype. A, mRNA expression of a number of cytokines, chemokines, and VCAM-1 was not upregulated in PH-SMCs compared with that in CO-SMCs. Number of cell populations analyzed: PH-SMCs (n = 7), CO-SMCs (n = 9). B, PH-Fibs consistently exhibited markedly higher mRNA expression levels of a number of cytokines, chemokines, and VCAM-1, than those of PH-SMCs. No significant difference was observed for mRNA expression levels of TNF-α, GM-CSF, CCR7, and CD40L. Number of cell populations analyzed: PH-Fibs (n = 5), PH-SMCs (n = 7). *p < 0.05, **p < 0.001, ***p < 0.0001.

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PH-Fibs also increased the migration of primary murine BMDMs (2.10 ± 0.06-fold) (Fig. 7A, right panel). We also sought to determine if PH-Fibs exhibited increased adhesion for THP-1 monocytes and show that PH-Fibs induced (1.6 ± 0.04)-fold higher adhesion of monocytes, compared with that of CO-Fibs (Fig. 7B).

We also evaluated whether proinflammatory PH-Fibs could promote the activation of monocytes and macrophages toward a proinflammatory phenotype. Incubation of both human THP-1
monocytes and murine BMDMs with PH-Fib-CM, but not with CO-Fib-CM, resulted in markedly upregulated mRNA expression of canonical proinflammatory mediators and profibrotic molecules (Fig. 7C, 7D). Specifically, in response to PH-Fib-CM, THP-1 monocytes showed increased mRNA expression of IL-1β (8.04 ± 2.1-fold increase compared with that in response to CO-Fib-CM), IL-6 (2.32 ± 0.23-fold increase), CCL2/MCP-1 (7.44 ± 1.27-fold increase), and type 1 collagen (COL1A1) (3.95 ± 0.65-fold increase) (Fig. 7C). In murine BMDMs incubated with PH-Fib-CM, mRNA expression of IL-1β increased (6.56 ± 0.35-fold) compared with that of BMDMs incubated with CO-Fib-CM, IL-6 increased (12.0 ± 0.81-fold), CCL2/MCP-1 increased (2.7 ± 0.11-fold), Col1a1 increased (1.32 ± 0.06)-fold (Fig. 7D), and TIMP1 increased 2-fold (data not shown).

**Inhibition of HDACs results in attenuation of PH-Fibs functional activities**

We next asked if the attenuation of the proinflammatory phenotype in PH-Fibs observed after application of HDAC inhibitors was associated with reduced functional abilities of PH-Fibs to recruit and activate monocytes/macrophages. Indeed, treatment of PH-Fibs with SAHA and apicidin decreased the chemotactic activity of PH-Fib-CM for the monocytes (Fig. 8A) and significantly attenuated their ability to activate proinflammatory cytokine production in THP-1 monocytes (Fig. 8B). Induction of IL-6 or COL1A1 expression by PH-CM was not affected by class I HDAC inhibitors at the time point tested (data not shown).

**Discussion**

The present study demonstrates that hypoxia-induced pulmonary vascular remodeling is characterized by the emergence of a distinct adventitial fibroblast population (termed here PH-Fibs) that exhibits a constitutively activated “imprinted” proinflammatory phenotype that is capable of inducing recruitment, retention, and proinflammatory activation of monocytes and macrophages. Remarkably, this proinflammatory phenotype of PH-Fibs was characterized by high expression levels of canonical proinflammatory cytokines (IL-1β and IL-6), macrophage chemoattractant cytokines [CCL2/MCP-1, CXCL12/SDF-1, and CCL5(RANTES)], macrophage growth factor (GM-CSF), and a costimulatory molecule capable of activating macrophages (CD40L) as well as by increased expression of the adhesion protein VCAM-1 in the absence of any exogenous stimulation. In contrast, SMCs isolated from the same arteries of hypertensive animals did not exhibit a proinflammatory phenotype. Our study supports the hypothesis that, mechanistically, the phenotype of PH-Fibs was due to epigenetic alterations, as demonstrated by increased catalytic activity and protein expression of class I HDACs. This hypothesis was supported further by our observations that apicidin, a specific class I HDAC inhibitor, preferentially and dramatically decreased expression of a specific subset of proinflammatory mediators and caused a marked reduction in the ability of PH-Fibs to induce monocyte migration and activation.

### Table III. Fold change in mRNA expression levels between PH-Fibs and PH-SMCs

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</table>

**FIGURE 6.** PH-Fibs exhibit increased HDAC activity and protein expression, which contribute to their proinflammatory phenotype. Aa. Class I HDAC catalytic activity was increased significantly in PH-Fibs compared with that in CO-Fibs. Ab and Ac. Increased class I HDAC (HDAC1, HDAC2, and HDAC3) protein expression was demonstrated by Western blot assay (image of a representative blot and densitometric quantification of three experiments are shown in Ab, and quantification is presented in Ac). B. Incubation of PH-Fibs with the pan-HDAC inhibitor SAHA (10 μM) or with the selective class I HDAC inhibitor apicidin (3 μM) resulted in markedly decreased mRNA levels of several cytokines, chemokines, and VCAM-1. n = 4. *p < 0.05.
PH is characterized by dramatic changes in the structure of pulmonary arteries and the phenotype of vascular wall cells. In several forms of PH, including the calf model of severe hypoxia-induced PH presented in this study, the adventitia displays dramatic thickening, which was assumed originally to be exclusively caused by excessive accumulation of fibroblasts and myofibroblasts. New experimental data, however, have expanded this concept by demonstrating dramatic perivascular accumulation of inflammatory cells, suggesting that inflammation correlates with and constitutes an essential part of vascular remodeling in many diseases, including experimental PH and PAH in humans (1, 4, 6, 30–32). In the current study, using interspecies (rat, calf, and human) analysis of pulmonary perivascular cellular composition in several forms of PH (hypoxia-induced and monocrotaline-induced experimental PH in animal models as well as iPAH in humans), we documented and confirmed consistent accumulation of monocytes/macrophages in the pulmonary adventitia. These observations raise questions as to what specific vascular cell type is responsible for inducing inflammatory cell accumulation and activation in the adventitia. The presented data suggest that a specific population of proinflammatory pulmonary adventitial fibroblasts is the candidate cell type.

Functionally, proinflammatory PH-Fibs produced soluble factors that were capable of recruiting, retaining, and activating monocytes (i.e., THP-1) and macrophages (BMDMs). Candidate factors produced by PH-Fibs responsible for recruitment of monocytes/macrophages both in vivo and in vitro are CCL2(MCP-1), a canonical monocyte attractant cytokine, and CCL12(SDF-1). CCL2-dependent recruitment of monocytes/macrophages has been implicated recently in the pathogenesis of a number of chronic inflammatory conditions characterized by vascular remodeling (32, 33). CCL12(SDF-1) has been found to be upregulated in hypoxic lung and to be critical for recruitment of CXCR4-positive macrophages, which play critical roles in vascular remodeling (34, 35). Importantly, the persistence of inflammatory cells in pulmonary adventitia observed under sustained hypoxic exposure

**FIGURE 7.** PH-Fibs induce increased migration, adhesion, and activation of monocytes/macrophages. A. Media conditioned by PH-Fibs (PH-Fib-CM) induced increased transwell migration of THP-1 monocytes (left panel, 1 h time point is shown) and of murine BMDMs (right panel, 24 h time point is shown), as compared with media conditioned by CO-Fibs (CO-Fib-CM). PH-Fib-CM, obtained from four PH-Fibs cell populations; CO-Fib-CM, obtained from three CO-Fibs cell populations. B. PH-Fibs induced increased adhesion of THP-1 monocytes, compared with CO-Fibs. Number of cell populations analyzed: PH-Fibs (n = 4), CO-Fibs (n = 3). C. In response to serum-free media conditioned by PH-Fibs (PH-Fib-CM), human THP-1 monocytes upregulated mRNA expression of proinflammatory mediators IL-1β, IL-6, and CCL2(MCP-1) and of a profibrotic marker COL1A1. n = 4. D. In murine BMDMs incubated with PH-Fib-CM, increased mRNA expression levels of IL-1β, IL-6, and CCL2(MCP-1) and of a profibrotic marker, Col1a1, were detected. *p < 0.05, **p < 0.001, ***p < 0.0001.
requires upregulation of specific molecules capable of retaining circulating cells in the local microenvironment. PH-Fibs generated GM-CSF, a macrophage growth and survival factor that has been shown to control retention of macrophages in the tissue and that has been suggested to be a critical cytokine in regulating a variety of tissue inflammatory responses including lung inflammation (36, 37). Moreover, PH-Fibs exhibited increased expression of both CXCL12(SDF-1) and VCAM-1, which have been shown as crucial in the retention of hematopoietic progenitor cells within the tissue (e.g., bone marrow) (38). The current study also demonstrates that PH-Fibs produced soluble factors that induced a phenotypic alteration in monocytes/macrophages that was compatible with innate immune activation. Monocytes and macrophages exposed to culture supernatant from PH-Fibs exhibited a proinflammatory [IL-1β, IL-6, and CCL2(MCP-1)] gene transcription profile indicative of activated TLR/NF-κB signaling pathways. These findings are of particular interest, because, in patients with idiopathic and familial PAH, increased expression levels of IL-1β, IL-6, and CCL2(MCP-1) have been proposed to predict survival (7, 39–41). Proinflammatory IL-6 has been implicated recently in the pathogenesis of hypoxia-induced lung inflammation and pulmonary vascular remodeling using experimental animal models (42, 43). Additionally, generation of IL-6 and IL-1β by monocytes/macrophages is compatible with activation of TLR and inflammasome signaling pathways activated by danger-associated molecular patterns (44). Such danger-associated molecular patterns likely degenerately activate TLR/inflammasome pathways across species, which would account for the observation that soluble factors derived from bovine PH-Fibs activated human (THP-1) and murine (BMDMs) monocytes/macrophages. Alternatively, IL-6 and IL-1β secreted by PH-Fibs could activate STAT-dependent and NF-κB–dependent signaling pathways that lead to the activation of macrophages (25, 45).

Intriguingly, PH-Fibs induced expression of NF-κB and AP1 target profibrogenic genes, COL1A1 and TIMP1, in THP-1 monocytes and BMDMs macrophages, which is consistent with a previously described profibrogenic macrophage phenotype characteristic of chronic inflammation and tissue remodeling in PH and other diseases (46–48). In pulmonary circulation, in adult atherosclerotic pulmonary arteries, Liptay et al. (47) proposed that extracellular matrix gene expression (fibronecin and type I procollagen) was associated intimately with nonfoamy neointimal macrophages. Expression of virtually all types of collagens as well as of TIMP1, at both mRNA and protein levels, has been reported recently in macrophages, where the authors propose that collagen synthesis in macrophages may represent a specific feature of a hitherto unrecognized profibrogenic macrophage phenotype that adds to a spectrum of macrophage functional heterogeneity (49). Thus, our data are consistent with previous reports and suggest a phenotypic switch of monocytes/macrophages, exposed to soluble factors from PH-Fibs, toward not only a proinflammatory but also a potentially profibrogenic phenotype.

We have made the novel observation that, in the setting of severe hypoxic PH, pulmonary adventitial fibroblasts express a distinct profibrogenic phenotype that is stable in culture for numerous passages. Similar findings of persistently activated fibroblasts were reported for diseased tissues in other organs, including synovial fibroblasts from patients with rheumatoid arthritis, tumor-associated fibroblasts, fibroblasts from systemic sclerosis patients, and lung fibroblasts from patients with idiopathic pulmonary fibrosis (50–52). Although the molecular basis for such a distinct and stable phenotype remains unclear, a potential role for epigenetic modulation has been suggested. In the current study, PH-Fibs, isolated from severely hypertensive animals, were found to exhibit significantly elevated catalytic activity of HDACs, a family of enzymes that are known to play critical roles in the control of epigenetics (53). Specifically, class I HDACs (HDAC1, HDAC2, and HDAC3), which primarily localize to nuclei, are linked to epigenetics through their ability to efficiently deacetylate nucleosomal histones. Class I HDAC catalytic activity was increased in PH-Fibs, and specific catalytic inhibition of class I HDACs was sufficient to suppress production of proinflammatory mediators by PH-Fibs. The data thus suggest that transcriptional changes due to epigenetics, which are mitotically heritable and occur in the absence of underlying changes in DNA sequence, could mechanistically explain the stable, profibrogenic phenotype of PH-Fibs. Although some studies have reported decreased HDAC activity in lung diseases such as chronic obstructive pulmonary disease and bronchial asthma (54) as well as in rheumatoid arthritis (55), recent reports have demonstrated that both the total HDAC activity and the expression of HDAC1 specifically are increased significantly in rheumatoid arthritis, both whole tissues and synovial fibroblasts (19, 20). Importantly, the data of Kawabata et al. (19) in rheumatoid arthritis are very similar to our results with regard to increases in class I HDAC activity and protein expression. Kawabata et al. (19) discuss in detail the possible explanations for the discrepancies of their results from those reported earlier (55). Similar results (i.e., increased HDAC1 synovial fibroblasts from rheumatoid arthritis patients) also have been shown by Horiuichi et al. (20) who suggested that increased HDAC1 activity might be involved in rheumatoid arthritis pathogenesis by regulating cell cycle and sur-
vival in synovial tissues. Furthermore, anti-inflammatory effects of HDAC inhibitors (consistent with our results) have been shown both in vitro and in vivo in various inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus, asthma, inflammatory lung diseases, atherosclerosis, hemorrhagic shock, diabetes, inflammatory bowel diseases, osteoporosis, macular degeneration, and neurodegenerative and CNS diseases (16). In vascular disease models, recent publications have demonstrated that HDAC inhibition can decrease neointima formation (56) and decrease inflammation (21). The possibility that specific class I HDAC inhibitors may be beneficial in cardiovascular disease has been suggested by us previously (17).

Collectively, these results imply unfoseen potential for class I HDAC-selective small-molecule inhibitors for the treatment of pathological vascular remodeling in the setting of some forms of PH. In this regard, numerous HDAC inhibitors are in preclinical and clinical development, including compounds that selectively inhibit class I HDACs (22).

Vascular inflammation has been considered traditionally an “inside-out” response centered on monocyte/macrophage recruitment to the intima of blood vessels, wherein injured vascular endothelial cells produce inflammatory mediators and express surface adhesion molecules that participate in monocyte homing to the luminal surface and their transmigration into the intima and/or media. However, growing experimental evidence supports a new paradigm of an “outside-in” hypothesis, in which the adventitial compartment is viewed as a critical regulator of vessel wall function, with vascular inflammation being initiated in the adventitia and then progressing inward toward the media and intima (8). In support of this “outside-in” hypothesis is the observation, in a wide variety of systemic vascular injuries, of an almost immediate influx of monocytes/macrophages into the adventitial compartment (8, 11, 57–60). The findings of the current study support the “outside-in” hypothesis and provide novel information demonstrating that severe hypoxia-induced PH is associated with the emergence of adventitial fibroblasts with a proinflammatory phenotype that may orchestrate recruitment, retention, and activation of circulating inflammatory cells. Thus, even though vascular endothelial cells are well known as producers of cytokines and chemokines essential in the recruitment of inflammatory cells (33), it is becoming increasingly clear that the adventitial fibroblast is capable of a wide array of responses, including production of mediators controlling inflammatory responses and activation and differentiation of the recruited leukocytes.

Our findings of a uniquely distinct adventitial fibroblast in PH raise questions regarding the potential origin of this cell. Our previous work, using animal models of chronic hypoxic PH, demonstrated dramatic perivascular accumulation of circulating fibrocytes (mesenchymal cells of a monocyte/macrophage lineage) that produced collagen, expressed α-SM-actin, and actively proliferated in the pulmonary adventitia (i.e., a phenotype closely related to that described for PH-Fibs in the current study). In contrast to tissue fibroblasts, fibrocytes lack THY1 expression (61), an observation similar to that described here for PH-Fibs. Thus, the possibility that the PH-Fibs population arose from a circulating, hematopoietic rather than resident origin cannot be excluded. However, it should be noted that the cells used for the study did not express hematopoietic/progenitor markers, at least at the time point in the culture analyzed. Other potential origins of these cells may include the endothelial–mesenchymal transition from the adventitial vasa vasorum endothelial cells (62) or even from resident vascular progenitor cells (63).

In conclusion, our study has identified a novel adventitial fibroblast population with a stable proinflammatory phenotype that likely results from epigenetic modifications brought about by HDACs. Our data support the “outside-in” hypothesis for the pathogenesis of PH and suggest that adventitial proinflammatory fibroblasts orchestrate recruitment, retention, and activation of monocytes/macrophages toward a proinflammatory phenotype. These findings therefore begin to explain the very common observations of pulmonary perivascular inflammation in humans with PAH and in animal models of PH and suggest a pivotal role for fibroblast/macrophage interactions in the PH disease process.

Our study also highlights synthetic HDAC inhibitors or tailored immunomodulatory agents that target innate signaling pathways in fibroblasts and/or macrophages as promising candidates for therapy and prevention of PH.

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Disclosures
The authors have no financial conflicts of interest.

References
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