Review Article

Targeting the adventitial microenvironment in pulmonary hypertension: A potential approach to therapy that considers epigenetic change

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ABSTRACT

Experimental data indicate that the adventitial compartment of blood vessels, in both the pulmonary and systemic circulations, like the connective tissue stroma in tissues throughout the body, is a critical regulator of vessel wall function in health and disease. It is clear that adventitial cells, and in particular the adventitial fibroblast, are activated early following vascular injury, and play essential roles in regulating vascular wall structure and function through production of chemokines, cytokines, growth factors, and reactive oxygen species (ROS). The recognition of the ability of these cells to generate and maintain inflammatory responses within the vessel wall provides insight into why vascular inflammatory responses, in certain situations, fail to resolve. It is also clear that the activated adventitial fibroblast plays an important role in regulating vasa vasorum growth, which can contribute to ongoing vascular remodeling by acting as a conduit for delivery of inflammatory and progenitor cells. These functions of the fibroblast clearly support the idea that targeting chemokine, cytokine, adhesion molecule, and growth factor production in activated fibroblasts could be helpful in abrogating vascular inflammatory responses and thus in ameliorating vascular disease. Further, the recent observations that fibroblasts in vascular and fibrotic diseases may maintain their activated state through epigenetic alterations in key inflammatory and pro-fibrotic genes suggests that current therapies used to treat pulmonary hypertension may not be sufficient to induce apoptosis or to inhibit key inflammatory signaling pathways in these fibroblasts. New therapies targeted at reversing changes in the acetylation or methylation status of key transcriptional networks may be needed. At present, therapies specifically targeting abnormalities of histone deacetylase (HDAC) activity in fibroblast-like cells appear to hold promise.

Key Words: adventitial, fibroblasts, epigenetics, pulmonary hypertension

INTRODUCTION

An increasing volume of experimental data indicate that the adventitial compartment of blood vessels, in both the pulmonary and systemic circulations, like the connective tissue stroma in tissues throughout the body, is a critical regulator of vessel wall function in health and disease.[1-4] A rapidly emerging concept is that the vascular adventitia acts as a biological processing center for the retrieval, integration, storage and release of key regulators of vessel wall function. Indeed, the adventitial compartment has been suggested to be the principal "injury-sensing tissue" of the vessel wall, and the adventitial fibroblast to be a "sentinel cell."[5] In response to hormonal, inflammatory and environmental stresses such as hypoxia/ischemia, or vascular distention, resident adventitial fibroblasts are the first vascular wall cells to exhibit evidence of "activation." Such activation is characterized by increases in cellular proliferation, the expression of contractile (a-SM-actin) and extracellular matrix proteins, as well as in the secretion of chemokines, cytokines, growth, and angiogenic factors capable of

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directly affecting resident vascular wall cell growth and of initiating inflammation in a manner that influences overall vascular tone and wall structure. A particularly intriguing concept, given the fact that pulmonary hypertension in many cases is considered a chronic inflammatory disease, is that the fibroblast plays an active role in the persistence of inflammation. In fact, fibroblast-leukocyte interactions at sites of chronic inflammation appear to promote sustained leukocyte survival and retention resulting in failure to resolve the inflammatory lesion.[6-9] This has prompted some to consider fibroblasts and/or fibroblast-leukocyte interactions as a target for therapy in chronic inflammation and fibro-proliferative diseases.

The purpose of this review is to provide evidence that pulmonary vascular adventitial fibroblasts (1) initiate and perpetuate chronic vascular inflammation through the production of soluble factors such as chemokines that facilitate recruitment of circulating leukocytes and progenitor cells to the vessel wall, and cytokines that subsequently promote retention and activation of the recruited cells; (2) synthesize and release angiogenic factors which support neovascular growth of the vasa vasorum, creating a conduit that serves to perpetuate the inflammatory response; (3) undergo epigenetic alterations that drive the fibroblast towards a persistent pro-inflammatory activation state, thus preventing them from returning to a resting phenotype as would occur in physiological wound healing; and (4) may provide a promising therapeutic target to mitigate inflammation and fibrosis in the vessel wall.

ADVENTITIAL FIBROBLASTS: ROLE IN VASCULAR INFLAMMATION

Vascular inflammation (and remodeling in general) has traditionally been considered an "inside-out" response centered on leukocyte/monocyte recruitment to the intima of blood vessels. In this hypothesis, injured vascular cells on the intimal surface of blood vessels express surface adhesion molecules and inflammatory mediators that participate in monocyte homing to the endothelium and eventual transmigration into the media and/or intima.[10] In contrast, growing experimental evidence supports a new paradigm of an "outside-in" hypothesis in which vascular inflammation is initiated in the adventitia and progresses inward toward the intima (Fig. 1). For a long period of time, most immunologists

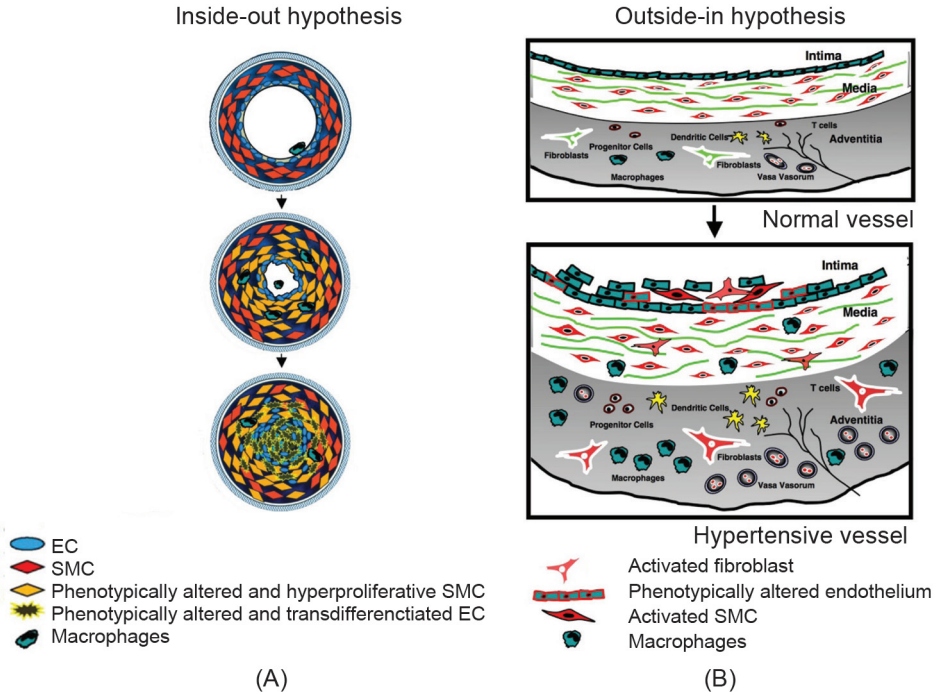

Figure 1: Contrasting hypotheses regarding origin and perpetuation of vascular remodeling and inflammation. (A) Traditionally, remodeling and inflammation have been considered an "Inside/Out" response centered on endothelial injury, leukocyte/monocyte recruitment to the intima of blood vessels, followed by activation of medial smooth muscle cells. In this hypothesis, remodeling is largely thought to be mediated by endothelial cell activation, injury or death, abnormalities in endothelial-smooth muscle communication and resultant hyper-proliferation of either/or both cell types and recruitment of inflammatory cells, which are directed from the lumen of the pulmonary artery. (B) The "Outside-In" hypothesis suggests that vascular inflammation because of resident professional (dendritic cells, macrophages and lymphocytes) and nonprofessional (fibroblasts) immune cells occurs early and persists in the adventitia. Fibroblast activation, leukocyte and progenitor cell accumulation and retention lead to remodeling not only of the adventitia, but cause subsequent changes in the media and ultimately even the intima. Thus, the adventitia, as opposed to its' usual depiction (Panel A) of an unimportant simple support structure, is actually a highly cellular, metabolically active, regulatory compartment of the vessel wall, capable of controlling tone, structure, and inflammation from the outside-in.

regarded fibroblast activation as relatively insignificant in regulating immune responses and concentrated primarily on interactions between lymphocytes, macrophages, and dendritic cells. However, it is now becoming clear that local immune responses are initiated by a variety of exogenous and endogenous danger signals that engage pattern recognition receptors, and thus a more integrated concept focuses on an extended local immune response in which tissue stromal cells, including fibroblasts, play a key role in modulating both innate and adaptive immune responses. It is now established that fibroblasts indeed express pattern recognition receptors, such as the toll-like receptors (TLRs), NOD-like receptors (NLRs) and the receptor for advanced glycosylation end-products (RAGE).[11-13] Fibroblasts are thus equipped with the necessary machinery to recognize exogenous and endogenous danger signals and to function as an innate immune cell (Fig. 2). In addition, small peptides released during activation of the coagulation or complement cascades as well as during remodeling of the extracellular matrix can profoundly affect fibroblast immune responses.[5,8,14,15] Activated fibroblasts have been shown to regulate the functional phenotype of hematopoietic cells that have been recruited to damaged tissues through a number of mechanisms, including CD40/CD40-ligand interactions and the production of chemokines and cytokines, including MCP-1, RANTES, IL-6, and SDF-1.[5,16-21] Consequently, upon activation, fibroblasts are not only stimulated to proliferate and differentiate into myofibroblasts and to produce extracellular matrix proteins, but also to adapt innate immune functions characterized by generation of pro-inflammatory cytokines, chemokines, adhesion molecules, and mediators of tissue remodeling such as TIMPS and MMPs (Fig. 2).[12]

In many models of arterial injury in the systemic circulation, early adventitial inflammation and accumulation of activated monocytes/macrophages is consistently observed.[15,19,22-24] Additional experimental evidence supporting the postulated "outside-in" hypothesis regarding adventitial regulation of vascular is as follows: (1) Zhou et al. demonstration that inward remodeling in a mouse carotid flow reduction model required early adventitial accumulation of CXCR3-positive macrophages,[25] both IP10 and Mig transcripts having rapidly increased following carotid flow reduction surgery and were required for subsequent accumulation of a unique subset of macrophages that are essential in promoting tissue remodeling; (2) Tang et al. demonstration that macrophage depletion prevented flow dependent inward remodeling in this mouse carotid artery model[24] and this inward remodeling was associated with adventitial macrophage activation and superoxide stimulated pro-inflammatory cytokine production (e.g., IL-1beta, IL-6, IP-10, and Mig); (3) the demonstration that inward remodeling was dependent on adventitial cell expression of MyD88, an essential TLR signaling pathway-associated adapter for activation of the transcription factor NFkβ;[24] and (4) studies showing that angiotensin II-mediated hypertension was blunted in RAG1^{-}/$^{-}$ mice that lack adaptive immunity (i.e., B and T lymphocytes), and in this model, adoptive transfer of T-cells but not B cells restored angiotensin II induced hypertensive responses.[21] These studies showed that low doses of angiotensin II stimulated the vessel wall cells to produce RANTES as well as other chemokines that recruit lymphocytes and leukocytes to the adventitia and adjacent peri-adventitial tissues. Infusion of angiotensin II also stimulated the production of IL-6 and MCP-1 by adventitial cells.[20] Increased expression of IL-6 and MCP-1 correlated

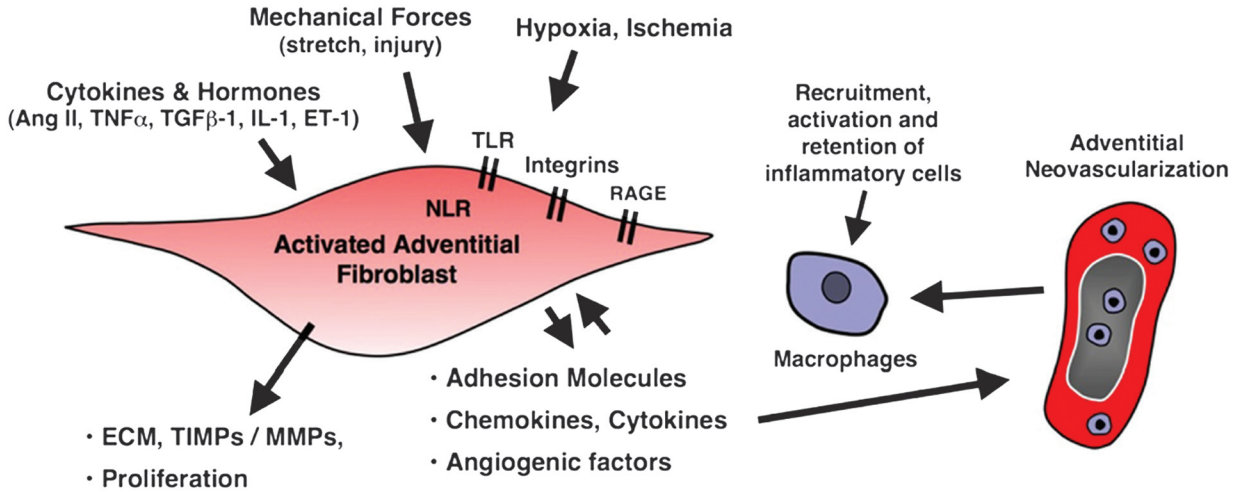

Figure 2: The activated adventitial fibroblast plays a pivotal role in the recruitment and retention of inflammatory cells in the vascular wall. In response to a variety of environmental stimuli, the adventitial fibroblast, potentially through a number of cell surface receptors, including Toll-like receptors (TLR), integrins, and receptors for advanced glycosolation end products (RAGE) is activated to produce extracellular matrix proteins, matricellular proteins, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). In addition, the fibroblast upregulates production of chemokines and cytokines, adhesion molecules, and angiogenic factors. This leads to the recruitment of leukocytes and ultimately to an increase in vasa vasorum density or adventitial neovascularization, which perpetuates the inflammatory process.

with adventitial thickening, monocyte/macrophage recruitment and activation, as well as aortic wall remodeling. When monocytes and aortic adventitial fibroblasts were co-cultured in vitro, increased levels of IL-6 and MCP-1 were detected in the conditioned medium. Importantly, this conditioned medium promoted the differentiation of monocytes into macrophages and enhanced expression of MCP-1 and MMP-9 by adventitial fibroblasts.[20]

Other studies have shown that in inflammatory vascular disease, increased macrophage and fibroblast p47phox (NADH oxidase) activity is increased in the adventitia, stimulating superoxide production that scavenges locally produced NO in the vessel wall leading to excessive vasoconstriction. Moreover, fibroblasts and macrophages can release TNFa and other cytokines that stimulate NADH oxidase activity in SMCs, further reducing NO levels and increasing vascular SM contractile tone.[2] Superoxide as well as other cytokines released by the activated stromal cells can also stimulate SMC proliferation and activation of adventitial progenitor cells, which may play a role in intimal lesion formation.[26,27]

Collectively, these findings provide strong support for the postulated role of the arterial adventitia and specifically the adventitial fibroblast as an initiator and mediator of inflammatory processes that predispose vessel walls to excessive vasoconstriction as well as pathogenic remodeling in adventitial, medial, and even intimal layers of the systemic vasculature.

Though not investigated to the same extent, studies in the pulmonary circulation have also shown marked adventitial/perivascular inflammation in animal models of pulmonary hypertension. In the two most commonly used models of pulmonary hypertension, hypoxia and monocrotaline, early appearance and subsequent persistence of inflammatory/progenitor cells followed by prominent remodeling in the adventitia and media of both large and small pulmonary arteries are consistently observed (Fig. 3A).[28-33] In fact,

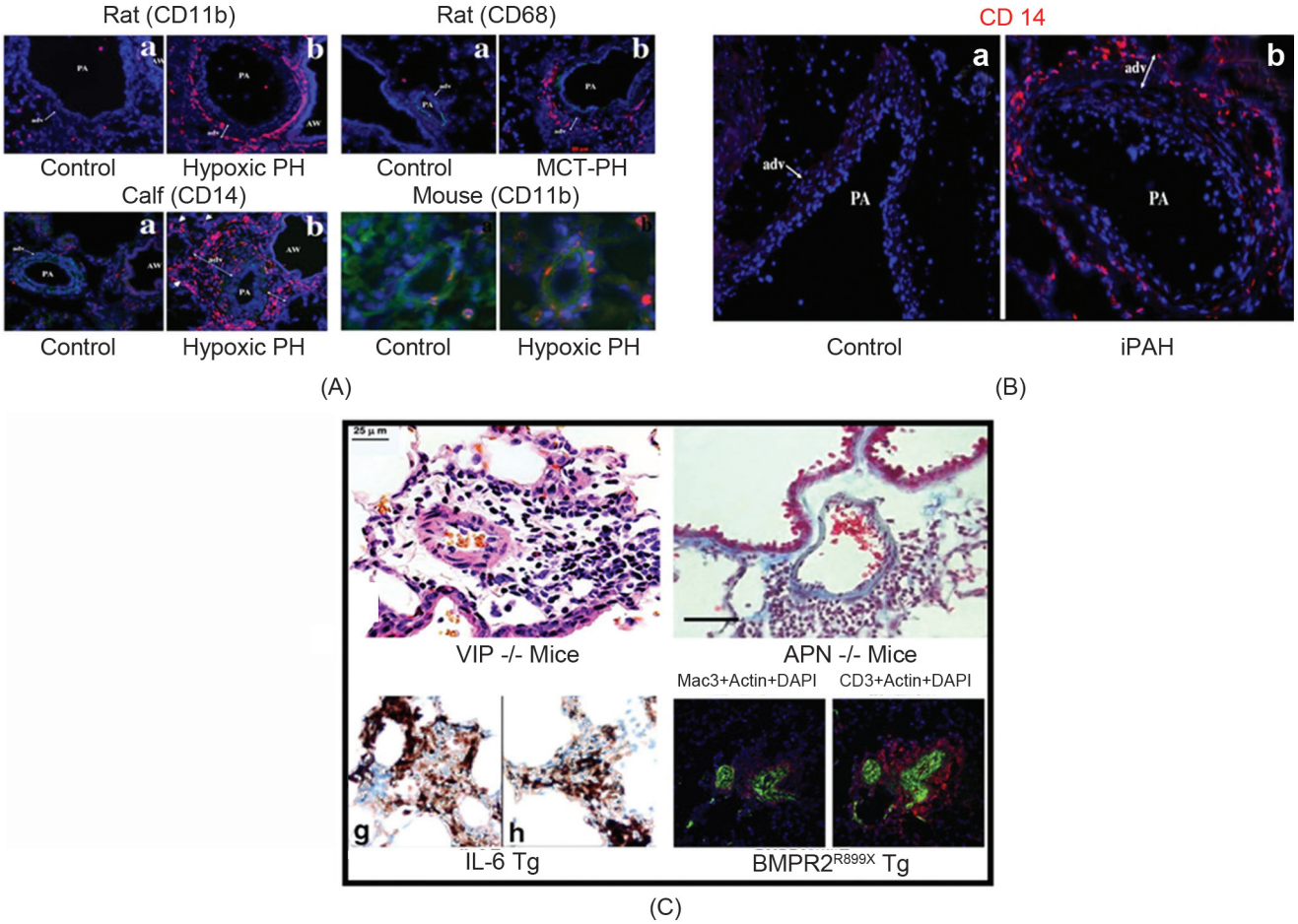

Figure 3: Animal models of pulmonary hypertension are consistently characterized by adventitial/peri-vascular accumulation of leukocytes, and in particular mononuclear cells. (A) Illustrates the accumulation of mononuclear cells/macrophages in the adventitial/ perivascular regions of chronically hypoxic rats, calves, and mice. (B) Perivascular accumulation of mononuclear cells (CD14+ cells) is observed in the human patient with iPAH. (C) Mouse models of pulmonary hypertension, including transgenic VIP−/− mice, adiponectin (APN) deficient mice, IL-6 transgenic mice, and the BMPR2 mutated mouse model all exhibit perivascular accumulation of leukocytes.[28-38]

it appears that virtually all animal models of pulmonary hypertension, including those in genetically engineered mice including BMPR2 mutations, IL-6 overexpression, Neprilysin and VIP knockouts, S100A4 overexpression, and schistosomiasis as well as human PAH are characterized by adventitial/peri-vascular inflammation (Fig. 3B and C).[34-38]

Recent studies have begun to elucidate the factors contributing to this inflammatory response. Using laser capture microdissection (LCM) in the hypoxic rat model of pulmonary hypertension, Burke et al. demonstrated a complex, time-dependent and pulmonary artery-specific upregulation of several cytokines, chemokines, their cognate receptors, as well as adhesion molecules, which appeared to be primarily produced/expressed by resident adventitial fibroblasts and macrophages and are likely involved in the initiation and perpetuation of the inflammatory response.[39] Chemotactic factors that were increased in the adventitia during the development of hypoxia-induced pulmonary hypertension included SDF-1, MCP-1, VEGF, ET-1, TGF-b, and osteopontin.[39,40] As mentioned above, all of these factors have also been shown to be upregulated in the adventitia in systemic vascular injury models. Similar findings using LCM of the pulmonary vessels in the monocrotaline models have been produced (personal communication, Scott Barman, David Fulton). Thus, there are likely numerous cytokines and chemokines that fibroblasts release in a stimulus-specific and temporal manner that create a microenvironment tailored to regulating the influx and persistence of specific leukocyte subtypes and subsequent fine-tuning of their functional phenotype. The newly recruited and activated leukocytes subsequently produce a variety of mediators such as ROS and cytokines, which in turn activate adventitial fibroblasts and/or the underlying SMC, thus augmenting and perpetuating the inflammatory response. This transition to a chronic nonresolving inflammatory tissue response requires changes in the expression repertoire of adhesion molecules, cytokines, chemokines, and cognate receptors on both fibroblasts and leukocytes. As such, fibroblasts do indeed express and upregulate adhesion molecules, including ICAM-1 and VCAM-1 that facilitate cell-adhesion of leukocytes in response to a variety of stimuli.[39,41] Moreover, secretion of cytokines, including TGF-b, by the activated fibroblast causes activation and upregulation of receptors such as CXCR4 on newlyrecruited hematopoetic cells.[42] Activated fibroblasts also secrete SDF-1, the cognate ligand for CXCR4. Thus, in chronically inflamed tissues, the fibroblast-generated stroma/adventitial microenvironment appears to serve as a "foster home" for leukocytes leading to their prolonged retention, survival, and aberrant functional activation.[14,41] As in the systemic circulation, this bidirectional signaling between fibroblasts, macrophages, and T-cells likely promotes nonresolving inflammation and remodeling.

ADVENTITIAL FIBROBLASTS AND THE VASA VASORUM

Recently, there has been great interest in vascular diseases with prominent expansion of the vasa vasorum, which occurs in many vascular diseases, prompting the hypothesis that the vasa vasorum contributes to vascular remodeling and may do so specifically by acting as a conduit for delivery of leukocytes and progenitor cells.[3,43-45] It has been shown that hypertension not only induces medial and adventitial thickening, but also significantly increases adventitial vasa vasorum density.[3,44,46,47] Additional studies have emphasized the involvement of vasa vasorum in the inflammation and progression associated with atherosclerosis.[43,44,48] Moulton et al. showed that blocking neovascularization with angiostatin reduces progression of advanced atherosclerosis.[49] There are additional data indicating that the inflammatory reactions observed in vasculitis appear to begin in the adventitia where inflammatory cells are located close to the vasa vasorum.[50]

Since chronic inflammation and fibrosis are known to be associated with angiogenic responses, the possibility exists that the vascular remodeling associated with at least some forms of pulmonary hypertension would involve angiogenic responses in or around the vessel wall. In the systemic circulation, the adventitial vasa vasorum undergoes marked neovascularization in a number of vasculopathies, including atherosclerosis, type II diabetes, metabolic syndrome, restenosis, and vasculitis.[44,49,51-54] This neovascularization is thought to play a direct role in the remodeling process.[44] In the setting of pulmonary hypertension, expansion of the vasa vasorum network in the adventitia and media has also been described.[40,55,56] In fact, expansion of the pulmonary artery vasa vasorum is commonly observed in the setting of pulmonary artery obstruction.[56] For instance, Kimura et al. reported that in patients with chronic thromboembolic obstruction of the pulmonary arteries, the volume of pulmonary adventitia vasa vasorum increases and the core of the nonresolving clots is recannulized by neovascular endothelialized structures that originate from the vasa vasorum.[57] Marked increases in the density of vasa vasorum have also been reported in idiopathic PAH (Fig. 4).[58] Increased density of capillaries in the adventitial and peri-adventitial regions of pulmonary arteries in patients with severe idiopathic pulmonary fibrosis and pulmonary hypertension has also been described.[59] In an animal model (i.e., the hypoxic neonatal calf) of severe hypoxia-induced pulmonary hypertension, marked expansion of the vasa vasorum network in the adventitia and within the outer aspects of the media of vessels all along the longitudinal axis of the pulmonary circulation has been described (Fig. 4).[40]

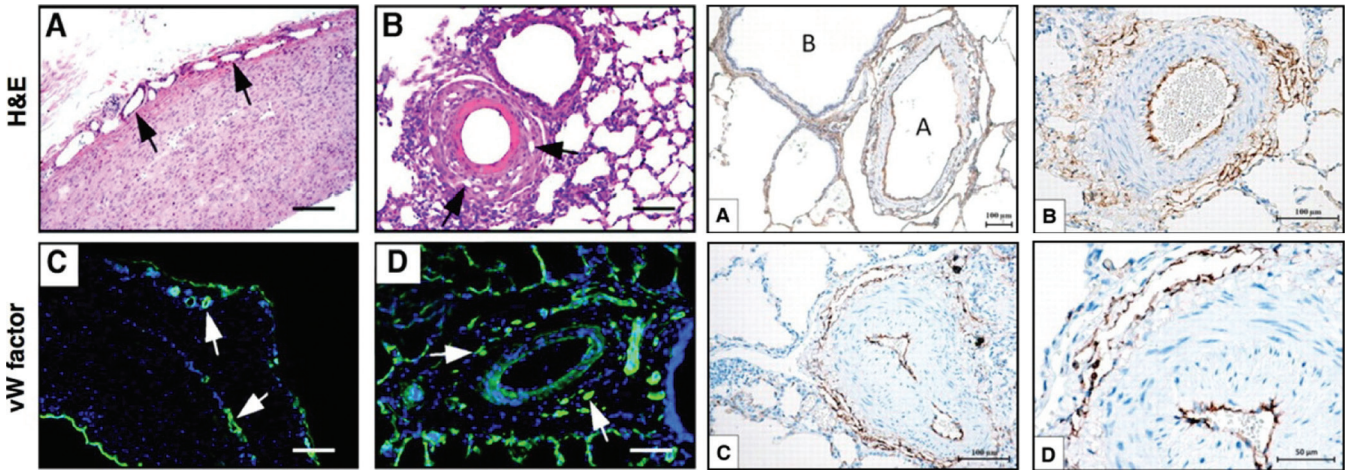

Figure 4: Vasa vasorum expansion in the hypoxic calf model of pulmonary hypertension and in iPAH. (A) In both large (panels A and C) and small pulmonary arteries (panels B and D) of the chronically hypoxic calf, marked expansion of the vasa vasorum is observed. Panels A and B demonstrate H and E staining. Panels C and D demonstrate von Willebrand Factor expression. (B) In pulmonary arterial lesions of patients with iPAH a large expansion of CD34+ vasa vasorum vessels around the remodeled pulmonary arteries is observed (panels B, C, and D). Rare CD 34+ vessels around pulmonary arteries of control subjects are observed (panel A).[40,58]

The mechanisms controlling expansion of the vasa vasorum network in the pulmonary and systemic circulations are not well understood. However, it is increasingly appreciated that activation of fibroblasts must play a critical role since stromal fibroblasts have been clearly implicated in the angiogenesis that accompanies tumor progression in cancers of epithelial origin and in chronic inflammatory diseases such as rheumatoid arthritis.[60-64] As noted, fibroblasts are capable of producing many factors involved in angiogenic responses, including VEGF, PDGF, ET-1, and TGF-b. Indeed, several angiogenic factors are observed in the hypoxic adventitia, including VEGF, fibronectin, thrombin, ET-1, and S100A4.[39,40] Davie et al. carried out experiments to determine if fibroblasts were involved in the expansion of the vasa vasorum seen in pulmonary hypertensive vessels. Using both co-culture and conditioned media approaches, they found that adventitial fibroblasts, especially those from pulmonary hypertensive animals, were capable of stimulating vasa vasorum endothelial cell (VVEC) proliferation.[55] Furthermore, conditioned media from adventitial fibroblasts was capable of augmenting both the self-assembly and the integrity of cord-like networks formed by VVEC in Matrigel. Exposure to hypoxia (3% O_2) augmented all these responses. Interestingly, all these molecules have been found to be upregulated in the pulmonary arteries of human patients with various forms of PAH.[32,65] In addition, studies in the systemic circulation have also suggested that ET-1, a factor well known to be upregulated in pulmonary hypertension, plays a critical role in coronary vasa vasorum neovascularization in the setting of experimental hypercholesterolemia through local upregulation of VEGF.[66,67] These observations are in accordance with a number of previous studies in which hypoxic conditions have been shown to induce angiogenic phenotypes in a number of stromal cell types.[68-70] Moreover, these observations are consistent with the well-established paradigm that hypoxia is a common feature of many pathological conditions associated with neovascular growth. Importantly, previous studies in our laboratory have established that adventitial fibroblasts exhibit the earliest and most dramatic activation responses among all cells in the vessel wall.[71] These data are consistent with the emerging concept that the endothelium of *de novo* forming microvessels receives and integrates pro-angiogenic signals from a number of nonendothelial cells, including fibroblasts.[52,72-75]

Fibroblasts, cultured on ECM proteins, have been shown to secrete cytokines and pro-angiogenic growth factors that regulate the formation of capillary-like networks by human umbilical vein endothelial cells and systemically derived microvascular endothelial cells. [52,73,76,77] Other studies have shown that stromal cells, including fibroblast-like cells, not only provide initial stimuli for the angiogenic cascade but also provide a stabilizin g force to newlyformed vessels. [52,72-77] Tissue fibroblasts have also been described to exhibit pro-angiogenic capabilities at sites of wound healing and inflammation. These cells respond to chemotactic cytokines released in the tissue environment and are frequently the first cell type to migrate to the wound site where they orchestrate reparative neovascularization. [72] Thus, activated adventitial fibroblasts may regulate angiogenic responses of the resident endothelial cells in the adventitia and stimulate a process of neovascular growth, be it normal or disordered. It is now appreciated that this vascular network can serve as a conduit for continued delivery of leukocytes and progenitor cells to the vessel wall. Thus, inhibiting or turning off fibroblast-produced

pro-angiogenic factors may be beneficial in certain inflammatory vascular diseases (Fig. 2).

EPIGENETIC CONTROL OF THE ACTIVATED FIBROBLAST PHENOTYPE: POTENTIAL FOR NEW THERAPY

As noted, there is good evidence that adventitial fibroblasts in the pulmonary hypertensive vessel wall exhibit a hyperproliferative, inflammatory, and invasive phenotype. Questions arise as to origins and mechanisms regulating this phenotype. Intriguingly, this phenotype resembles, in certain ways, the phenotypic characteristics of rheumatoid arthritis (RA) synovial fibroblasts (RASFs), cancer-associated fibroblasts and fibroblasts derived from the fibrotic lung, kidney and liver. It has been demonstrated that synovial fibroblasts (SF), perhaps more than other types of fibroblasts, acquire phenotypic characteristics commonly associated with transformed cells.[78,79]

RASFs show "spontaneous" or "constitutive" activities associated with aggressive behavior and they differ from SFs of patients with osteoarthritis or normal SFs. For example, RASFs upregulate proto-oncogenes, matrix-specific degrading enzymes (MMPs), adhesion molecules, and cytokines, thus exhibiting a distinct "imprinted" phenotype which is stable over many passages in culture.[8,79-82]

Similarly, primary fibroblasts isolated from fibrotic kidneys maintain their "activated" pro-fibrotic state even when cultured in vitro.[83] Additionally, there are convincing data that demonstrate stable phenotypic differences in fibroblasts obtained from the lungs of patients with idiopathic pulmonary fibrosis (IPF). IPF fibroblasts are more resistant to apoptosis compared to fibroblasts isolated from nonfibrotic tissues.[84] Fibroblasts isolated from the lungs of IPF patients also have documented increases in the expression of IL-13 receptor subunits.[85] Another pathway in which phenotypic differences in receptor expression have been reported includes the CCL2:CCR2 pathway. Fibroblasts isolated from sites of scleroderma, including the lung, have increased CCR2 expression.[86] It has also been demonstrated that IPF derived fibroblasts are hyper-responsive to cytokines, including TGFb, IL-13 and CCL2.[87] Consistent with these observations is work from our laboratory, which demonstrates that hypoxia-induced pulmonary vascular remodeling is characterized by the emergence of a distinct adventitial fibroblast population that exhibits a constitutively activated, or "imprinted," pro-inflammatory phenotype that is capable of inducing recruitment, retention and pro-inflammatory activation of monocytes/macrophages (Fig. 5).[41] Importantly, in the absence of any exogenous stimulation, these constitutively-activated pro-inflammatory fibroblasts are equipped to generate a microenvironment characterized by high expression levels of pro-inflammatory cytokines such as IL-1beta and IL-6; macrophage chemo-attractant cytokines such as (CCL2/MCP1), CXCL12, SDF1, and CCL5 (RANTES), macrophage growth and activation factor GM-CSF, co-simulatory molecules capable of activating macrophages such as CD40L, as well as the adhesion molecule VCAM-1. Smooth muscle cells isolated from the same arteries of hypertensive animals exhibited either no or a far lesser degree of activation of all the aforementioned molecules and mediators.[41]

The acquisition of stable, functional phenotypic changes in mesenchymal cells, such as the fibroblast described in the aforementioned conditions, probably requires epigenetic processes such as might occur in response to altered histone actelytation, DNA methylation and/or changes in micro-RNA expression profiles.[88,89] Histone-dependent packaging of genomic DNA into chromatin is a central mechanism for gene regulation. Expression of inflammatory genes, DNA repair genes and proliferation genes is controlled by the degree of acetylation of histone and nonhistone proteins produced by histoneacetyltransferase (HAT) and histone deacetylase (HDACs).[90-92] Several reports have documented such changes in HDAC activity in fibroblasts in rheumatoid arthritis (RA) and juvenile idiopathic arthritis, with recent reports demonstrating specific increases in HDAC-1 activity.[92,93] Additional reports have demonstrated anti-inflammatory effects of small molecule HDAC inhibitors in animal models of inflammatory diseases, fibrotic vascular disease and in cancer.[94,95] We recently reported that adventitial fibroblasts isolated from severely hypertensive, chronically hypoxic calves (described above) exhibited significantly elevated catalytic activity of HDACs, specifically Class 1 HDAC 1-3, which primarily localized to the nuclei and were linked to epigenetics through their ability to efficiently deacetylate nucleosomal histones.[41] Most importantly, we found that specific catalytic inhibition of Class 1 HDACs was sufficient to suppress production of the constitutivelyexpressed pro-inflammatory mediators expressed by activated fibroblasts.[41] These data 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 pro-inflammatory phenotype of these adventitial fibroblasts. These findings are consistent with those of Kawabata et al. in RA with regard to specific increases in Class 1 HDAC activity and protein expression (Fig. 6).[92]

HDAC inhibitors have been shown to exert antiinflammatory effects both in vitro and in vivo in various inflammatory diseases, including RA, systemic lupus erythematosus, asthma, inflammatory lung disease, atherosclerosis, hemorrhage shock, diabetes, inflammatory bowel disease,

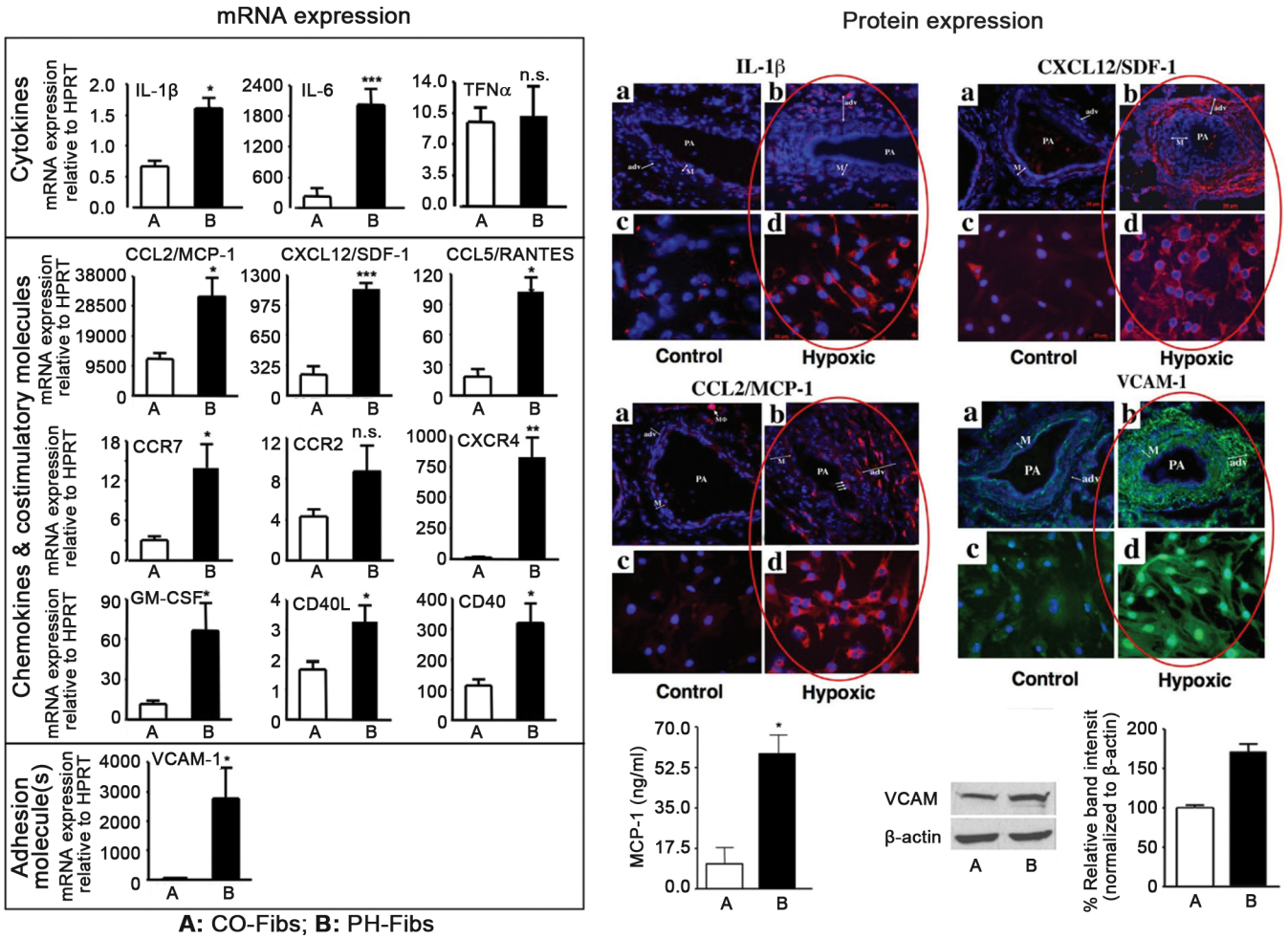

Figure 5: Fibroblasts from the chronically hypoxic pulmonary artery express an "imprinted" pro-inflammatory phenotype. Fibroblasts derived from control and hypoxic pulmonary hypertensive calves were cultured and in the absence of any exogenous stimuli, the fibroblast from pulmonary hypertensive animals (PH-Fibs) exhibited a constitutively activated "imprinted" phenotype characterized by overexpression of cytokines, chemokines and adhesion molecules. This phenotype was confirmed at the mRNA level and at the protein level both in vivo and in vitro. At the protein level IL-1β, SDF-1, MCP-1, and VECAM are dramatically upregulated both in vivo and maintain this phenotype in vitro. Confirmation of MCP-1 and VECAM are shown in the bar graphs below.[41]

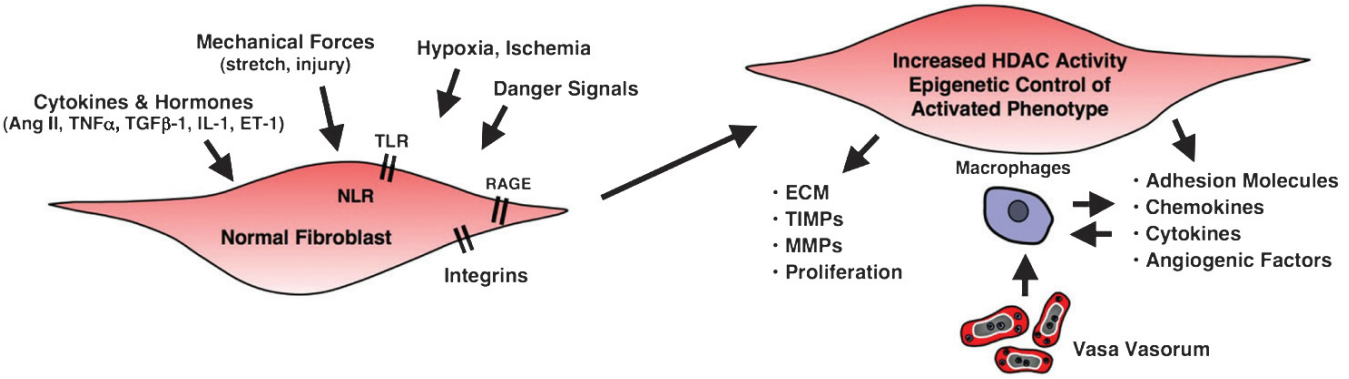

Figure 6: The constitutively activated "imprinted" phenotype of fibroblasts is due, at least in part, to increased HDAC activity.

osteoporosis, and macular degeneration.[96] In vascular disease models, recent publications have demonstrated that HDAC inhibition can decrease neointima formation and decrease inflammation.[94,97] Collectively, these results imply unforeseen potential for Class 1 HDAC-selective small molecule inhibitors for the treatment of pathologic 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 1 HDACs (Fig. 7).[95]

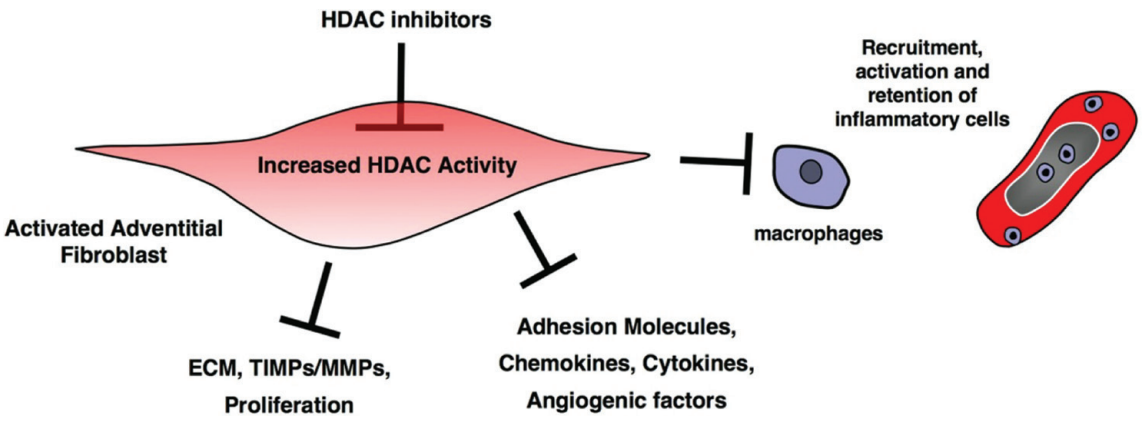

Figure 7: HDAC inhibitors attenuate the pro-liferative and -inflammatory phenotype exhibited by the constitutively activated fibroblast from pulmonary hypertensive animals.

Another highly important mechanism through which cells become epigenetically altered is through DNA methylation changes. DNA methylation refers to the covalent attachment of a methyl group to the C5 position of cytosine residues in CpG dinucleotide sequences that are called CpG islands. DNA islands are often in the promoter or enhancer regions of genes and methylation of these sites can alter transcription. DNA methylation is involved in normal cellular control of gene expression and is dynamically regulated. However, changes in DNA methylation are also relevant to disease and may be of particular relevance to the changes in fibroblast phenotype that are observed in chronic fibrotic disorders. Five-methylcytosine DNA levels are reduced in RASF tissues and in cultured RASFs.[79,98] Specifically, the promoter of an L1 element was partially demethylated, confirming a global genomic hypomethylation in RASFs. It was proposed that the hyper-aggressive and pro-inflammatory phenotype of RASFs was the result of a progressive loss of methylation marks and tissue-specific transcription factors, which are not normally expressed, are upregulated and are responsible for activation of many genes involved in the pathogenesis of rheumatoid arthritis. This concept was confirmed in experiments where 5-azaC (α DNA-hypomethylator) treatment of normal SFs lead to a phenotype identical to RASFs. Over 186 genes were upregulated in 5-azaC treated cells by greater than 2-fold, including growth factors and growth factor receptors, extracellular matrix proteins, adhesion molecules and matrix degrading enzymes. Furthermore, hypomethylation of certain receptors, specifically the death receptor, could explain the relative resistance to apoptosis, which has been reported in RASFs in certain patients.[79,99] Additional data suggest that global genomic hypomethylation can be accompanied or followed by specific promoter hypermethylation.[100]

There is growing evidence for abnormalities in DNA methylation in fibroblasts in other chronic fibrotic diseases, such as are observed in the lung and kidney.[101,102] A recent study demonstrated epigenetic silencing of Thy-1 by DNA hypermethylation specifically within fibroblast foci in patients with IPF, suggesting that this may be an important mechanism for pathogenetic fibroblast alterations since absence of Thy-1 correlates with a pro-fibrotic phenotype.[103] Importantly, treatment with DNA methyltransferase restored Thy-1 expression in Thy-1-negative fibroblasts. Interestingly, the adventitial fibroblasts from hypertensive calves described above are Thy-1-negative while adventitial fibroblasts from healthy control calves are Thy-1-positive. Furthermore, a recent study showed that hypermethylation of RASALI, encoding an inhibitor or the Ras oncoprotein, is associated with the perpetuation of fibroblast activation and fibrogenesis in the kidney,[83] and that kidney fibrosis is ameliorated in mice heterozygous for DNA (cytosine-5)-methyltransferase 1 (Dnmt1). Very intriguing data came from a recent study using lung fibroblasts that convincingly indicated that alterations of histone modifications alter DNA methylation.[104] Sanders et al. showed that treatment with the HDAC inhibitor trichostatin (TSA) restored Thy-1 expression in Thy-1-negative cells in a time and concentration-dependent fashion and was associated with enrichment of histone acetylation. Bisulfite sequencing of the Thy-1 promoter region revealed demethylation of the previously hypermethylated CpG site in response to treatment with TSA.[104] TSA treatment also upregulated total methyltransferase activity in these cells. The experimental observation that treatment with an HDAC inhibitor can restore Thy-1 (a proposed "fibrosis-suppressor" gene) expression in fibroblasts in fibrotic disease and change the phenotype (it decreased a-SM-actin expression) suggests that HDACs could be used as a therapeutic target for the treatment of fibrotic diseases such as IPF with or without pulmonary hypertension.

Because of the well-documented antiproliferative and anti-inflammatory properties of Class I HDACs in many cell types, including vascular wall and cardiac cells, we tested the effects of specific Class I HDAC inhibitors in a hypoxic model of PH. We found that two Class I HDAC inhibitors, MGCD0103 and MS-275, reduced hypoxia-mediated PH in rats in a manner

that correlated with suppression of medial thickening of pulmonary arteries and inhibition of SMC proliferation in these vessels.[105] Reduced SMC proliferation upon Class I HDAC inhibition was due, in part, to upregulation of the antiproliferative transcription factor, FoxO3a. Importantly, we also demonstrated that RV function was maintained in the face of Class I HDAC inhibition, and that indices of adverse ventricular remodeling (e.g., myocyte apoptosis and inflammation) were blunted by selective inhibition of Class I HDACs. This is in contrast to what was previously observed with the pan-HDAC inhibitor, trichostatin A (TSA) in a pulmonary artery banding model, and supports the hypothesis that isoform-selective HDAC inhibition will be safer than general HDAC inhibition in the setting of RV pressure overload. Both MGCD0103 (Mocetinostat) and MS-275 (Entinostat) are in clinical development for cancer and are well tolerated by humans, thus highlighting the translational potential of the present findings.

Two other reports have addressed effects of HDAC inhibitors in models of PH and RV remodeling. Valproic acid was shown to block RV cardiac hypertrophy in response to pulmonary artery banding (PAB) as well as in the setting of pulmonary hypertension caused by monocrotaline-induced lung injury.[106] In contrast, TSA failed to block hypertrophy in response to PAB, and actually appeared to worsen RV function.[107]

TSA is a potent, pan-HDAC inhibitor.[108] The deleterious effects of this compound on the RV (e.g., decreased cardiac output, increased RV dilatation and apoptosis) could be a reflection of a protective role for HDAC(s) in this chamber of the heart. It is interesting to note that valproic acid, which exhibits selectivity for Class I HDACs, did not cause adverse effects in the PAB model.[107] Our findings suggested that, with regard to the PH and RV, isoform-selective HDAC inhibition was safer than nonselective suppression of HDAC activity. This was evidenced by the ability of MGCD0103 to block RV apoptosis and inflammation and maintain RV contractile function in chronically hypoxic rats.[105] Nonetheless, it should be noted that the model used for our studies (3 weeks of hypoxia in SD rats) was mild with regard to RV remodeling, and represented a model of PH caused by interstitial lung disease and/or hypoxemia (World Health Organization [WHO] Group III PH). It will be important to extend the current findings to more severe models of PH and RV dysfunction, such as the SUGEN plus hypoxia model, to determine whether the beneficial effects of Class I HDAC inhibitors are generalizable to other forms of PH, including WHO Group I iPAH.

SUMMARY

It is clear that adventitial cells, and in particular the adventitial fibroblast, are activated early following vascular injury and play essential roles in regulating vascular wall structure and function through production of chemokines, cytokines, growth factors and ROS. The recognition of their ability to generate and maintain inflammatory responses within the vessel wall provides insight into why vascular inflammatory responses, in certain situations, fail to resolve. It is also clear that the activated adventitial fibroblast plays an important role in regulating vasa vasorum growth, which can contribute to ongoing vascular remodeling by acting as a conduit for delivery of inflammatory and progenitor cells. These functions of the fibroblast clearly support the idea that targeting chemokine, cytokine, adhesion molecule, and growth factor production in activated fibroblasts could be beneficial in abrogating vascular inflammatory responses and thus in ameliorating vascular disease. Further, recent observations that fibroblasts in vascular and fibrotic diseases may maintain their activated state through epigenetic alterations in key inflammatory and pro-fibrotic genes suggests that current therapies used to treat pulmonary hypertension may not be sufficient to induce apoptosis or to inhibit inflammatory signaling pathways in these cells. New therapies targeted at reversing changes in the acetylation or methylation status of key transcriptional networks may be needed. At present, therapies specifically targeting abnormalities of HDAC activity in fibroblasts appear to hold promise.

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