Lung Vascular Cell Heterogeneity
Endothelium, Smooth Muscle, and Fibroblasts

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The pulmonary circulation represents a unique vascular bed, receiving 100% of the cardiac output while maintaining low blood pressure. Multiple different cell types, including endothelium, smooth muscle, and fibroblasts, contribute to normal vascular function, and to the vascular response to injury. Our understanding of the basic cell biology of these various cell types, and the roles they play in vascular homeostasis and disease, remains quite limited despite several decades of study. Recent advances in approaches that enable the mapping of cell origin and the study of the molecular basis of structure and function have resulted in a rapid accumulation of new information that is essential to vascular biology. A recent National Institutes of Health workshop was held to discuss emerging concepts in lung vascular biology. The findings of this workshop are summarized in this article.

Keywords: pulmonary circulation; progenitor cells; proliferation; apoptosis; permeability

Significant strides have been made in the past 10 years by pulmonary vascular biologists. In recognition of this progress, and in recognition of the need to reevaluate new frontiers of research in vascular biology, the National Institutes of Health convened a workshop to evaluate lung cell heterogeneity. A range of lung vascular biologists discussed recent findings, and they posed the following questions and challenges to our research community: How many lung vascular cells exist? How many phenotypes exist within any specific cell population? What are the molecular cues that govern cell phenotype and memory? Can we map cell phenotypes, either *in vitro* or *in vivo*? How is a "disease" cell phenotype generated? The following article addresses these issues in lung endothelium, smooth muscle, and fibroblast cell types.

ENDOTHELIAL CELLS

Although endothelium has historically been considered a homogeneous cell layer, there is increasing appreciation that it exhibits a rich diversity in structure and function (1–3). Heterogeneity is apparent between endothelial cells in different organs, in endothelial cells along a single vascular segment within an organ, and in those between immediately adjacent cells. Endothelial cells along the arterial–capillary–venous axis display remarkable anatomic distinctions (4, 5). As an example, pulmonary artery endothelial cells reside on a thick basement membrane that separates the intima from underlying smooth muscle layers, whereas only a thin basement membrane separates capillary endothelial cells from interacting with nearby type I pneumonocytes. Pulmonary artery endothelia interact with as many as six adjacent endothelial cells, whereas capillary endothelial cells interact with just one neighbor. Although the former cells are aligned in the direction of blood flow, capillary endothelium exhibits no such flow alignment. Although these (and other) anatomic distinctions have been recognized for a number of years, the mechanisms responsible for maintaining such heterogeneity, and the functional consequences of heterogeneity have largely remained an enigma.

Infusion of fluorescently labeled lectins into the pulmonary circulation reveals a distinct border at approximately 25 μm, where pulmonary artery endothelial cells change their phenotype and become microvascular (capillary) endothelial cells (6). *Helix pomatia* lectin interacts prominently with pulmonary artery endothelial cells, whereas *Griffonia simplicifolia* primarily interacts with pulmonary microvascular endothelial cells. Functional studies in the intact circulation have demonstrated that capillary endothelial cells possess a highly resistant barrier function when compared with pulmonary artery and vein endothelial cells (7–9). Moreover, inflammatory agonists discretely target arterial, capillary, and venous endothelium to increase permeability, resulting in site-specific edema formation. A striking example of this heterogeneity is seen using two discrete calcium agonists. Thapsigargin is a plant alkaloid that directly activates calcium entry through store-operated calcium entry channels. Application of thapsigargin to the intact pulmonary circulation increases endothelial cell permeability (10, 11). However, the increase in permeability is due to interendothelial cell gap formation only in extraalveolar arterial and venous endothelial cells (12). In contrast, 4 alpha-phorbol 12, 13-didecanoate (4αPDD) is a phorbol ester that directly activates the transient receptor potential 4 channel in the vanilloid family of ion channels (TRPV4). Application of 4αPDD to the intact pulmonary circulation increases endothelial cell permeability (13). The increase in permeability is due, at least in part, to loss of cell–matrix adhesion in capillary endothelial cells, and is not due to gap formation in extraalveolar endothelial cells. Thus, whereas thapsigargin increases extraalveolar endothelial cell permeability, 4αPDD increases capillary endothelial cell permeability (14).

The discrete nature of extraalveolar and alveolar vascular compartments can be further divulged using animal models of heart failure (15). Chronic heart failure leads to hydrostatic edema. However, these animals fail to respond to thapsigargin with an increase in permeability, and the store-operated calcium entry channels that are responsible for increasing extraalveolar permeability are not expressed at normal levels (i.e., down-regulated). In contrast, the 4αPDD-induced increase in permeability is retained in animals with heart failure, and the TRPV4

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channel that is responsible for increasing alveolar permeability is expressed at normal levels.

These findings exemplify how little we know about the cellular and molecular events that govern site-specific endothelial cell function, and highlight the importance of gaining a better understanding of how phenotypically distinct endothelial cell populations control the local physiology, and how regionally restricted environmental factors impact on the phenotype of endothelial cells within a given vascular location. As an example, thapsigargin induces the accumulation of large fluid cuffs around extraalveolar vessels, reminiscent of what has been reported in heart failure, high-altitude pulmonary edema, acute lung injury (which also displays alveolar edema), and asthma. Although we have known for some time that fluid collects in these vascular cuffs, the impact of such cuffs on lung mechanics has not been thoroughly studied. Recent findings indicate that perivascular cuffs decrease lung compliance, and negatively impact the functional coupling of bronchioles to lung parenchyma (14). These new findings indicate that factors other than surfactant inactivation decrease lung compliance, and suggest that edema accumulation can decrease lung compliance without concomitant hypoxemia. In this scenario, extraalveolar endothelium, and not capillary endothelium, represents a suitable clinical target; improving extraalveolar barrier integrity would decrease permeability, potentially eradicate cuff formation, and improve lung compliance. Although endothelium is an increasingly important target cell type for therapeutics, we have, to this point, not accepted the importance or impact of phenotypic heterogeneity on therapeutic targeting.

Both pulmonary artery and microvascular endothelial cells can be isolated from the intact circulation, and cultured *in vitro*. Even in *in vitro* studies, pulmonary microvascular endothelial cells exhibit a more resistant barrier function than do pulmonary artery endothelial cells, and they uniquely respond to different inflammatory agonists with an increase in permeability, just as in the intact circulation (16, 17) (*see* Table 1). These collective findings have led to an appreciation of the stable, distinct nature of extraalveolar and alveolar endothelial cell phenotypes, and have challenged the idea that environmental pressure is the only important determinant of phenotype specification. Indeed, interactions between cell specification and environmental pressure determine cell phenotype (1, 3).

Although there is an increasing appreciation for the functional diversity of endothelium along the arterial–capillary–venous axis, heterogeneity exists within a cell population, and between adjacent cells as well. The basis for such interendothelial cell heterogeneity is poorly understood, and has partly been ascribed to environmental factors, such as production of autocrine and paracrine factors, and mechanical forces, such as shear stress and pressure (1, 3). In addition, pacemaker cells have been described within cell populations, in which a leading cell entrains adjacent population members to cyclically shift membrane potential or ion concentrations, as in calcium oscillations (18, 19). Such pacemaker activity has been described both *in vitro* and *in vivo*. The presence of pacemaker cells within a population suggests the presence of some intrinsic, imprinted intercellular heterogeneity.

Recently, Ingram and colleagues (20, 21) have addressed this possibility by adapting a single-cell cloning approach commonly used in the hematology field and applying it to the study of endothelium. In these studies, endothelial cells are seeded at single-cell density, and growth is observed over a 2-week time course. Most aortic and human umbilical cord endothelial cells that are seeded in the single-cell clonogenic assay do not divide (≈75%), and are considered to be differentiated endothelium. A smaller proportion (≈25%) divide, but to different degrees, most often growing to no more than 500–2,000 cells. Few single cells (<3%) grow to more than 10,000 cells. Interestingly, when these highly proliferative cells are reseeded at the single-cell density, they repopulate the entire hierarchy of growth potentials. The slower growing cells, in contrast, cannot repopulate the entire hierarchy of growth potentials. Thus, highly proliferative potential cells fulfill the criterion for "progenitor" cells, because they divide at high rates and are able to renew the entire cell population. Moreover, these progenitor cells are angio-vasculogenic, and thus fulfill an important, defining endothelial attribute.

TABLE 1. SUMMARY OF THE PRINCIPAL ENDOTHELIAL CELL ATTRIBUTES

	Pulmonary Artery Endothelium*	Pulmonary Microvascular Endothelium*
Endothelial cell "markers"		
eNOS	√	√
PECAM-1	√	√
VE-cadherin	√	√
LDL uptake	√	√
Lectin-binding criterion		
Helix pomatia	√	—
Griffonia simplicifolia	—	√
Glycine max	—	√
Adherens junction protein expression		
VE-cadherin	√	√
E-cadherin	—	√
Relative barrier strength	+	+++
Permeability response to agonists (normal subjects)		
Thapsigargin	√	—
4αPDD	—	√
Permeability response to agonists (heart failure)		
Thapsigargin	—	—
4αPDD	—	√
Relative number of progenitors within the cell population	+	+++
Relative angiogenic potential	+	+++

Dashes indicate negligible interaction; *plus signs* indicate positive interaction.

Definition of abbreviations: eNOS = endothelial nitric oxide synthase; E-cadherin = epithelial cadherin; LDL = low-density lipoprotein; PECAM = platelet-endothelial cell adhesion molecule; VE-cadherin = vascular endothelial cadherin.

* Although both pulmonary artery endothelial cells and pulmonary microvascular endothelial cells express classical markers of endothelium, they differ in their lectin-binding criteria, and in critical functions, such as barrier function and angiogenic potential.

Not all endothelial cell populations possess the same number of high-proliferating progenitors. Pulmonary artery and microvascular endothelial cell growth potentials were assessed using the single-cell approach (22). Remarkably, nearly 50% of the individual microvascular endothelial cells could expand into large cell colonies, whereas only approximately 3% of pulmonary artery endothelial cells exhibited such profound proliferative capacity (22). Reseeding the large colonies in the single-cell assay revealed that the high proliferative potential cells repopulate the entire hierarchy of endothelial cell growth potentials, consistent with the idea that the high proliferative potential cells are progenitor cells. Perhaps most interesting, pulmonary artery– and microvascular-derived progenitor cells retain both endothelial-specific and segment-specific attributes, based on functional assays and surface antigen expression. These findings therefore suggest that the microcirculation is enriched with progenitor cells that are phenotypically related to their vascular origin.

It is important to identify a molecular basis for the high proliferative potential of lung microvascular endothelium. Global expression profiling resolved higher expression of nucleosome

assembly protein (NAP)-1 in microvascular endothelial cells, when compared with pulmonary artery endothelial cells. NAP-1, and the related NAP-1 family, has recently been shown to control proliferation, most notably in yeast, of *Xenopus*, and *Arabidopsis thaliana* (23–30). Pulmonary microvascular endothelial cells express more NAP-1 than do pulmonary artery endothelial cells (31). Overexpressing NAP-1 in pulmonary artery endothelial cells increases their growth, and decreasing NAP-1 expression in pulmonary microvascular endothelial cells decreases their growth. Moreover, whereas microvascular endothelial cells form more blood vessels in *in vivo* Matrigel vasculogenesis assays than do pulmonary artery endothelial cells, overexpressing NAP-1 in the conduit-derived cells normalizes this vasculogenic potential. Thus, there is considerable cellular diversity within extraalveolar and alveolar endothelial cell populations. Pulmonary microvascular endothelial cells, in particular, are enriched in progenitors, and NAP-1 contributes to the pro-proliferative and vasculogenic phenotype of these cells.

The study of endothelial progenitor cells has been exciting, and may offer new insights into our understanding of vascular development, homeostasis, and repair after injury. It is not presently clear whether such rapidly growing cells contribute to vascular disease, as in the plexiform lesion in pulmonary hypertension. Recent studies from Xu and colleagues and Masri and associates (32–34) have illustrated that the endothelium of patients with idiopathic pulmonary hypertension displays hyperproliferative, apoptosis-resistant growth characteristics. Interestingly, these cell features are retained in culture. It is unclear whether this vascular disease originates from genetic (i.e., somatic mutation) and epigenetic causes, or whether vascular disease selects for a resident cell type, such as an endothelial progenitor cell (35).

Although it is exciting to consider that resident progenitor cells exist within populations of endothelium, we still know very little about these cells. How do we identify them *in vivo*? What is their relationship to pacemaker cells? What is their origin, and how do they relate to stem cells and progenitor cells found in the bone marrow and elsewhere? What is their level of differentiation? How do they retain a memory? What is their role in vascular development, homeostasis, and repair after vascular injury? What role do these cells play in vascular disease? Clearly, we have only scratched the surface; the next 10 years stand to redefine our understanding of endothelial cell biology.

SMOOTH MUSCLE CELLS AND ADVENTITIAL FIBROBLASTS

Our definition and functional classification of smooth muscle cells (SMCs) and fibroblasts have changed significantly over the past two decades as a result of extensive *in vivo* and *ex vivo* experimental findings. No longer can either of these cell types be considered as a single distinct entity but rather both are now known to comprise a diverse group of cell phenotypes, each sharing major common characteristics but concurrently displaying a spectrum of biochemical and functional differences. Thus, several important issues need to be considered when defining the phenotype of SMCs or fibroblasts and in assessing their roles in both the normal and diseased pulmonary circulation.

SMCs

One of the primary goals of this workshop was to identify molecular "markers" useful in defining SMCs. It has become readily apparent that α-smooth muscle actin can no longer serve as a reliable smooth muscle (SM)–specific marker, because a variety of other cell types can express it either under normal or pathologic conditions. Similarly, the intermediate filament desmin, another marker which has been used to "detect" SMCs, has clearly been found to be expressed by some, yet not all, vascular SMCs. Current experimental evidence would suggest that SM–myosin heavy chain (SM-MHC) is, thus far, one of the most reliable markers capable of distinguishing between SMCs and at least cells of a fibroblast phenotype. Several other SM-related proteins, such as alternatively spliced variants of SM-MHC (SM-2, SM-B isoforms), metavinculin, high-molecular-weight caldesmon, can help to define a more "differentiated" SM phenotype. Smoothelin has also been suggested to be a marker expressed by differentiated SMCs and not by "synthetic" or dedifferentiated SMCs or myofibroblasts (36, 37). Unfortunately, few studies have addressed the relationship between the expression patterns of these proteins to specific SMC functions (e.g., contractility). Therefore, the precise relationship between expression of proteins believed to identify the SMCs and function remains unclear. It may be that panels of markers should be applied to characterize SMCs *in vivo* as well as when the cells are used in *ex vivo* studies. Definition of the exact source of the cells (i.e., vascular location from which they are isolated) is important because it may determine cell phenotype (*see below*), as is a description of the methods through which cells were expanded in culture (e.g., different growth media have different effects on SMC protein expression and function). These considerations are important in light of the fact that so little is known regarding the origin of SMCs in the pulmonary circulation and that many different SMC subpopulations with different origins and different functional capabilities exist.

Vascular SMCs, which were previously believed to be nearly uniform in nature and distributed seamlessly throughout vascular beds, are now increasingly appreciated as being highly mosaic in nature, because many distinct SMC subtypes can be identified within a common arterial tree. Excellent studies based on fate-mapping techniques have demonstrated the existence of numerous vascular SMC subtypes, each with different embryonic origins and each exhibiting distinct phenotypic and functional characteristics (38–40). Origins of vascular SMCs include the neural crest, proepicardium, mesothelium, secondary heart field, somites, mesangioblasts, and stem/progenitor cells (38). The boundaries between SMCs of different origins within a vessel are often remarkably sharp with little or no intermixing. Moreover, SMCs from different embryonic origins respond in lineage-specific ways to common stimuli, even when tested under identical conditions *in vivo* or *in vitro* (40, 41).

The vast majority of studies evaluating SMC diversity and its potential importance in vascular disease have been in the systemic circulation. SMC lineage maps were used to isolate neural crest–derived SMCs from the aortic arch and mesoderm-derived SMCs from the abdominal aorta of chick embryos. When compared under identical conditions *in vitro*, these two distinct types of aortic SMCs exhibited striking differences in growth and transcriptional responses to transforming growth factor (TGF)-β1 (40, 41). DNA synthesis and cell proliferation were increased by TGF-β1 in neural crest–derived SMCs, whereas mesoderm-derived SMCs isolated from the same vessels were growth inhibited by the same concentrations of TGF-β1. In other experiments, it was shown that TGF-β1 produced greater contractile responses in mesoderm-derived SMCs compared with neural crest–derived SMCs (42). Studies using a series of aortic homograft transplantations also support cell lineage–dependent, rather than environmental influences, in the development of atherosclerosis. Abdominal aortic segments (which are "atherosclerosis prone") were relocated to atherosclerosis-resistant positions in the circulation, either the

thoracic aorta or jugular vein, and the displaced thoracic aorta or jugular vein segments were repositioned to the abdominal aorta. Animals were then fed an atherogenic diet for 1 year. The atherosclerosis-prone abdominal aortic segments developed severe atherosclerotic lesions in all locations. Atherosclerosis-resistant segments failed to develop intimal lesions even when transplanted into the atherosclerosis-prone environments of the abdominal aorta (43, 44). Segments of abdominal aorta were also transplanted into the disease-resistant pulmonary artery and significant atherosclerosis developed in the usually atherosclerosis-resistant pulmonary circulation (45). These studies show that intrinsic differences in vessel wall cells themselves, rather than position-dependent hemodynamic effects, contribute to susceptibility to disease. Large-scale studies in the humans have also led many to conclude that arterial bed–specific differences in disease susceptibility are attributable to "genetic difference in the composition of the vessel wall" (46). Collectively, these and other studies suggest that different types of SMCs found within a common vessel wall or along the axis of an arterial bed in an organ can respond in lineage-specific ways to both environmental stress and soluble factors that control development, growths and remodeling of the vessel wall.

There are very limited studies addressing this issue of lineage-dependent cell-specific responses in the pulmonary circulation. Studies of large proximal pulmonary arteries have demonstrated the presence of phenotypically distinct SMC subpopulations (47–49). A limited (non–fate-mapping) developmental analysis of these cells suggests distinct embryonic origins. These different SMC populations exhibit very stable and distinct functional capabilities both *in vitro* and *in vivo*, again supporting the idea of different embryonic origins for each SMC subtype. Interestingly, the morphologic appearance and biochemical characteristics of these cells and their functional capabilities also change remarkably during development. Compared with airway SMCs, which undergo an early process of differentiation, the SMCs in the vasculature differentiate at different rates and times (50). There are very clear differences in differentiation levels among early fetal, late fetal, neonatal, and mature vascular SMCs. These differences are important to consider because the responses to pathophysiologic stress in SMCs are age dependent (49). The phenotype of SMCs in the distal circulation (based on morphologic, biochemical, and functional characteristics) also appears to be distinct from the SMC subtypes that exist in the more proximal circulation. However, to our knowledge, no fate-mapping analysis regarding the origin of these different cells has ever been undertaken.

Studies are needed to define the biochemical and functional phenotypes of SMC that might exist at different locations in the pulmonary vasculature. Lineage analysis and fate-mapping studies, similar to those performed in the systemic circulation, would help define the developmental origin of pulmonary vascular SMCs at different sites (proximal, mid-, and distal arteries as well as in veins). Comparing and contrasting the origin of these cells to airway and bronchial artery SMCs will be of great importance. In addition, more studies are necessary to define the role of vascular SMC phenotypic and functional heterogeneity in both normal homeostatic functions as well as in pathophysiologic responses to injury.

Studies are also needed to define the role of stem/progenitor cells in lung vascular development and in disease. It is now commonly accepted that a variety of different types of self-renewing progenitor cells can form SMCs *in vitro* and *in vivo*. Mouse embryonic stem cells (ESCs) cultured on type IV collagen and then sorted for the expression of fetal liver kinase (Flk)-1 have been reported to differentiate into either SMCs or endothelial cells depending on the type of growth factor to which they are exposed. In the presence of platelet-derived growth factor (PDGF)-BB, more then 95% of the Flk-1–positive cells expressed α-smooth muscle actin and acquired a spindle-shaped characteristic of vascular SMCs (51). Under other conditions, ESCs produced colonies consisting of both endothelial cells and SMCs. Thus, it is now accepted by many that there exist within embryonic development bipotential progenitor cells, which are essential in vascular development (52). Interestingly, progenitor cells with a capacity for SMC differentiation have also been identified in adult arteries. Using flow cytometry, Sainz and colleagues reported isolation of "side population" (SP) cells from an intima-media digest, which were not present in adventitial tissues (39). The tunica media–derived SP cells expressed a Sca-1^+, c-Kit^{low}, Lin^-, $CD34^{low}$ profile and were reported to make up 6% of the total media cell population. These aortic SP cells expressed an SMC phenotype when exposed to either TGF-β1 or PDGF-BB and an endothelial phenotype when cultured in the presence of vascular endothelial growth factor (39, 53). Adult aortic SP cells lacked erythroid, lymphoid, or myloid potential, and were thus different from marrow-derived SP cells identified in skeletal muscle (39, 53). A second distinct population of SMC progenitors have also been described in the arterial adventitia (54). These cells expressed the Sca-1 marker and were particularly abundant in the adventitia surrounding the aortic root. When these adventitial Sca-1 progenitor cells from Rosa26 mice were transplanted to the adventitial side of vein grafts in apolipoprotein-E–deficient mice, β-gal–positive cells were found in the graft neointima and were positive for SM markers (54). Therefore, it appears that several distinct types of SMC progenitors may normally reside in the adult artery wall, with an ability to respond to injury- or disease-promoting stimuli, and can differentiate into SM-like cells *in vivo*. Cells with the capability of differentiating into SMCs have also been described as existing within the circulation. These cells can be recruited to the vessel wall under pathologic conditions and can differentiate into SM-like cells (55). Thus, at least in the systemic circulation, it is suggested that the formation of new vascular SMCs from undifferentiated progenitors is not limited to embryogenesis and that the quiescent adult artery wall contains resident progenitor cells that can differentiate into SMCs or SMC-like cells *in vivo*. In addition, under certain circumstances, there may be recruitment of bone marrow–derived circulating cells, which are also capable of differentiating into SM-like cells (55, 56).

At present, it is not known if the pulmonary vascular wall contains resident SMC progenitors. It is also unclear whether circulating, bone marrow–derived progenitor cells contribute to the accumulation of SMCs in the injured or remodeled pulmonary arterial wall. In this regard, the "definition" of what constitutes an SMC (discussed *above*) is important. How resident or recruited SMC precursors function, upon their differentiation, in specific locations is also unclear. Whether environmental cues at specific locations allow them to function in ways similar to those of the surrounding "resident" cells is also unclear but important, based on the studies described above regarding functional differences of SMC subtypes. It will also be important to determine whether the vascular media renews its resident SMC populations. If so, what are the mechanisms involved in SMC turnover and what are the contributions of local SMC proliferation versus contribution of nonresident or even resident SMC precursors?

Adventitial Cells

Until recently, the adventitial fibroblast, the principal cell type in the adventitia, has been largely overlooked as a cell type important in contributing to normal vascular homeostasis or to vascular remodeling under pathologic conditions. However, an increasing volume of experimental data indicates that the

adventitial compartment of blood vessels, in both the pulmonary and systemic circulations, is a critical regulator of vessel wall function in health and disease. 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. In fact, the adventitial compartment may be considered a principal injury-sensing tissue of the vessel wall. In response to environmental stresses, such as hypoxia or vascular distention, or vascular injury with or without inflammation, resident fibroblasts are activated and undergo a variety of functional changes. Increases in cell proliferation, expression of contractile and extracellular matrix proteins, as well as in the secretion of factors capable of directly affecting vascular tone and growth, occur in fibroblast in a manner that influences overall vascular tone and structure (57–59).

Given the importance of the adventitial fibroblast, it is clear that, as with SMCs, we must define what exactly constitute markers of the "fibroblast" phenotype. At present, precise identification of fibroblasts *in vivo*, and *in vitro*, is difficult because markers frequently used to identify fibroblasts, such as vimentin and α-smooth muscle actin, lack specificity or sensitivity. Vimentin, a frequently used marker for fibroblasts, unfortunately does not distinguish fibroblasts from other cells of mesenchymal origin. α-Smooth muscle actin is not generally observed in resting fibroblasts but is often used to identify myofibroblasts. This has led to confusion in differentiating myofibroblasts from certain SMCs or SMC-like cells because α-smooth muscle actin is expressed in de-differentiating SMCs and in circulating or resident SMC progenitors undergoing a differentiation process. Fibroblast-specific protein (FSP-1), also known as S100A4, is an 11-kD protein that belongs to the S100 superfamily of intracellular binding proteins. It has been suggested as a specific marker of fibroblasts, although the specificity of FSP-1 as a fibroblastic marker remains extremely unclear and highly unlikely at the present time (60). Further study is needed to identify fibroblast-specific markers and to develop a better definition of what constitutes a "fibroblast."

Another issue complicating the phenotyping of fibroblast is that, similar to SMCs, there is a significant diversity in the fibroblast populations in the adventitia of both systemic and pulmonary vessels (59, 61, 62). This is perhaps not surprising because heterogeneity of fibroblast populations within tissues, including the lung, is well documented. Differences among fibroblasts have been identified on the basis of surface markers (including Thy-1), cytoskeletal composition, lipid content, and cytokine profile (59, 61, 62). Numerous studies have demonstrated that selective expansion of specific subsets of fibroblasts occurs in response to injury. For instance, fibroblasts isolated from lungs with active fibrotic disease have increased proliferative capacity, are capable of anchorage-independent growth, and are morphologically distinct (61, 63). Interestingly, myofibroblasts in fibroblastic foci in lungs from individuals with idiopathic pulmonary fibrosis/usual interstitial pneumonia are Thy-1 negative, despite the fact that the majority of fibroblasts in normal lungs are Thy-1 positive. Studies of fibroblasts derived from the adventitia of pulmonary arteries also suggest a tremendous heterogeneity in their functional characteristics and support the possibility of selective expansion of specific subsets during remodeling (64).

The mechanisms contributing to fibroblast heterogeneity need to be studied. Lineage analysis or fate-mapping studies to identify the origins of adventitial fibroblasts will be extremely important. In addition, more work regarding the role of specific fibroblast populations in the pathophysiology of pulmonary vascular disease should be undertaken. It is becoming increasingly clear that cells exhibiting myofibroblast-like characteristics are the most common cell type found in the pulmonary vascular remodeling that characterizes all forms of pulmonary hypertension. These cells have very distinct contractile and relaxant capabilities. Therapies directed at specifically modulating their function may be important in pulmonary vascular disease.

It is also essential to recognize that there are many potential sources of fibroblasts/myofibroblasts in pathologic conditions beyond local expansion of resident populations of fibroblasts (65). Endothelial and epithelial cells have been shown to undergo a process of transdifferentiation into fibroblasts or myofibroblasts (66–68). Furthermore, circulating fibroblast precursors, including fibrocytes, likely contribute to accumulation of fibroblast/myofibroblast-like cells in vascular pathologies (69, 70). Frid and colleagues have recently demonstrated an important role for fibrocytes (circulating mesenchymal or fibroblast precursors) in hypoxia-induced pulmonary vascular remodeling (71). The development of mouse models, which allow specific lineage tracking of endothelial, epithelial, and precursor cells has allowed evaluation of the relative contributions of these cells and processes to fibrosis in organs such as liver, kidney, lung, and skin (68). Because the myofibroblast is so predominant and can affect both directly and indirectly so many structural and functional aspects of the pulmonary vessel wall, it is imperative that the potential sources of fibroblasts be evaluated in the setting of pulmonary vascular diseases. Identification of the origin of the cells involved in remodeling and fibrosis may allow specific therapies to be developed to prevent or reverse the accumulation of fibroblasts under different conditions. Therefore, a better understanding of fibroblasts and how they accumulate in the diseased vessel wall will be critical for a better understanding of pulmonary vascular pathology.

FIBROBLAST/FIBROBLAST-LIKE CELLS IN LUNG INJURY, INFLAMMATION, AND REMODELING/FIBROSIS

Although it is accepted that fibroblasts play key roles in fibrotic diseases of the lung, as well as in lung development, the full spectrum of their role in various processes related to both lung disease and development remains to be fully elucidated. With respect to response to injury, the ability of such cells to elaborate inflammatory cytokines implicates them in immune and inflammatory responses, whereas their role in extracellular matrix deposition is a key factor in healing and remodeling (65, 72). Their participation in cross-talk with epithelial, endothelial, and other lung cellular elements is also considered to be critical in multiple pulmonary diseases, such as asthma and interstitial lung diseases. The role of specific fibroblast populations, such as the myofibroblast, in fibroproliferative disorders of the airway and more distal parenchyma, including the vascular wall in pulmonary hypertension, has been extensively described (65). Yet there is little information on the origin and genesis of these cells to guide translational investigation on their potential significance as novel therapeutic targets. The current evidence is controversial, with multiple origins suggested. Although increasing evidence suggests contribution from bone marrow progenitors and circulating fibrocytes, endogenous sources, such as from resident lung mesenchymal stem cells and epithelial (and endothelial)–mesenchymal transition, have also been proposed (73–77). However, the actual impact or role of these cells in fibroproliferative lung diseases remains to be elucidated.

Recent studies have highlighted the developmental importance of fibroblast phenotype as a function of tissue localization, which extends to determination of the phenotype of adjacent epithelial cells (78, 79). This argues for the potential importance

of the fibroblast with respect to development as well as its effect on epithelial or endothelial phenotype in response to lung injury. Although the role of the epithelial cell has been suggested, the importance of the fibroblast in determining epithelial cell phenotype itself has not been fully investigated. A similar situation exists vis-à-vis potential fibroblast regulation of endothelial phenotype.

Despite these observations, there remain significant gaps in current understanding of lung fibroblast biology and pathobiology, which hampers further advances in understanding pathogenesis of many pulmonary diseases. As implied above, some of these issues relate to the origin of pulmonary fibroblasts in normal and diseased lungs. In addition to resident lung progenitors, the issue of potential extrapulmonary progenitors requires further investigation. The identity and phenotype of the resident lung as well as extrapulmonary progenitor cells require further careful study, not least of which is the need to obtain a suitable set of rigorous criteria for their identification as progenitors. It goes without saying that part of this task would include identifying the tools to be used for tracking these cells as they undergo differentiation or transdifferentiation. A related issue is the determination of the distinct subtypes of fibroblasts that may exist in the normal versus fibrotic lung. There is evidence of distinct, relatively stable phenotypes (80–83), but it is unclear if they have different origins/progenitors, or are derived from a single or few progenitors with interconversion/transdifferentiation possible. With respect to epithelial (and endothelial)–mesenchymal transition much of the molecular mechanism remains to be elucidated. Thus, a significant number of issues remain that require future investigation for clarification.

STEM CELLS IN PULMONARY VASCULAR BIOLOGY

The lung is constantly subjected to a variety of stimuli and insults originating from the organism's environment and from peripheral systems, which reach the organ through the pulmonary circulation. Homeostasis of pulmonary vessels is critical for maintaining gas exchange and adequate blood gas balance, which can be maintained through the organism's lifespan in the absence of disease. However, little is known about the rate of turnover for pulmonary-associated cells, such as endothelial cells, SMCs, fibroblasts, and pericytes, and much of what we know is related to disease. In the context of disease, a variety of insults that result in postnatal endothelial injury can resolve by restoration of functional vasculature. In contrast to the beneficial role of restoring injured vasculature cells, augmented proliferation of pulmonary vascular cells can result in adverse effects, as observed during pulmonary hypertension, in which smooth muscle cells and endothelial cells, or cells with endothelium-like attributes proliferate (84–86). Collectively, these observations suggest that during health and disease, regeneration of pulmonary vascular cells occurs postnatally, and can have beneficial roles, such as those observed during homeostasis and recovery from vascular injury, or can contribute to disease and have deleterious effects, such as that observed during pulmonary hypertension. The origin and identity of pulmonary vascular progenitors is not well understood, and remains a critical area of pulmonary vascular research.

Recent evidence indicates that vascular progenitors can arise from a pool of resident vessel wall–derived progenitor cells or from a variety of circulating bone marrow–derived phenotypes (20, 21, 87, 88). Early studies from Schwartz and Benditt (89) revealed that within the tunica intima of the aorta exist multiple foci of endothelial cells with progenitor capacity, as evidenced by the rapid uptake and label retention of [^{3}H]thymidine in *ex vivo* explants. Recent studies by Ingram and colleagues (20, 21) validate this notion that resident endothelial progenitor cells represent a subpopulation of cells that can be resolved within the vessel wall. These progenitor cells exhibit high replicative behavior and the capacity to self-renew after they are subjected to a single-cell clonogenic assay (20, 21). Because no specific surface marker(s) has been described to select resident endothelial progenitor cells from a greater population of cells, it is clear that the use of functional assays constitutes an important tool for studying progenitor phenotypes. Endothelial heterogeneity along the pulmonary vascular axis brings into question whether individual vascular segments possess a select niche of endothelial progenitors, in which endothelial progenitor cells display segment-specific molecular imprints (31), or whether resident endothelial progenitor cells constitute a universal pool of progenitor cells that lack segmental specification. Functional *in vitro* studies constitute an important tool to identify endothelial progenitor cell populations and to examine their gene profiles, to determine whether unique molecular imprints can be resolved *in vivo*. Use of laser capture microdissection followed by molecular studies that reveal gene expression (polymerase chain reaction, *in situ* hybridization) constitutes an important tool that can be used for identifying endothelial progenitors *in situ* and generating selective clones that can be expanded *ex vivo*.

Bone marrow–derived cells have been shown to incorporate into the pulmonary vasculature, as demonstrated using gender mismatch and bone marrow transplant studies, or by identification of "selective" progenitor markers (90, 91). However, although there is controversy about the frequency of "engraftment" of bone marrow–derived progenitor cells, the use of selective markers that can be expressed alternatively by different phenotypes hampers conclusions that are more definitive. Although a capacity to differentiate into different lineages has been demonstrated in some bone marrow–derived populations, it is not clear whether those cells first home to and engraft in response to specific signaling cues and whether they can adapt segmental specification and retain progenitor capacity with commitment toward the endothelial lineage upon time. Thus, chronic *in vivo* studies that identify or track cell lineages *in situ* become critical, and a variety of approaches that include cre-recombinase technology capable of determining cell origin and cell fate (i.e., fate mapping) will be valuable. Laser capture microdissection with genetic profiling represents an important tool for identification of cell fate and clone generation (86). Quantitative approaches and cell–cell interactions between endothelial progenitors and their progeny, and the "native" resident populations, by confocal microscopy complement a list of studies that can be outlined to study the biology of endothelial progenitors.

Cell reprogramming has been recently demonstrated in which fully differentiated cells become pluripotent after *ex vivo* gene transfer (92, 93). It will be interesting to determine whether differentiated or mature endothelial cells possess the intrinsic capacity to dedifferentiate to a more immature phenotype while retaining lineage commitment. Generation of specific clones that can be subjected to different environmental conditions followed by examination of cell fate based on functional behavior can delineate another mechanism yielding pulmonary endothelial progenitors.

CONCLUSIONS

We are in an exciting, and perhaps unprecedented, time in vascular biology. Steady strides have been made in our understanding of fundamental endothelial cell, smooth muscle cell, and fibroblast biology. However, new areas of investigation have now unraveled as we begin to appreciate the significant cell

heterogeneity that is prominent within each of these cell phenotypes, and as we begin to probe new questions regarding the epigenetic imprinting of cell phenotypes, and the contribution that such imprinting has on health and disease. Indeed, we have only a rudimentary understanding of how cell memory is conditioned, the molecular cues that govern cell differentiation, and whether cell differentiation (the extent to which it is differentiated) is dynamic and reversible. The broader field of stem cell biology directly and indirectly impacts on vascular cell biology. Resolution of progenitor cells within populations of vascular cells raises important new questions about the origin of these committed progenitors, how they arise, and how they impact on development, homeostasis, and repair from injury. Moreover, the roles of genetics and epigenetics in a "disease" cell phenotype take on new meaning, with broader questions being raised regarding the role of progenitor versus nonprogenitor cells in vascular disease states. New questions, new resources, and new approaches will, indeed, bring about a new depth of understanding in vascular biology in the years to come.

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