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SPOTLIGHT REVIEW

Dynamic and diverse changes in the functional properties of vascular smooth muscle cells in pulmonary hypertension

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Abstract

Pulmonary hypertension (PH) is the end result of interaction between pulmonary vascular tone and a complex series of cellular and molecular events termed 'vascular remodelling'. The remodelling process, which can involve the entirety of pulmonary arterial vasculature, almost universally involves medial thickening, driven by increased numbers and hypertrophy of its principal cellular constituent, smooth muscle cells (SMCs). It is noted, however that SMCs comprise heterogeneous populations of cells, which can exhibit markedly different proliferative, inflammatory, and extracellular matrix production changes during remodelling. We further consider that these functional changes in SMCs of different phenotype and their role in PH are dynamic and may undergo significant changes over time (which we will refer to as cellular plasticity); no single property can account for the complexity of the contribution of SMC to pulmonary vascular remodelling. Thus, the approaches used to pharmacologically manipulate PH by targeting the SMC phenotype(s) must take into account processes that underlie dominant phenotypes that drive the disease. We present evidence for time- and location-specific changes in SMC proliferation in various animal models of PH; we highlight the transient nature (rather than continuous) of SMC proliferation, emphasizing that the heterogenic SMC populations that reside in different locations along the pulmonary vascular tree exhibit distinct responses to the stresses associated with the development of PH. We also consider that cells that have often been termed 'SMCs' may arise from many origins, including endothelial cells, fibroblasts and resident or circulating progenitors, and thus may contribute via distinct signalling pathways to the remodelling process. Ultimately, PH is characterized by long-lived, apoptosis-resistant SMC. In line with this key pathogenic characteristic, we address the acquisition of a pro-inflammatory phenotype by SMC that is essential to the development of PH. We present evidence that metabolic alterations akin to those observed in cancer cells (cytoplasmic and mitochondrial) directly contribute to the phenotype of the SM and SM-like cells involved in PH. Finally, we raise the possibility that SMCs transition from a proliferative to a senescent, pro-inflammatory and metabolically active phenotype over time.

Keywords Progenitor cells • Endothelial mesenchymal transition • Inflammation • Metabolism • Senescence

This article is part of the Spotlight Issue on Novel concepts for the role of smooth muscle cells in vascular disease.

1. Introduction

Pulmonary hypertension (PH) is the end result of interaction between pulmonary vascular tone and a complex series of cellular and molecular events termed 'vascular remodelling'. This remodelling involves the intimal, medial, and adventitial compartments of the pulmonary (arterial and venous) vessel wall and often the perivascular space as well. The remodelling process almost universally involves medial thickening, driven by increased numbers and hypertrophy of its principal cellular constituent, smooth muscle cells (SMCs); concomitant with these processes, SMCs exhibit enhanced chemokine and cytokine production, and alterations in extracellular matrix protein production and degradation. We consider that these structural and functional changes in SMCs and their role in PH are dynamic and may undergo significant changes over time (which we will refer to as cellular plasticity); no single property can account for the complexity of the contribution of SMCs to pulmonary vascular remodelling. Thus, the approaches used to pharmacologically manipulate PH by targeting the SMC phenotype(s) must take into account processes that underlie dominant phenotypes that drive the disease.

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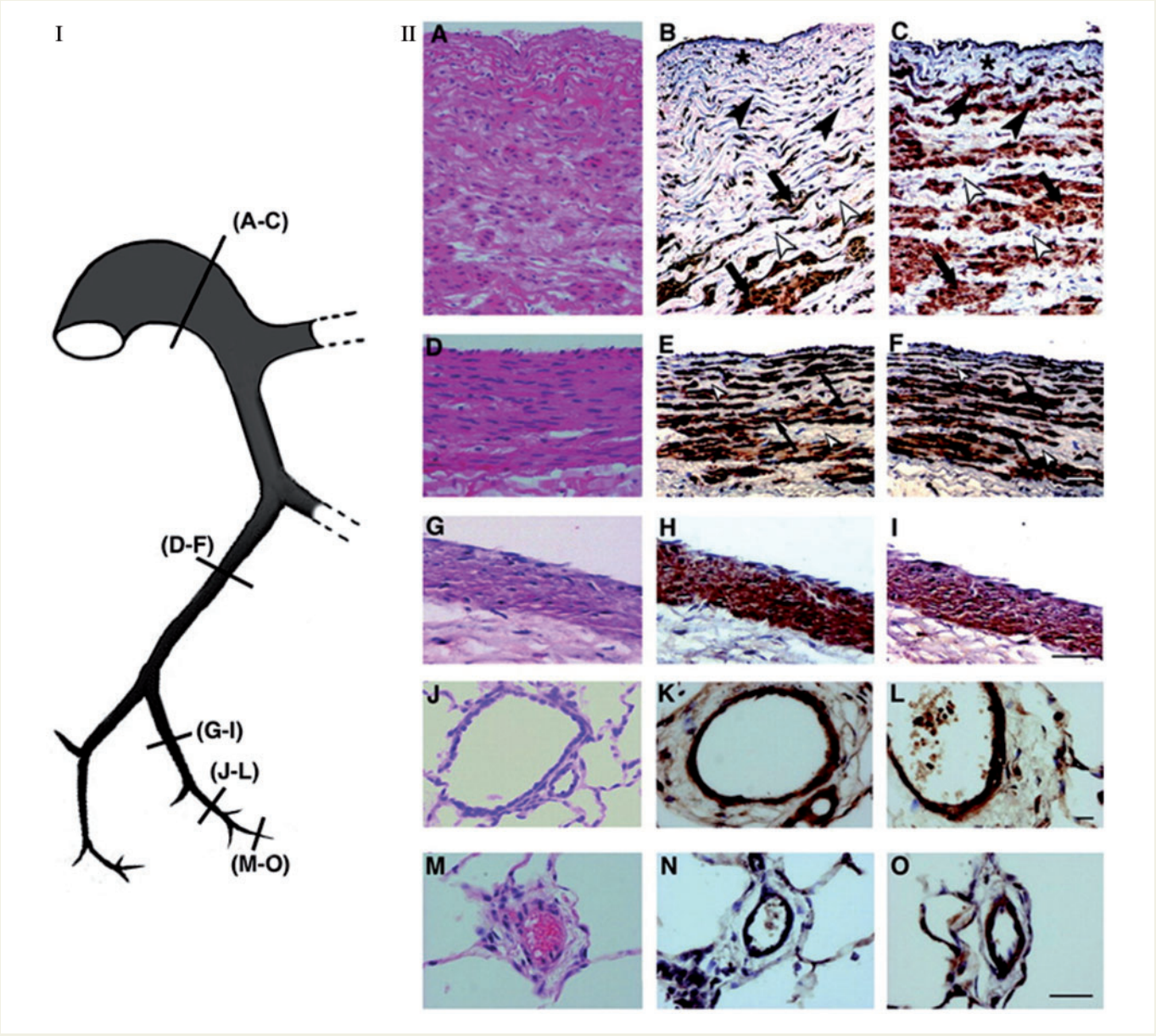

Figure 1 (I) Schematic representation of the pulmonary arterial tree depicting levels, at which the sections were obtained. (II) The cellular composition of the bovine arterial media gradually changes along the longitudinal axis from phenotypically heterogeneous (proximal arteries) to more uniform (distal arteries). This is not immediately apparent on histologically stained tissue (*H&E*; *left A, D, G, J, M*), but becomes evident via immunostaining for SM-specific differentiation markers: meta-vinculin (*middle*: *B, E, H, K, N*) and smooth muscle myosin heavy chain B isoform (SM-MHC-B; *right*: *C, F, I, L, O*). From *top* to *bottom*: MPA (*A–C*), intra-lobar PAs with inner diameters of: 3000 μm (*D–F*), 1, 500 μm (*G–I*), 500 μm (*J–L*), and 100 μm (*M–O*). Scale, 100 μm. Adapted from reference.[6]

SMCs are heterogeneous in the normal pulmonary circulation. It has been shown that pulmonary artery (PA) SMCs are not uniform in phenotype throughout the pulmonary circulation, but rather are heterogeneous at both a single anatomical site (proximal vs. distal PAs) and along a specific vascular segment, exhibiting site-specific and unique responses to pathologic hypertensive stimuli.[1–5] For instance, the cellular composition of the bovine PA media changes along its proximal to distal longitudinal axis from being phenotypically heterogeneous in the pulmonary trunk to being phenotypically uniform in distal resistance vessels (see *Figure 1*).[6] The media of the proximal large PAs, including the main PA (MPA), is composed of a mosaic of more differentiated SMCs and less differentiated 'SM-like' cells (characterized by expression of α-SM-actin but not by other differentiation markers), while in the distal muscular PAs, the arterial media is composed of a phenotypically uniform and apparently well-differentiated SMC phenotype (including expression of SM-myosin heavy chains, SM h-caldesmon and metavinculin). These observations *in vivo* provided a 'cellular basis' for the different functional properties of vessels along the vascular tree and are consistent with the classic physiologic studies of Burton *et al.*[7] These studies outlined that great arteries are structured to provide tensile strength and withstand high wall tension along with a certain degree of distensibility to accommodate stroke volume. The small arteries, on the other hand are

exposed to much less wall stress and pulse pressure than the large arteries. In these distal arteries, under homeostatic conditions, the interaction of endothelial cells with contractile SMCs provide the tone essential for regulation of blood pressure and flow in the pulmonary circulation.

The purpose of this perspective is to (i) review the evidence for time- and location-specific changes in SMC proliferation in various models of PH, with special emphasis on the transient nature of SMC proliferation; (ii) emphasize that the heterogenic SMC populations that reside in different locations along the pulmonary vascular tree exhibit distinct responses to the stresses associated with the development of PH; (iii) review the potential sources of SM or SM-like cells that acquire a proliferative phenotype and/or contribute in other ways to the remodelling process, including endothelial cells (throughout the process of endothelial–mesenchymal transition (EndMT)), fibroblasts, and resident and circulating progenitors; (iv) review evidence of the pro-inflammatory phenotype of SMCs in PH; (v) present evidence that metabolic alterations (cytoplasmic and mitochondrial), which are akin to those observed in cancer cells directly contribute to the phenotype of the SM and SM-like cells involved; and (vi) present evidence supporting the possibility that SMCs transition from a proliferative to a senescent, pro-inflammatory and metabolically active phenotype over time.

2. SMC proliferation and hypertrophy in experimental animal models and in human PH

2.1 Time, location and species dependent effects

Increased medial thickness of the PAs is a well-established feature of PH in animal models and humans. Proliferation of resident medial SMCs is widely believed to play an important role in this thickening. Seminal studies in the late '70 s by Dr Meyrick and Dr Reid established site- and time-specific changes in the medial thickening and SMC proliferation that occurred in animal models of PH.[8–11] In the hypoxic rat model of PH (Sprague-Dawley rats @ 230 g at hypoxia initiation), the first increases in thymidine incorporation occurred in the hilar vessels, yet these were predominately in the adventitial and endothelial compartments, with only minimal change (<2% labelling index) in the medial compartment. The authors also showed that thickening of the large vessels appeared to consistently follow the increase in PA pressure. However, medial changes and SMC proliferation in the intra-acinar arteries and vessels of the alveolar wall occurred later. Similar, but not identical observations were made in the monocrotaline rat (Sprague-Dawley) model of PH.[9] Changes in SMC proliferation in hilar vessels also appeared to follow increases in PA pressure, but occurred later, whereas the time course of changes in the intra-acinar vessels occurred earlier because the authors speculated that the timing of SMC proliferation in this models PH was due to more severe endothelial injury by the alkaloid monocrotaline and associated interstitial inflammation than those observed in the hypoxic model. The most consistent observation with regard to SMCs in these two experimental PH models was extension of the 'sleeve of SM-like cells to vessels that had been previously non-muscularized'. This response was believed, at the time, to be mediated by proliferation of intermediate 'SMC-like cells' and/or pericytes.[8–11]

Subsequent studies in other animal models of PH have largely supported these findings: in hypoxic mice, proliferation of SMCs was greatest in larger hilar vessels and occurred early (at 3–4 days) but then gradually diminished, and, as most studies demonstrated, after 4–5 weeks of hypoxia cell proliferation was minimal.[12–14] Distal muscularization occurred early and consistently in the hypoxic mouse models, similar to that in rat.[15,16] The *Schistosoma* mouse model also has proliferation of cells in the medial layer, and again SMC proliferation decreases with time.[17] Further, these mouse and rat models are characterized by PAs in which the endothelial cells, though dysfunctional, line the PAs as single layer. This structural characteristic is also present in human disease associated with left ventricular dysfunction (WHO Group 2), hypoxia and interstitial injury (WHO Group 3). However, the paradigmatic pathology of severe PH in humans, characteristic of idiopathic and BMPR2 mutation associated pulmonary arterial hypertension, characterized by excessive luminal growth of endothelial cells, forming plexiform lesions, and significant but notably more mild expansion of the medial layer is not observed in these animal models.[18,19] These findings underscore that PH is not a monolithic disease and much can be gained from dissecting the commonalities and differences among the multiple of forms of PH.

Recently, a Sugen + Hypoxia model of PH (based on the combination of the VEGF receptor blocker SU5416 and chronic hypoxia[20,21]) has been increasingly thought of as one of the better models to study human pulmonary arterial hypertension (PAH), combining suprasystemic levels of PA pressures with progressive plexiform-like lesions. It is noteworthy that, in this model, there is again evidence for only transient proliferation of SMCs in the large as well as small vessels, notably early in the course of the disease; importantly, this proliferation response wanes with time and is insignificant at later stages (*Figure 2*). This observation challenges the dominant dogma that SMC proliferation is the root cause of PH, most importantly, of the persistence and chronicity of SMC in vascular remodelling; it rather suggests that SMC proliferation may indeed be only a transient response and that, in established disease, alternative SMC intermediate phenotypes might play key roles in established disease.

Consistent with the observations in animal models wherein proliferation, particularly in SM-actin$^+$ cells, seems to wane with time are studies of cellular proliferation using lung tissues from both idiopathic PAH (iPAH) and familial PAH (FPAH) patients compared with controls. The authors report, based on the pathological evaluation of a large numbers of lungs with PAH (and Group 3 PH), no evidence of SMC proliferation in established PH, with no significant Ki67 expression in α-SM-actin$^+$ SMCs.[22] Instead, other cell populations, including endothelial cells and inflammatory cells that concentrate around and within the pulmonary vascular lesions in PH.[18] exhibit evidence of ongoing proliferation. Interestingly, there were significant perivascular accumulations of CD45$^+$ and CD133$^+$ cells, observations that have been supported by other studies,[23] which were proliferating but without significant fusion with recruited circulating cells.[22] This is consistent with the observations of Rich et al. in a patient with iPAH, where they used double-immunolabelling techniques for α-SM-actin and PCNA (marker of cell cycle progression), and showed that proliferation occurred almost exclusively in non-α-SM-actin expressing cells.[24] Collectively, these observations challenge the concept that SMC proliferation continues unabated, while raising important questions as to what are the phenotypes of the proliferating cells in chronically hypertensive vessel wall, as these appear to contribute to sustaining the large cellular mass and ultimately to vascular obstruction.

2.2 Age and development effects on SMC proliferation

There are also age and/or development specific differences in the proliferative responses of pulmonary vascular wall cells to various stimuli

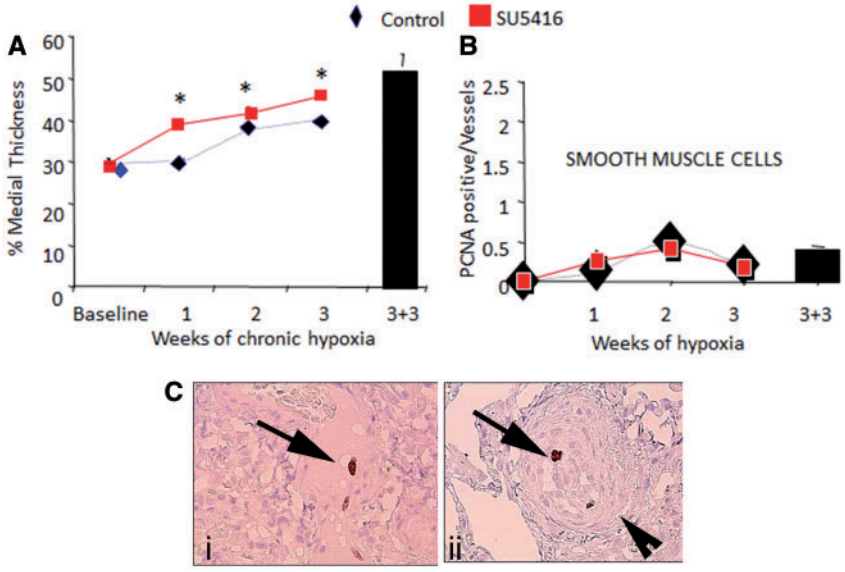

Figure 2 SMC proliferation is minimal in the Sugen + Hypoxia model of PH. (*A*) Percent medial thickness in PAs of rats treated with the VEGF receptor blocker SU5416 and exposed to chronic hypoxia for 1–3 weeks; (3 + 3) indicates the group of rats treated with SU5416+chronic hypoxia and then exposed to room air (Denver altitude) for an additional three weeks. *$P < 0.01$ (*t*-test) ($n = 3$ per time point and experimental group). (*B*) Expression of a nuclear proliferation marker PCNA is minimal, if any, in medial SMCs of SU5416+Hypoxia or Control rats, as outlined in *A* ($n = 3$ per time point and experimental group). (*C*) Staining for MIB1 (nuclear proliferation marker) in (i) a control human PA shows rare proliferating endothelial cells (arrow) and (ii) endothelial cells in a plexiform lesion (arrow) in a lung of a patient with idiopathic pulmonary arterial hypertension. Note lack of immunoreactivity (i.e. lack of proliferation) in the SMCs of the arterial media (arrowhead). *$P < 0.01$ (students *t*-test); Adapted from reference.[20]

involved in hypoxia induced PH. In neonatal calf models, SMCs have a much higher capacity for proliferation than cells from later stages of development in both large and small vessels compared with older weanling calves.[25–28] It should be noted that there is a relatively high rate of proliferation in both the media (and adventitia) at birth, which decline over the first 14 days following birth.[29] If this transition is interrupted by exposure of the animals to hypobaric hypoxia, there are very significant increases in both medial and adventitial cell proliferation (assessed by labelling index), which exceed the proliferative responses usually observed in rats exposed to hypoxia, even in rapidly growing rats (e.g. 8-weeks of age). Interestingly, in this neonatal calf model, at least within the medial compartment, the highest rates of proliferation occur in small muscular arteries and not in the larger hilar elastic vessels.[29] Based on observations in this calf model and in SMC responses in young vs. old rats, matched neonatal and adult bovine SMCs were isolated from young and older animals and tested for differences in many parameters associated with growth capacity. Neonatal PA SMCs were smaller, grew faster, reached a higher plateau density and were less susceptible to senescence than adult PA SMCs. Further, they were more resistant to serum withdrawal, had spontaneous autocrine growth capacity, and were more responsive to IGF-1, PMA, and the combination.[25,26] Studies in older calves also provided interesting information regarding the effects of more prolonged hypoxia that suggested that with time the structural changes in the media became more and more significant and actually were associated with the failure of PA pressure to return to normal upon oxygen breathing or vasodilator administration.[27,30] Interestingly, medial SMCs of proximal and intermediate arteries were occasionally found to be in 'advanced stages of degeneration', with observations of sarcoplasm filled with large vacuoles, perinuclear oedema, degenerating mitochondria, myelin whorls and small intracellular particles of varying size and electron density. At this stage, cells appeared compressed perhaps by the marked accumulation of increased amounts of extracellular collagen and elastin fibres in the subintima and media. Concentric laminae of basement membrane material were sometimes noted surrounding SMCs. Similar changes in SMCs have been described in humans with PAH[31] and in systemic hypertension.[32] These observations raise questions regarding the state of SMCs in more advanced stages of PH where vascular reactivity is lost.[27,30] They also support the idea that models of PH should include the use of animals across the spectrum of developmental stages, including later stages of the life span.

2.3 Heterogenic SMC subpopulations exhibit distinct growth capabilities *in vivo* and *in vitro*

As noted earlier, the vascular media in large mammals and humans has been demonstrated to be comprised of phenotypically heterogeneous SMC populations that potentially serve diverse roles in vascular homeostasis.[4] *In vivo*, these cells have distinctly different growth responses to hypoxia and growth factors, which drive most if not all pulmonary hypertensive responses, including pulmonary vascular remodelling[2,5,6,33] (*Figure 3*). When isolated in culture, these cell subpopulations continue, at least for the first couple of passages, to maintain their distinct phenotype, as well as to display markedly different rates of proliferation[6] (see *Figure 4*). These phenotypically distinct vascular SM-like cell populations

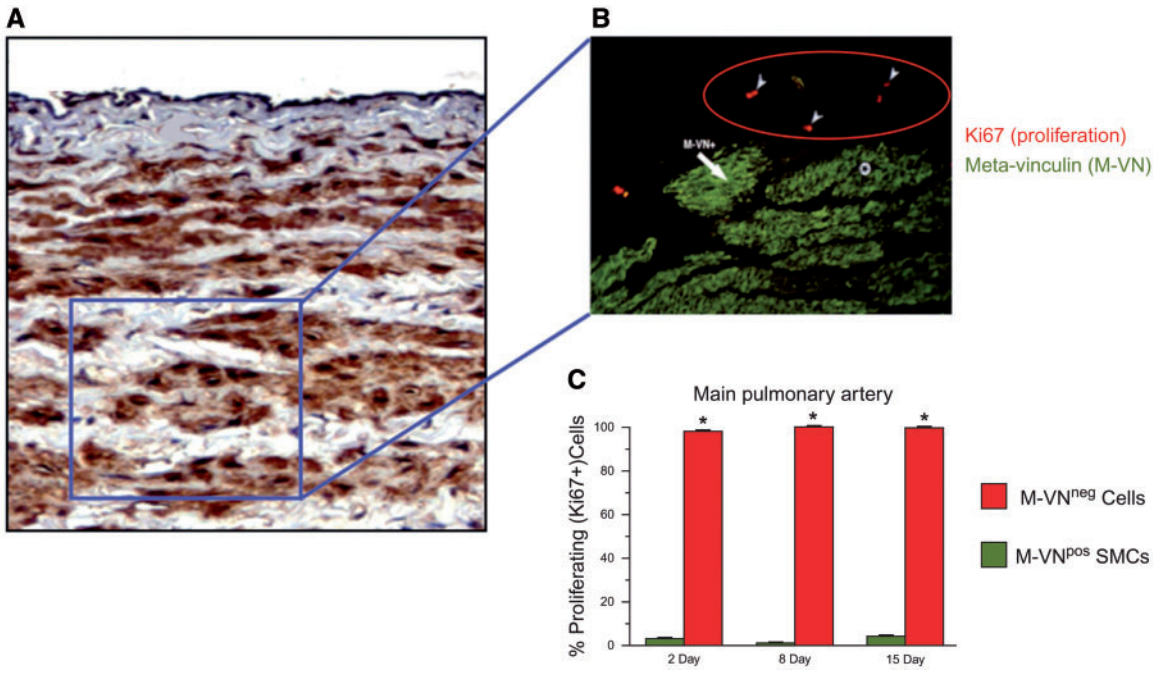

Figure 3 Phenotypically distinct SMC populations within the pulmonary arterial media of calves exhibit markedly different proliferative responses to hypoxia. (*A*) Phenotypic heterogeneity of pulmonary arterial media is demonstrated by immunostaining with antibodies against SM-myosin heavy chains. (*B*) Double-labelling with antibodies against a nuclear proliferative marker Ki67 (red, marked by arrowheads) and SMC differentiation marker meta-vinculin (green, marked by an asterisk) demonstrates that, in the PAs of calves with hypoxia-induced PH, proliferation (Ki67-positive cells) occurs almost exclusively in a less differentiated (metavinculin-negative) SMC-like subpopulation. (*C*) Quantitative analysis of proliferating cells expressing two distinct phenotypes, metavinculin-positive (green bars) and metavinculin-negative (red bars) (*$P < 0.001$; $n = 3$ calves in each group at each time point). Adapted from reference.[33]

appear to subserve different functions within the vascular media, based on observations of distinct ion channel expression and proliferative and matrix-producing capabilities in response to various stimuli, including hypoxia.[1,2,6,33–37] Because each of these populations express α-SM-actin in culture, any one of these rapidly proliferating cells could contribute to 'the highly proliferative phenotype' that is often said to characterize 'SMCs' in the patient with PH.

There is evidence to support the argument that these heterogenic cells are derived from distinct lineages and are not simply a common cell, exhibiting different states of differentiation.[4,38,39] Little is known regarding the mechanisms that confer unique proliferative characteristics to specific cell populations that exist in the large PAs. It has been demonstrated that less differentiated, more proliferation-prone medial cells are characterized by exuberant responses to G-protein coupled receptor (GPCR) agonists, compared with differentiated medial SMCs that do not exhibit proliferative responses to hypoxia. For example, hypoxia-induced activation of GPCR, with subsequent signalling through Gαi and Gq-mediated activation of extracellular signal regulated kinase 1/2 (ERK1/2), has been shown as necessary for hypoxia-induced proliferation in a specific subset of cells (SM-like) in the subendothelial media.[2] These observations support the idea that there are differences in receptor expression and/or intracellular signalling among various medial cell types.[2] Differences in endothelin production and receptor expression and responses have been described in distinct cell populations derived from the ovine PA.[40,41] Changes in the engagement of intracellular signalling pathways have also been described. For instance, increases in cAMP response element-binding protein expression have been shown to function as molecular determinants of SMC proliferative capability under hypoxic conditions, as have differences in protein kinase C.[42,43] Collectively, these observations support the existence of phenotypically distinct medial cell populations with unique proliferative responses to environmental stimuli; these responses are driven by membrane bound receptors sensitive to hypoxic activation that engage specific intracellular signalling pathways. Thus, in various segments of the vessel wall, there may reside distinct SMC populations that can respond differently to injury or environmental queues, and thus play diverse roles in maintaining vascular homeostasis.

Studies of cell heterogeneity in the bovine and human species are supported by recent elegant studies in mice, using clonal cell labelling strategies with multi-color reporters to probe the behaviour and potential of individual and sibling mesenchymal cells during lung development.[44] These studies demonstrate a surprising diversity of mesenchymal progenitor populations with different locations, patterns of migrations, recruitment mechanisms, and lineage boundaries. Even a more recent report by Majesky et al. suggests that heterogeneity exists among vascular cells even as they differentiate into what appear to be a 'uniform' vascular SMC phenotype.[45] Thus, future studies are needed to determine the cell-autonomous and microenvironmental cues that orchestrate an assembly of diverse SMC populations with different proliferative capabilities at a particular site. Most importantly, it will be essential to determine the fate of these cells as they cease dividing even under the continuous presence of injurious stimuli.

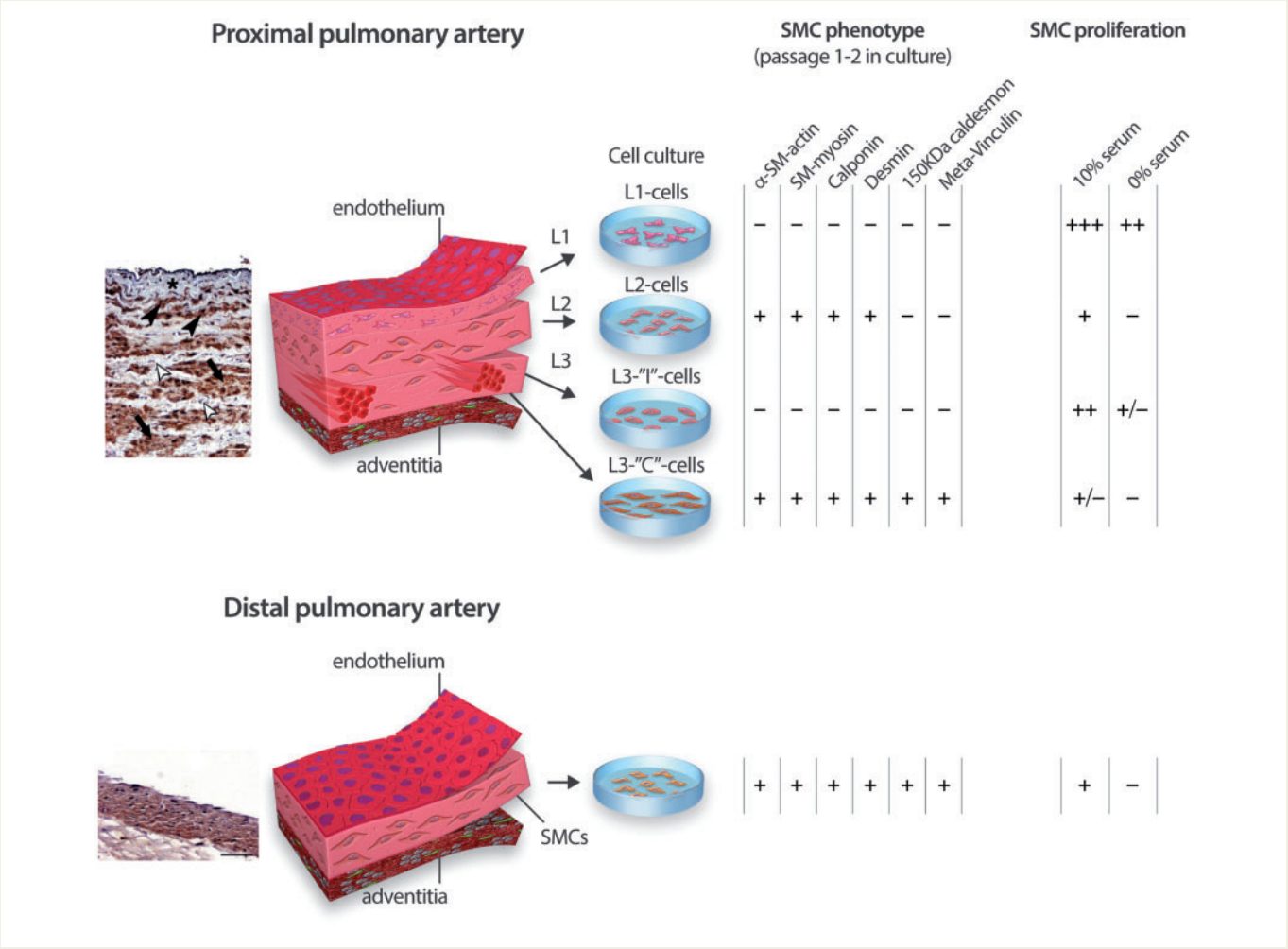

Figure 4 Cellular composition of tunica media of large proximal PA markedly differs from that of distal PA. Proximal (main) PA (top row) is characterized by profound heterogeneity of SMC populations, as reflected in cell morphology, phenotype and proliferative capabilities. In contrast, cellular composition and functional responses (proliferation) of distal PA (bottom row) are generally uniform. In the MPA, the heterogeneous pattern of cell arrangement allows the arterial media to be subdivided into three cellular 'layers': subendothelial (L1), middle (L2) and outer (L3). The outer media (L3) is comprised of two differently arranged cell populations: cells forming compact 'clusters' ('C') are oriented longitudinally, and cells in 'interstitial' ('I') areas between the clusters are oriented circumferentially. Adapted from reference.[4]

2.4 Heterogeneity of matrix-producing capabilities of SMCs in PH

Marked increases in accumulation of collagen, elastin, fibronectin, tenascin-C and other ECM proteins are observed in humans with PAH and in experimental animal models of PH. The cellular mechanisms responsible for these changes appear to be dependent on the species examined and resident cellular composition of the arterial wall. For instance, in the rat model of hypoxic PH (and similarly, in the mouse), adventitial thickening of the large PAs occurs early, whereas thickening of the vascular media lags behind.[12,46] The observed medial thickening in rodents has been suggested to be due primarily to SMC hypertrophy and increased matrix (elastin and collagen) deposition. In contrast, hypoxic PH models of larger mammals (e.g. calf and pig) demonstrate predominating early and dramatic medial thickening,[37,47] which is believed to be due, in part, to increases in proliferation and thus expansion of phenotypically distinct, less differentiated medial SMC subpopulations.[33] The species-specific differences may be explained by the fact that the cellular composition of proximal arteries in large mammals (including cow, lamb, pig and human) is far more complex than that of the rodent species.[4,37,38,48] The arterial media in large mammals is a complex organ-like structure with multiple phenotypically distinct SMC populations, which subserve diverse cellular functions in health and disease.[4,37,38,48] These findings support the notion that the heterogeneity of SMCs in pulmonary vasculature governs, at least in part, the pattern of abnormal matrix-producing capabilities that characterize chronic hypoxic forms of PH. For example, as we have demonstrated in our previous *in vitro* studies, using phenotypically distinct SMC populations isolated from the MPA of chronically hypoxic hypertensive neonatal calves, less differentiated PA-SMCs exhibited higher elastogenic responses to chronic hypoxic exposure.[34]

Importantly, various intrinsic (genetic, developmental, and epigenetic) differences in matrix-producing capabilities, as well as local and regional phenotypic heterogeneity of PA-SMCs, also regulate the pattern of remodelling of the tunica media in PH.

3. Non-medial origins of SM-like cells in the pulmonary vascular wall

3.1 Endothelial-to-mesenchymal transition

Increases in the accumulation and persistence of SM-like cells in pulmonary circulation clearly occur in all settings of PH. However, the idea that these cells derive solely from local proliferation of one of the resident cell populations described earlier, has been challenged in recent years by experimental data demonstrating many possible sources of SM-like cells not only in the lung, but also in the heart, kidney and liver. These include endothelial cells undergoing EndoMT, resident vascular progenitor cells in the process of their differentiation, recruited circulating progenitors, and/or multifunctional pro-fibrotic/inflammatory cells (fibrocytes).[49,50]

The observation that certain vascular endothelial cells are capable of undergoing an EndoMT was initially demonstrated by Frid *et al.*[51] More recently, studies by Ranchoux et al. and Hopper et al. combined to provide convincing experimental evidence that EndoMT occurs in the setting of PH in both humans and experimental animal models, and potentially constitutes a target against which specific therapeutic agents could be used to abrogate the process.[52,53] Regulation of EndoMT can involve changes in growth factors, inflammatory signalling, and the mechanical *in situ* environment.[54] At present, the best-described inducers of EndoMT are members of the transforming growth factor-beta (TGF-β) super family.[55] Interestingly, these more recent studies have observed a link between BMPR2 deficiency and EndoMT. This is important because decreases in pulmonary BMPR2 expression, which can be observed not only in familial but also in acquired forms of PH, appear to precede EndoMT and severe vascular remodelling, as has been demonstrated in at least the monocrotaline model of PH.[2,56] Yet, it remains unclear as to whether EndoMT is an essential and important contributor to the accumulation of SM-like cells in obstructive lung vascular lesions. For instance, histological assessment of lungs from patients with systemic sclerosis-associated PAH and from the sugen/hypoxia murine PH model, identify the presence of von Willebrand factor/α-SM-actin-positive transitional endothelial cells in only ∼5% of pulmonary vessels.[57] Since, during EndoMT, endothelial markers are ultimately lost in newly generated mesenchymal cells making them almost indistinguishable from other SM-like cells, there is a possibility, as will be further discussed later, that mesenchymal cells in occlusive or even plexigenic lesions may also have been derived via the EndoMT process.

3.2 Resident and circulating stem cells

In the diseased systemic vessel wall, the notion that at least some SM-like cells arise from resident and/or circulating progenitor cells is now well established.[50,58–60] Similarly, in pulmonary circulation, in the pathogenesis of vascular remodelling in PH, there is now good evidence for participation of both resident and circulating progenitors.

As noted at the beginning of this review, the appearance of new muscle in normally non-muscular regions of peripheral intra-acinar arteries seems to be universal in experimental animal models used to study PH. This was first thought to arise from multiplication of SMCs in the peripheral vasculature and their migration down the pulmonary vascular tree. However, Hislop and Reid,[61] using electron microscopy, demonstrated that the new muscle more likely represented hypertrophy and/or metaplasia of SMC precursors normally present in the non-muscular regions of the artery. These pioneering observations paved the way for more recent elegant studies by the Grief lab, using a lineage tracing approach and showing that the new precursor cells participating in the extension of SM were actually a subset of PDGFR-β-expressing SM precursors.[15,16] These investigators showed that, in mice, SM progenitor cells gave rise to the distal musculature as if 'primed' to do so.[15,16] An important finding of these studies is the demonstration that the process of distal muscularization in hypoxic mice is clonal in nature; this offers unique opportunities to identify molecular signals that drive specific clones to expand. Moreover, these findings also suggest SMCs with distinct potentials for growth (like progenitor cells) vs. cell growth arrest, as seen with fully differentiated and long-lived cells.

Recently, Dierick *et al.* identified resident *PW1*$^+$ progenitor cell populations located in both the lung parenchyma and in perivascular zones of small peripheral vessels, which show an early recruitment and a potential to differentiate into SM-like cells during chronic hypoxia-induced PH.[62–64] *PW1* encodes a zinc finger protein that has been shown to regulate cell cycle and cell stress responses caused by inflammation and p53.[62–64] Interestingly, *PW1* has also been shown to function as a transcription factor with a DNA-binding motif regulating a large array of genes involved in metabolic homeostasis.[65] These cells are also characterized by the expression of CD34, c-Kit and PDGFRα, all markers used to identify vascular progenitor cells with the capability of differentiating into SM-like cells. Importantly, these studies confirmed a resident origin for at least these new SMCs that were not derived from circulating BM-derived progenitors. It appears that *PW1* identifies multiple progenitor populations. Further studies are needed to determine the respective role of each of the *PW1*-expressing cell populations, with the ultimate goal of inhibiting mobilization, proliferation or differentiation of these cells as a potential therapeutic avenue to an early treatment of PH.

Davie *et al.*[66] were among the first to examine the possibility that circulating progenitor cells contribute to the PH-associated pulmonary vascular remodelling. These initial studies documented the recruitment of cKit$^+$ cells to the adventitial compartment but did not demonstrate unequivocally that these cells acquired SM-like properties. Shortly thereafter, Hayashida et al. used green fluorescent protein bone marrow (BM) transplanted (GFP-BMT) chimeric mice to investigate the possible role of BM-derived cells in hypoxia-induced pulmonary vascular remodelling.[67] They demonstrated a significant increase in the number of GFP$^+$ BM-derived cells in the hypoxic PA wall and showed an increase in the number of GFP/α-SMA expressing cells within the vascular media and adventitia. Subsequently, utilizing the calf model of PH, where, in the distal PA, control animals express a phenotypically uniform SMC composition, it was noted that the remodelled distal PA media of calves with severe hypoxia-induced PH comprises cells with a distinct phenotype characterized by the expression of haematopoietic (CD45), leukocyte/monocytic (CD11b, CD14), progenitor (cKit), and motility-associated (S100A4) cell markers[68] (*Figure 5*). Consistent with these *in vivo* observations, primary cultures generated from the distal PA media of hypertensive calves yielded both differentiated SMCs as well as smaller, morphologically 'rhomboidal' cells that transiently expressed CD11b, expressed progenitor markers cKit, CD34, CD73 and, with time in culture, gained expression of α-SM-actin. It is possible that these progenitor cells account for the disease-acquired heterogeneity of the distal pulmonary vasculature.[68] These SM-like cells (termed 'R'-cells for their morphological rhomboidal appearance) exhibit higher mRNA expression for IL6, CCL2/MCP1 (*Figure 6*), and S100A4 (not shown) and are hyperproliferative, even under serum-deprived conditions, compared with a population of differentiated SMCs grown from the same vessel (*Figure 6*). Thus, it is possible that cells from the circulation are recruited to the distal media that can differentiate to express α-SM-actin that remain distinct from resident medial SMCs. These cells also secrete growth factors

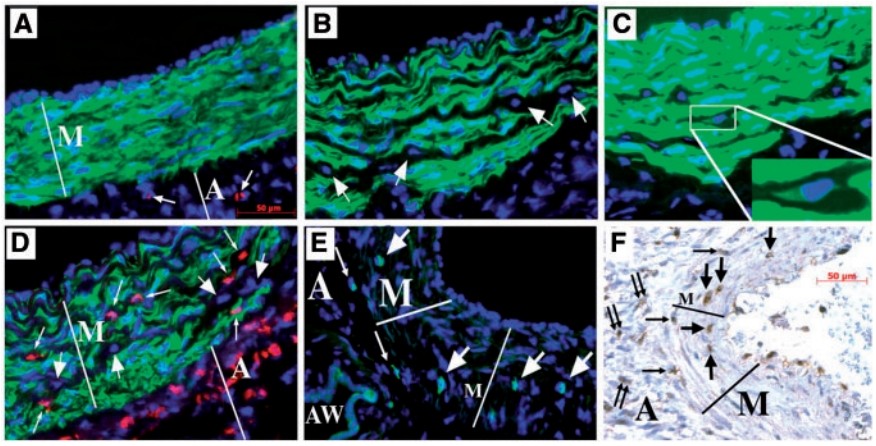

Figure 5 Exposure of neonatal calves to chronic hypoxia induces, within the media of distal PAs, the appearance of cells phenotypically distinct from differentiated SMCs. (*A*) in control normoxic calves, distal arterial media is comprised of a phenotypically uniform SMC population (α-SM-actin; green). (*B–F*) in chronically hypoxic calves, a markedly thickened media of distal PAs is composed of a phenotypically heterogeneous cell population, where certain cells do not express α-SM-actin (*B*, arrows), other express low levels of α-SM-actin (*C*, *inset*), some cells express a leukocytic marker CD45 (D, small arrows pointing to the red-labelled cells; and large arrows point to cells that do not express either α-SM-actin or CD45). There are a few cells that express a progenitor marker cKit (*E*, green) within the media (large arrows) or at the medial-adventitial border (small arrows). Several cells within the media express S100A4 (*F*, arrows pointing to brown cells). (*A–E*) Cell nuclei are labelled in blue (DAPI). M, media; A, adventitia, AW, airway. Adapted from reference.[68]

including PDGF and CXCL12 that stimulate proliferation of resident SMCs *ex vivo*.

Another population of circulating progenitor cells involved in pulmonary vascular remodelling is comprised by $CD133^+$ cells.[69–72] These cells are capable of differentiating into haematopoietic, endothelial, SM, and neuronal cell types. In response to chronic hypoxia, circulating BM-derived $CD34^+CD133^+Flk^+$ cells are recruited to the adventitial, medial, or intimal compartments where they assume mesenchymal, SM-like characteristics.[69–72] Intriguingly, *in vitro* these cells have been reported to acquire the morphology and phenotype of the cells with which they are co-cultured.[73] For example, when these $CD133^+$ cells were co-cultured with human PA segments, they migrated through the intima and differentiated into SMCs.

Interestingly, transplantation of BM-derived $CD133^+$ progenitor cells from patients with PAH into mice resulted in angio-proliferative pulmonary vascular remodelling, right ventricular failure and death.[74] Recent studies have begun to elucidate the molecular mechanisms that underlie the expansion and differentiation of $CD133^+$ cells into SM-like cells. Overexpression of glucose 6-phosphate dehydrogenase (G6PD) is required for and promotes $CD133^+$ cell proliferation.[75] G6PD activity upregulated expression of hypoxia inducible factor-1α (HIF1α), cyclin A and phospho-histone H3, thereby promoting CD133+ cell dedifferentiation and self-renewal.[75] Expression of SMC contractile proteins in these cells is also dependent on G6PD activity. Interestingly, administration of dehydroepiandrostrone, which is known to block G6PD activity, inhibited accumulation of $CD133^+$ cells around PAs and reduced formation of vascular obstructive lesions.[75,76]

Lastly, it should be noted, the BM-derived stem cells can effect vascular remodelling and stiffening through paracrine effects on SMCs or other cells in the vascular wall. Launay *et al.*[77] demonstrated a causal link between BM-derived stem cells and serotonin (5-HT) in PH. They established that BM-derived stem cells are critical for PH development and further established a causal link between recruited cells and 5-HT by showing that blocking 5-HT2β receptor receptors (or genetically knocking them out specifically only in BM cells) abrogated PA development. Studies in BMPR2 mutant mice treated with 5-HT2β inhibitor also showed that 5-HT signalling in BM-derived cells is critical in PH development.[78] Thus, more work interrogating BM-derived progenitor cell interactions with SMCs is needed in PH models.

4. Pro-inflammatory phenotype of SMCs in PH

It is now apparent that PH is associated with classical cellular and molecular components of inflammation, which may direct specific SMC populations in their contribution to pulmonary vascular remodelling. Current evidence supports the idea that the emergence of the hyperproliferative SM-like cell is nearly always observed in the context of local changes in the inflammatory milieu of the vessel wall; however, this same proinflammatory pulmonary environment can shape long-lived, non-proliferative SMCs as well (see discussion below on senescent phenotype). It is increasingly clear that inflammation is observed in all described experimental animal models of PH, as well as in humans with PAH and other forms of chronic PH.[50,79,80] Several studies have demonstrated that sustained hypoxia (perhaps the best studied animal model related to recruitment of inflammatory and progenitor cells) induces a robust accumulation of leukocytes and mesenchymal progenitor cells in perivascular areas of the PAs.[81–83] The majority of studies in PH have demonstrated that the principal inflammatory cell types recruited to and retained in the hypoxic lung vasculature are those of mononuclear origin. Recruitment and retention of these cells are critical for hypoxia-induced pulmonary vascular remodelling, as has been demonstrated by *in vivo* depletion of circulating mononuclear phagocytes using liposome-encapsulated clodronate, which abrogated the pulmonary vascular remodelling.[84] These observations are consistent with studies in the systemic circulation

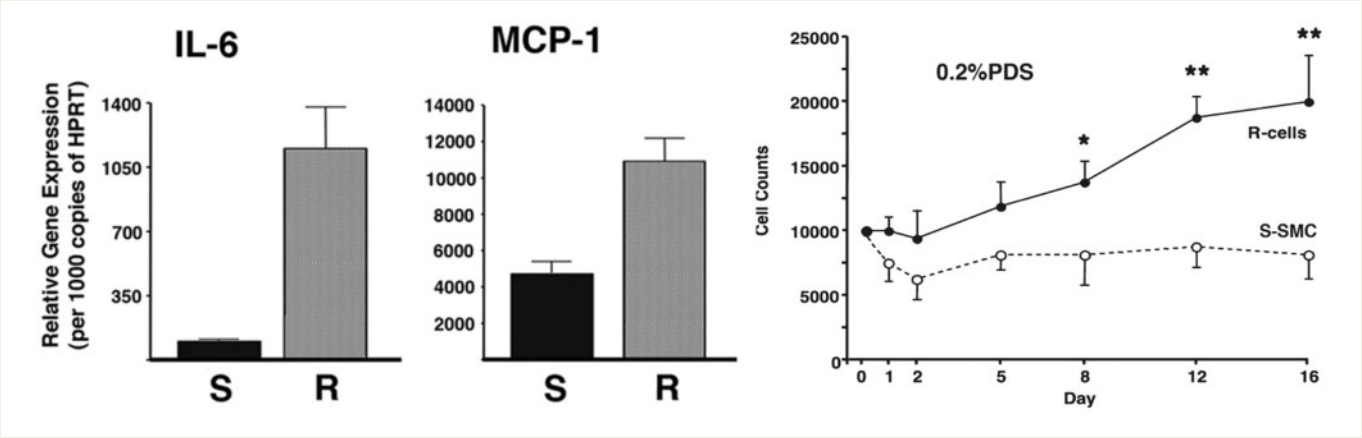

Figure 6 *Ex vivo*, morphologically 'rhomboidal' SM-like cells ('R'-cells) [corresponding to cells, shown in *Figure 5*, expressing α-SM-actin (but not SM-myosin), cKit and S100A4] express higher mRNA levels for inflammatory mediators IL6 and CCL2/MCP1 compared with differentiated SMCs ('S'-SMCs). Furthermore, R-cells exhibit autocrine, serum-independent growth (0.2% plasma-derived serum), whereas differentiated 'S'-SMCs remain growth-arrested under these conditions (*$P < 0.05$, **$P < 0.01$). Adapted from reference.[68]

showing that macrophages play an essential role in both outward and inward remodelling.[85–87] Burke *et al.*[83] used laser capture microdissection of the remodelled PAs from hypoxic hypertensive rats and found a progressive accumulation of monocytes/macrophages and dendritic cells but only a few T-cells and no B-cells or neutrophils. Upregulation of CXCL12/SDF-1, VEGF, C5, ICAM-1, osteopontin and TGF-β preceded mononuclear cell influx. The cellular source of these cytokines was not precisely determined in this study, but was likely a combination of both medial and adventitial cells. Both SMCs and fibroblasts produce chemokines and cytokines, including IL-1β, IL-6, CCL2/MCP-1 and CXCL10 capable of recruiting and activating monocytes/macrophages. However, by and large, in both the pulmonary and systemic circulations in the disease process, the macrophage infiltrate appears largely in the adventitia, sparing the media.[79,85,88,89] Studies by several laboratories support a mechanistic role for cytokines and inflammation in the disease process by showing that chemical inhibition and/or genetic knockout of CXCL12/SDF-12, C-C chemokine receptor type 5, CXCR7 and IL-1β/MyD88 all led to attenuation of hypoxia and/or monocrotaline-induced PH with reduced inflammatory cell recruitment and inflammation.[90–94]

It seems highly likely that the inflammatory capacities of the SM-like cells in the diseased vessel wall are heterogeneous, similar to that previously noted for proliferating SMCs, and depend on their precise origin. In support of this are observations of a cell population that accumulates in the media of the distal vasculature of hypoxic hypertensive calves, which originates from circulation yet acquires expression of α-SM-actin, and exhibits a distinct pro-inflammatory phenotype (with augmented expression of IL-6 and CCL2/MCP-1), compared with the resident SMCs within the vessel wall. It is possible that these cells contribute to the continued inflammation observed in the distal vasculature. More studies are needed to define the inflammatory capabilities of various heterogenic populations of SMCs contributing to vascular disease.

Collectively, these studies point to the fact that acute, and more importantly non-resolving, inflammation plays a prominent role in irreversible pulmonary hypertensive process. It is possible that as SMCs shift from a proliferating state to cell arrest, they are maintained in a distinct phenotype characterized by abnormal pro-inflammatory cytokine production and a locked altered metabolic state. This distinct phenotypic state, which is discussed below, has been described in so-called senescent cells; their potential critical role in chronic stages of PH is beginning to be appreciated.

5. Role of metabolism in SMC phenotypic changes

All WHO Groups of PH, but particularly those in Group 1, are characterized by a complex panvasculopathy that recapitulates features similar to those observed in cancer, including excessive proliferation, apoptosis resistance, inflammation and a dramatic remodelling of the extracellular matrix.[80,95–97] Borrowing from recent advancements in cancer[98–100] and immune research,[101–106] it has been suggested that PH phenotypes might be, in part, explained by a substantial metabolic/pro-inflammatory reprogramming of vascular wall cells, including adventitial fibroblasts and macrophages. In cancer research, it is now accepted that most oncogenes and mutated tumour suppressor genes play a primary role in metabolic regulation.[107] Conversely, aberrant metabolism can promote epigenetic control of oncogenic re-programming, even in the absence of specific mutations, in response to hypoxic or cytokine-triggered stimuli, promoting a pro-inflammatory and pro-fibrotic phenotype that favours both hyper-proliferation and prolonged survival of cancer and cancer-associated cells; of note, a similar overall picture also applies to immunity[99,104,105,108] Studies on the metabolic reprogramming of activated immune cells indicate that such a complex phenotype can be observed in the absence of specific genetic mutations.[104] This observation prompted cardiovascular investigators to wonder whether similar molecular underpinnings contribute to PH aetiology, fuelling the debate around the metabolic theory of PH.[109–111]

Importantly, a chronic shift in energy production from mitochondrial oxidative phosphorylation to glycolysis has been described to occur in SM-like cells, as well as in endothelial cells and fibroblasts. This phenotype, originally described in cancer and termed the Warburg Effect, contributes to both PA SMC hyper-proliferation (notably early in the disease) and to resistance to apoptosis (with established disease).[111,112] The observed metabolic shift towards glycolysis increases the availability

of non-oxidized amino acids, lipids, and sugars, which are all necessary for rapid cell proliferation. Further, it has been described that, in PH, PA SMCs have hyperpolarized mitochondria, which produce less ROS.[112,113] The reported decreases in mitochondrial ROS production are associated with the well described decrease in Kv channels, and attenuated activity and expression at the plasma membrane, increased intracellular calcium levels, and increased activity of transcription factors, including nuclear factor of activated T-cell and HIF-1α, leading to increased proliferation and decreased apoptosis. Again, these changes have been extensively reviewed and are relevant not only to the hyperproliferation state, but also to apoptosis resistance of SM-like cells, endothelial cells, and certain populations of adventitial fibroblasts.[114]

A potential driver of altered SMC cellular metabolism is increased TGF-β signalling, which is associated with a glycolytic shift in other contexts including cancer.[115,116] Altered TGF-β signalling via BMPR2 and other genetic mutations underlie the majority of familial cases of PAH. Kumar and colleagues recently reported that inflammation drives the recruitment of thrombospondin-1 (a known activator of latent TGF-β) expressing monocytes to the pulmonary adventitia, resulting in TGF-β activation and resulting in expansion of the pulmonary vessel medial layer.[117] The Isenberg group has documented another function of thrombospondin-1 is signalling through CD47 to block nitric oxide signalling, which may also lead to SMC dysfunction.[118] Sheppard and colleagues also recently showed that SMC-specific deletion of the TGF-βR2 receptor blocked development of the PH phenotype in a mouse model of scleroderma.[119]

6. Senescence phenotype of SMCs

Given that there is no evidence of continuous SMC proliferation in PH, it is conceivable that several of the early SMC mitogenic responders to a PH stimulus may eventually acquire a senescent phenotype. Senescent cells, which are long-lived, metabolically and functionally active cells, are unable to re-enter the mitotic cycle while resisting apoptosis or other cell death processes.

Senescence refers to a characteristic behaviour, first noticed in cultured cells, of long-lived cells, which are incapable of cell proliferation. Additional characteristics include increased cell size, expression of senescence associated β galactosidase activity (SA-βgal) and the cell cycle inhibitors $p16^{Ink4a}$ and $p21^{WAF1/Cip1}$, and potential presence of the DNA damage response (DDR).[120] There are several molecular processes linked to senescence, which however cannot account to the full range of experimental observations linked to this key cellular state. The most notable is the observation of telomere shortening in cultured cells that progressed to cell growth arrest. Telomere shortening likely triggers the DDR, as could also be triggered by strong mitogenic stimuli, and therefore causes senescence. The major pathophysiological implications of cellular senescence are the cell's inability to further proliferate and their acquisition of an inflammatory phenotype (labelled senescence-associated secretory phenotype or SASP), largely characterized by overexpression of IL-1, IL-6 and IL-8.[120]

At the organismal level, senescence has been observed and related to processes of aging, which can be defined as loss of fecundity, increased susceptibility to disease, and enhanced risk of death.[121] In summary, these events are due to loss of homeostatic mechanisms involved in organ structure and function. The relation between senescence and aging varies, being either considered as associative or contributory.[120] Many age-related diseases have increased expression of cells with markers of senescence and some experimental age-related disease can be attenuated with depletion of $p16^{Ink4a}$ expressing cells[122]; however, it is unclear if the senescent cells play a role in age-related human diseases. Moreover, senescence may represent a pathogenetically relevant *in vivo* response that per se may not be associated with increased age, as in diseases such as lung fibrosis and PH.

The roles of senescence in lung diseases were initially identified in chronic obstructive pulmonary disease (COPD), notably in emphysematous tissue[123] with subsequent support by evidence of telomere shortening in peripheral blood mononuclear cells from COPD patients.[124] More recently, the role of senescence was expanded to pulmonary fibrosis,[125] including a genetic link with the discovery of telomerase mutations in idiopathic pulmonary fibrosis.[126,127] A causal role of senescence in COPD and pulmonary fibrosis is anticipated given the close association with increasing age of affected individuals.[128] It is therefore not surprising that PH or pulmonary vascular remodelling in the setting of COPD has been linked to SMC senescence.[129] In this study, telomere length assessed in peripheral blood mononuclear cells correlated inversely with IL6 levels and PA pressures assessed by echocardiography. These findings are in line with the burden of oxidative stress in smoking (which leads to of DNA damage,[130,131] adduct-modified proteins[132] and telomere shortening[133]). As mentioned previously, DNA damage is a critical determinant of senescence.

A potential role for PA SMC senescence in PH or pulmonary vascular disease was inferred from studies on COPD patients, with some patients having echocardiography-based evidence of mild PH.[129] PAs of patients with mild COPD had increased numbers of SMCs positive for SA-βgal, $p16^{Ink4a}$ and $p21^{WAF1/Cip1}$, reaching up to almost 48% of counted cells. At the same time, almost 50% of SMCs were positive for Ki67, implying that they were undergoing mitosis. This dichotomy of SMCs either in senescence or proliferation is surprising, as one might anticipate that the senescence phenotype would be more restricted given the wide range of SMC phenotypes; moreover, it is unclear from the study which specific SMC cell populations gave rise to the senescent-marker expressing cells. The study goes on demonstrating that, in culture, SMCs originating from COPD-PAs developed senescence in earlier passages than control SMCs and produced the SASP of cytokines. These cytokines could then drive increased proliferation and migration of the non-senescent SMCs. Whether the process of cellular senescence applies to PAH (a more severe disease, usually clustered as WHO Group 1 PAH[134]) remains unclear.

PAH (as compared with PH associated with COPD, known as WHO Group 3 PH) is considered a more proliferative process, notably in endothelial cells as they form plexiform lesions.[135] Given the evidence of increased telomerase activity in proliferative processes (like malignancy and after vascular injury), one might predict that PAH SMCs might rather have increased telomerase activity as compared with controls or PH related to COPD. In PAH, telomerase reverse transcriptase (TERT) expression was increased in $CD44^{+}$ SMCs located in the outer rim of remodelled PAs, but was not expressed in α-SMC actin-positive cells or KI67-negative cells. No control SMCs expressed TERT.[136] Mice knocked out for TERT or wild type mice treated with a TERT inhibitor had attenuated PA pressures when challenged with hypoxia, while having increased expression of $p16^{Ink4a}$ and $p21^{WAF1/Cip1}$ in pulmonary vascular SMC. Of note, these results are in opposition to those presented in human studies of COPD remodelled PAs, in which senescence is increased and promotes SMC growth.[129] It is therefore unclear whether, in PAH, there is cellular senescence that might drive the overall pro-remodelling phenotype seen in PAH or even in hypoxia driven PH.

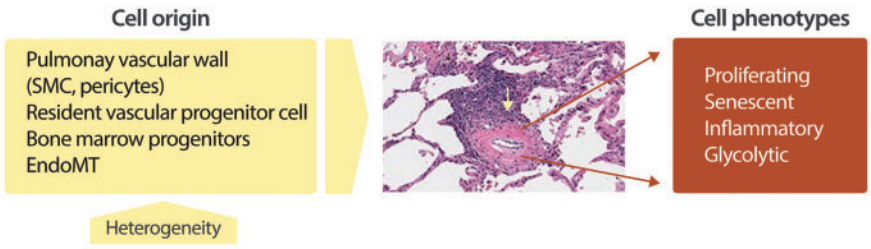

Figure 7 Potential SMC origins and phenotypes in PH. Central image shows vascular remodelling in medium-sized PA, characterized by thickening of tunica media (arrow) with lymphocytic cluster of inflammatory cells, largely lymphocytes (short arrow).

In summary, the potential conflictive roles of SMC senescence may be as conflictive as those delineated in the cancer field.[120] On one hand, in proliferation-prone states, like in specific SMC populations in PAH or even early hypoxic PH, senescence may be a protective mechanism by disengaging the proliferative SMC process. On the other hand, in late disease when proliferation may no longer play a critical role, SMC senescence may be pathological by promoting persistent inflammation, recruitment of progenitor cells and a switch to matrix production.

7. Metabolic characteristics of cellular senescence

As alluded previously, several key features of metabolic adaptation may participate in the phenotype of endothelial cells and SMC in PH PAs. The predominant metabolic features involve aerobic glycolysis, which have been reviewed previously.[97,137,138] As outlined earlier, hypertensive SMCs utilize glycolysis to generate a pro-inflammatory feedback interactions with pulmonary vascular fibroblasts, ultimately contributing to pulmonary vascular remodelling.

Interestingly, senescent cells are largely glycolytic as well.[139] This metabolic adaptation would serve, as in highly proliferating cells, to provide carbon intermediates for macromolecular synthesis, most notably of proteins and phospholipids [140] (as nucleic acids are dispensable due to growth arrest). However, the teleological reasoning behind the preferential use of glycolysis vs. mitochondria metabolism in the maintenance of senescence remains unclear. One unexplored reason would reside in the preservation of the SASP phenotype, notably of IL6 production and STAT3 activation.

Moreover, key metabolic alterations associated with or causing senescence shape the metabolic landscape of these cells. ADP and AMP levels increase in relation to ATP, causing activation of the enzyme AMPkinase. AMPkinase can promote cell growth arrest via activation of p53 and via promotion of $p16^{Ink4a}$ and $p21^{WAF1/Cip1}$ stabilization. Moreover, the levels of NAD^+ (in relation to its reduced form NADH) decrease and thus trigger a multi-pronged molecular process that favours senescence: reduction of NAD^+ leads to decrease the DNA damage repair activity of PARP; furthermore, reduced NAD^+ decreases the activity of SIRT 3 and 5 (which with SIRT6), which also contribute to senescence.

Mitochondria mass increases in senescent cells and plays a key role in the acquisition of the SASP phenotype, whereas depletion of mitochondria DNA, while promoting senescence, blocks SASP. However, the role of mitochondria in senescence is complex, in that ROS originating from mitochondrial oxidative phosphorylation promotes senescence, possibly via DDR.

As the role of senescence in accounting for the unique heterogeneity of SMC phenotypes in PH remains largely unexplored, the angle of the metabolic interface between the different SMC phenotypes might offer unique insights into how to dissect this complex interaction. Senescent cells define their organ microenvironment in that these cells lose proliferative and repopulation properties; however, they also affect via paracrine interactions how the microenvironment reacts to proliferative cues.

8. Conclusion

The spectrum of SMC origin and phenotype, particularly in disease, is evolving (*Figure 7*) and, though complex, may lend itself to potential therapeutic targeting. To reach this goal, it is critical that specific PA SMC phenotypes be ascribed specific roles in the disease process, including the temporal relation with the course of disease. Incorporation of cell fate mapping using key transcription markers characteristic of specific phenotypes allied to clonal markers, will provide important tools to delineate specific contributions and the relative important of the different types of PA SMCs. Other pulmonary fields, including those addressing origins and phenotypes of macrophages and type II cells, are paradigms that have utilized these tools, which can serve as a framework to be applied to PA SMCs. At present, we do not know of any studies, which have specifically tried to target the functional phenotype of a specific subset of SMC or SM-like cells in PH. Certainly there continues to be great interest in inhibiting SMC proliferation. Several agents have been utilized in animal models with success including inhibitors of receptor tyrosine kinase, mTOR, p38, CDK4/6 among others. Again, a subset of human iPAH patients responded clinically to Imatinib treatment. Future work will define the subset(s) of patients who may respond to these therapies at a point in time when SMC proliferation is contributing significantly to disease pathogenesis.

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