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Bovine distal pulmonary arterial media is composed of a uniform population of well-differentiated smooth muscle cells with low proliferative capabilities

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Stiebellehner, Leopold, Maria G. Frid, John T. Reeves, Robert B. Low, Meena Gnanasekharan, and Kurt R. Stenmark. Bovine distal pulmonary arterial media is composed of a uniform population of well-differentiated smooth muscle cells with low proliferative capabilities. *Am J Physiol Lung Cell Mol Physiol* 285: L819–L828, 2003. First published July 11, 2003; 10.1152/ajplung.00062.2003.—The media of the normal bovine main pulmonary artery (MPA) is composed of phenotypically heterogeneous smooth muscle cells (SMC) with markedly different proliferative capabilities in response to serum, mitogens, and hypoxia. Little, however, is known of the SMC phenotype in distal pulmonary arteries (PA), particularly in arterioles, which regulate the pulmonary circulation. With a panel of muscle-specific antibodies against α-smooth muscle (SM)-actin, SM-myosin heavy chains (SM-MHC), SM-MHC-B isoform, desmin, and meta-vinculin, we demonstrate a progressive increase in phenotypic uniformity and level of differentiation of SMC along the proximal-to-distal axis of normal adult bovine pulmonary circulation so that the media of distal PA (1,500- to 100-μm diameter) is composed of a phenotypically uniform population of "well-differentiated" SMC. Similarly, when isolated and assessed in vitro, distal PA-SMC is composed of a single, uniform population of differentiated SMC that exhibited minimal growth responses to a variety of mitogens while their cell size increased substantially in response to serum. Their growth was inhibited by hypoxic exposure under all conditions tested. Distal PA-SMC also differed from MPA-SMC by exhibiting a distinct pattern of DNA synthesis in response to serum and mitogens. Thus, in contrast to the MPA, distal PA media is composed of an apparently uniform population of well-differentiated SMC that are proliferation resistant and have a substantial capacity to hypertrophy in response to growth-promoting stimuli. We thus speculate that distinct SMC phenotypes present in distal vs. proximal PA may confer different response mechanisms during remodeling in conditions such as hypertension.

resistance arteries; pulmonary hypertension; smooth muscle heterogeneity; hypertrophy; hypoxia

UNDERSTANDING THE FUNCTIONS of the various segments of the normal pulmonary circulation requires, at least in part, a knowledge of the cellular composition of the vascular media at specific sites along the arterial axis. We have previously shown that, in the bovine pulmonary circulation, the normal media of the main pulmonary artery (MPA) is complex, being composed of several distinct cellular regions. Within these medial regions, the cells are remarkably different with regard to morphology, orientation, smooth muscle (SM) phenotype, and proliferative and matrix-producing capabilities (8–10). However, the pattern of medial smooth muscle cell (SMC) composition at more distal sites in the pulmonary circulation, particularly in the arterioles, the locus of pulmonary circulatory control, is not known.

It has long been proposed that the MPA exhibits multiple functions that may account for its complex structure and need for multiple SMC phenotypes (3). The primary function of the distal pulmonary arterioles is vasoconstriction, which calls for SMC with "contractile" phenotype. Thus we hypothesized that the normal media of distal arterioles is composed of a phenotypically uniform population of SMC with a "well-differentiated" so-called contractile phenotype. Important aspects of a well-differentiated SM phenotype include expression of SM proteins known to define a mature contractile SMC and a quiescent nonproliferative phenotype (19). When used in combination, these markers can help define "the level of differentiation" of SMC (9, 10, 19). Therefore, our approach was, first, to evaluate in vivo the phenotype of SMC at different loci along the proximal-to-distal axis of the pulmonary arterial circulation utilizing a panel of specific antibodies against cytodifferentiation-related proteins [α-SM-actin, SM-myosin heavy chains (SM-MHC), SM-MHC-B isoform (SM-MHC-B), desmin, and meta-vinculin]. Second, we isolated and cultured distal pulmonary arterial (PA)-SMC to evaluate their growth capabilities in response to mitogenic stimuli and hypoxia and to contrast them with SMC derived from the

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MPA. Our in vivo and in vitro data demonstrate that the normal media of the small PA, at least in bovine species, is composed of SMC of a uniform well-differentiated phenotype as defined by expression of several SM markers and low proliferative capability.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies against α-SM-actin (clone 1A4; Sigma Chemical, St. Louis, MO) and desmin (clone D33; Dako, Carpinteria, CA) were both used at 1:200 dilution. Affinity-purified rabbit polyclonal antibodies against bovine aortic SM-MHC were kindly provided by Dr. R. S. Adelstein (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) (14) and used at 1:2,000 dilution. These antibodies react strongly with SM-1 and SM-2 isoforms of SM-MHC in bovine arterial tissue and, at the dilutions used, do not recognize nonmuscle MHC isoforms (10). The rabbit polyclonal SM-MHC-B antibodies have been previously described (16, 17) and were used at 1:200 dilution. These antibodies identify specifically the seven-amino acid insert present in the SM-MHC-B and do not cross-react with either SM-MHC-A isoform, which lacks this seven-amino acid insert, or with nonmuscle MHC. Affinity-purified rabbit antibodies against meta-vinculin (used at 1:50 dilution) were generated as previously described and shown to react specifically with meta-vinculin but not vinculin (10).

Tissue Immunohistochemistry

The MPA and left lung were obtained at a commercial abattoir from 13 adult (~2-yr-old) bovine animals (9 males, 4 females). Samples from the MPA and extralobar PA were immersed in the alcohol-based Omnifix (Xenetics Biomedical, Tustin, CA) for 12 h. The left lower lung lobe was distended with the fixative at 25 cmH_2O and perfused through the PA for 12 h. After fixation, blocks of tissue were taken from the MPA, lobar PA, and lung tissue. The blocks were then embedded in paraffin with a standard procedure and cut into 4-μm-thick sections. Hematoxylin/eosin-stained sections were used to ensure that the chosen sections of lung tissue contained PA with an inner diameter of ~3,000 μm to ~1,000 μm and arterioles at the levels of the bronchoalveolar junction and alveolar duct. Histological sections were deparaffinized with xylene and rehydrated in a graded ethanol series. Endogenous peroxidase was quenched by incubation with 0.3% H_2O_2 (vol/vol) in absolute methanol. Sections were then treated with 0.01% Pronase (Sigma Chemical) in PBS. After being blocked with 5% serum in PBS for 20 min, the sections were incubated sequentially with the primary antibodies, the appropriate secondary antibodies (biotinylated anti-mouse or anti-rabbit IgG), and avidin-biotin complex (ABC)-peroxidase complex. Peroxidase binding was then visualized with 3,3′-diaminobenzidine substrate, and sections were counterstained with hematoxylin. As a control, serial sections underwent the same procedure with the substitution of the primary antibody with nonimmune rabbit serum or mouse ascites (Sigma Chemical).

Cell Isolation

Cell populations from the media of the MPA were obtained as previously described using modified explant techniques (9). For isolation of medial cells from the distal PA, 5-mm-long segments of distal arterioles from the distal lung were obtained. The proximal half of the vessel was frozen in optimum cutting temperature compound (Sakura Finetek USA, Torrance, CA) and used for phase-contrast microscopy to assess the internal diameter of the vessel (670–1,340 μm, mean 994 ± 25 μm). The distal half of the vessel was utilized for cell culture. The adventitia was removed under a dissecting microscope, the artery was opened, and the endothelial cells were gently scraped off with a flexible plastic cell scraper. The remaining media was cut into 1-mm^2 pieces, placed in a culture dish, and maintained in complete DMEM (Sigma Chemical) supplemented with 200 U/ml penicillin, 0.2 mg/ml streptomycin, and 10% calf serum (CS; HyClone Laboratories, Logan, UT). After 10 days, the explants were removed, and the primary SMC cultures were expanded (with split ratio of 1:4) using standard culture techniques. All cell cultures were studied at the same passage number and under similar culture conditions. With time, in 3 out of 30 primary cultures, the appearance of large, irregularly shaped, markedly vacuolated cells was noted. Because of their appearance and their failure to grow in subculture (even in 10% serum), these cultures were considered senescent (2) and were not taken for further experiments.

Immunofluorescence Analysis of Cultured Cells

Cells isolated from the media of distal PA were assessed for expression of muscle-specific contractile and cytoskeletal proteins, α-SM-actin, SM-MHC, and meta-vinculin using indirect single- and/or double-label immunofluorescence staining techniques (9). Briefly, cells were grown to confluence on Tissue-Tek chamber slides (Tissue-Tek Nunc) in 10% CS, growth arrested in 0.1% CS for 48 h, and fixed in absolute methanol at −20°C for 10 min, and nonspecific adsorption was blocked with serum for 20 min. Incubation with primary antibodies against α-SM-actin, SM-MHC, and meta-vinculin (at dilutions mentioned earlier) was for 1 h at room temperature. As secondary antibodies, biotinylated anti-mouse IgG (for α-SM-actin monoclonal antibody) or biotinylated anti-rabbit IgG (for SM-MHC and meta-vinculin polyclonal antibodies; both from Amersham) were used at dilutions recommended by the supplier. Controls were performed in which primary antibodies were replaced by nonimmune rabbit serum or nonimmune mouse ascites (Sigma Chemical). The stained cells were examined with a Nikon Optiphot epifluorescence photomicroscope. Photomicrographs were taken with Ektachrome 160T film.

DNA Synthesis and Cell Growth

DNA synthesis in response to serum, purified mitogens, and hypoxia was determined by measuring [^{3}H]thymidine incorporation as previously described (6). Briefly, cells were plated at 50×10^3 cells/well onto 24-multiwell plates in DMEM supplemented with 10% CS. The next day, cells were rinsed with PBS and growth arrested in 0.1% CS for 72 h. No significant differences in plating efficiency among the cell populations were observed. To assess DNA synthesis, serum-free DMEM supplemented with 0.5 μCi/ml of [^{3}H]thymidine (NEN Life Science Products, Boston, MA) was added for the 24-h period along with one of the following mitogens: platelet-derived growth factor-BB (PDGF-BB; 10 ng/ml), basic fibroblast growth factor (bFGF; 30 ng/ml), or IGF-I (100 ng/ml; all from R&D Systems, Minneapolis, MN). Concentrations were chosen based on prior results (6, 9). As positive and negative controls, [^{3}H]thymidine incorporation in 10% CS and 0.1% CS alone was assessed. Four wells with cells of the same subpopulation were assessed for each mitogen. Cell counts were concurrently obtained from four additional wells. Incorporation of [^{3}H]thymidine was expressed as dis-

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integrations per minute per 10^3 cells. For evaluation of the effects of hypoxia on DNA synthesis, cells were plated and growth arrested as described earlier, [^{3}H]thymidine was added, and cells were placed in sealed, humidified gas chambers (Bellco Glass, Vineland, NJ) that were purged with either 21 or 3% O_2 for 20 min (either gas contained 5% CO_2 and the balance N_2). Chambers were then placed in the 37°C incubator for 24 h, again based on prior results (6, 9). Measurement of [^{3}H]thymidine incorporation was then performed as described earlier. For assessment of cell growth and construction of growth curves, cells at *passages 5–6* were plated onto 24-multiwell plates at a density of 10×10^3 cells/well in DMEM supplemented with 10% CS. Four wells were trypsinized and counted in a standard Spotlite hemocytometer (Baxter Diagnostics, McGaw Park, IL) on *days 1, 3, 5, 7*, and *10* after seeding.

[^{35}S]methionine Incorporation

Cells were plated and growth arrested as described for DNA synthesis assay. Ten percent CS was then added along with [^{35}S]methionine (1 μCi/ml) for 24 h. Cells were then harvested by precipitation with trichloric acid followed by dissolution in 0.2 N NaOH. Incorporated [^{35}S]methionine was assessed in a liquid scintillation counter.

Cell Size Analysis

To assess the cell size in primary cultures, multiple explants from the media of distal PA were placed in a T-75 culture flask with DMEM supplemented with 10% CS. After 10 days, the explants were removed and the medium thereafter changed every third day. Before cells became confluent, they were washed with PBS, trypsinized, and diluted in Isoton reagent (Beckman-Coulter). Size distribution measurements were carried out with a Coulter counter. To assess changes in cell size after a period of serum stimulation, cells were seeded in two T-75 culture flasks at a density of 25×10^3 cells/cm^2. After 24 h, cells were growth arrested with 0.1% CS for 72 h. For controls, cells in one flask were rinsed with PBS, trypsinized, diluted in Isoton, and processed for size measurement. Concurrently, cells in the second flask were refed with 10% CS. The serum-containing medium was changed after 4 days. After a total of 7 days in 10% CS, cells were processed for size measurement as described earlier. Changes in size were expressed as percent change from the control (0.1% CS).

Statistical Analysis

Data were obtained from cell cultures isolated from a total of 30 arteries from 13 animals. Data are presented as means ± SE. Statistical analysis was performed by ANOVA, followed by Tukey-Kramer's multiple comparisons test, with significance accepted at $P < 0.05$.

RESULTS

Pulmonary Artery Medial Cell Composition Becomes More Uniform as a Function of Distance From the Heart

With the use of immunohistochemical staining with a panel of specific antibodies against markers of SM phenotype (α-SM-actin, SM-MHC, SM-MHC-B, desmin, and meta-vinculin), we compared the phenotype of cells composing the pulmonary arterial tree along its proximal-to-distal axis. For the present analysis, if the cells expressed all markers tested, they were considered well-differentiated SMC. If they did not express any of the markers, they were called "nonmuscle." If they expressed some but not all of the markers, their phenotype was considered "intermediate" SM. We have previously described that the MPA was characterized by marked cellular heterogeneity of the tunica media (10). In the present study, we analyzed the phenotype of SMC at different loci along the proximal-to-distal axis of the pulmonary arterial circulation and contrasted it with that of the MPA.

As we have previously described, three general regions could be identified within the media of MPA on the basis of cell composition and orientation. Cells in the subendothelial region did not express any SM markers and thus were considered nonmuscle cells (Ref. 10 and Fig. 1, *B* and *C*, and Table 1). Within the middle medial region, cells were uniformly positive for α-SM-actin and SM-MHC (Ref. 10 and Table 1), were heterogeneous in their staining for SM-MHC-B (not described previously, Fig. 1*C*, Table 1) and desmin (Table 1), and did not express meta-vinculin (Ref. 10 and Fig. 1*B*, Table 1). In the outer region of the media, at least two phenotypically distinct cell populations could be identified. Cells of one population stained positively for all SM markers analyzed (Ref. 10, Table 1, and Fig. 1, *B* and *C*) and were, therefore, defined as well-differentiated SMC. Another population of cells did not stain for any of the SM markers tested and, therefore, was defined as nonmuscle cells (Ref. 10, Table 1, and Fig. 1, *B* and *C*). The cellular composition of the media of the large extralobar PA was similar to that of the MPA. The only apparent difference between the MPA and the extralobar PA was that the latter had a thinner middle medial region with more SM cell "bundles" in the outer media (not shown).

Large intralobar PA (~3,000 μm in diameter) did not have easily identifiable medial regions such as those observed in the MPA and extralobar PA. However, the media of these arteries contained two phenotypically distinct cell populations, similar to those in the outer media region of the MPA, the well-differentiated and the nonmuscle phenotypes (Fig. 1, *D–F*, and Table 1). The phenotypes of these two cell populations were similar to those of cells within the outer media region of the MPA (Table 1); however, in contrast to the MPA, the well-differentiated SMC in the large intralobar PA were oriented circumferentially within the vessel wall.

PA ~1,500 μm or smaller in diameter, including those <100 μm, consistently demonstrated a uniform pattern of immunostaining with all the SM-specific antibodies tested (Fig. 1, *G–O*, and Table 1). Thus the distal PA SMC were considered to express a well-differentiated SM phenotype similar to that of the well-differentiated SMC population within the outer media of the MPA, extralobar PA, and large intralobar PA [Fig. 1, *B, C, E*, and *F* (cell populations marked with arrows), and Table 1].

The results of immunohistochemical staining suggest a progressive increase in phenotypic uniformity in the cellular composition of the tunica media along the

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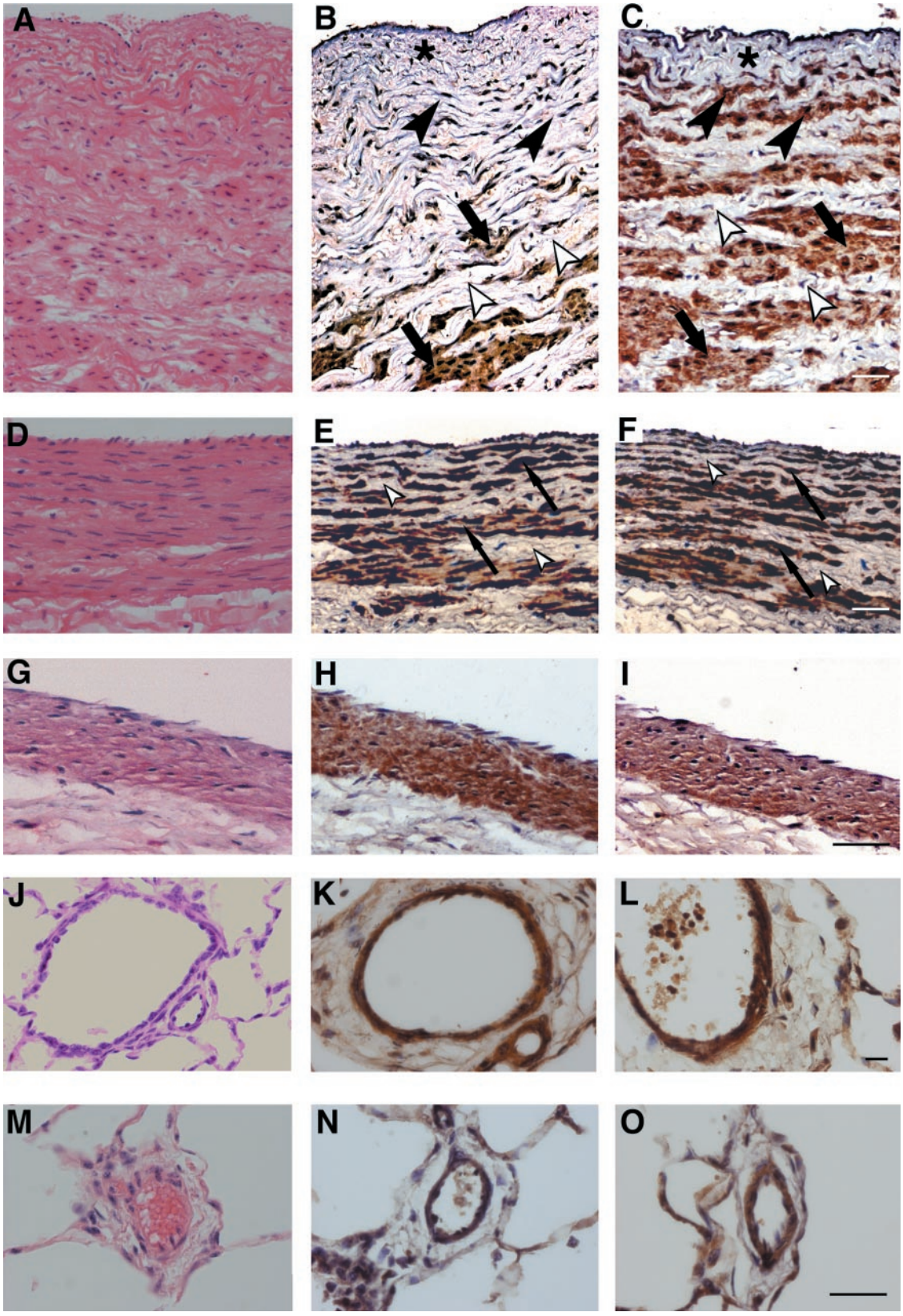

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Table 1. *Qualitative analysis of in vivo immunostaining characteristics of medial cells from the MPA, an extralobar PA, and an intralobar distal PA*

Region of the Media (if any)	Main PA			Extralobar PA			Intralobar PA		
	Subendothelial	Middle	Outer	Subendothelial	Middle	Outer	Distal PA 3,000 μm	Distal PA 1,500 μm	Distal PA 500-100 μm
Immunostaining									
α-SM-actin	−	+	+ −	−	+	+ −	− +	+	+
SM-MHC	−	+	+ −	−	+	+ −	− +	+	+
SM-MHC-B	−	+/−	+ −	−	+/−	+ −	− +	+	+
Desmin	−	+/−	+ −	−	+/−	+ −	− +	+	+
Meta-vinculin	−	−	+ −	−	−	+ −	− +	+	+

The outer media in the main pulmonary artery (MPA) and extralobar pulmonary artery (PA) is composed of 2 distinct cell subpopulations, each located in distinct areas (see RESULTS and as previously described in Ref. 10). +, Positive staining; −, no staining observed; +/−, heterogeneous staining (described in RESULTS); α-SM-actin, α-smooth muscle actin; SM-MHC, smooth muscle myosin heavy chains. $N = 6$ animals.

proximal-to-distal longitudinal axis of the pulmonary arterial tree. Thus, whereas the media of the MPA is composed of several cell phenotypes, including nonmuscle subtypes, the media of the distal muscular pulmonary arterioles appears to be phenotypically uniform and wholly composed of phenotypically well-differentiated SMC.

Distal PA Media Yield in Culture a Phenotypically Uniform Population of Well-Differentiated SMC

Recent studies have demonstrated that even tunica media composed of an apparently uniform cell phenotype in vivo can give rise to at least two phenotypically and functionally distinct cell populations in culture in certain species (9). Therefore, to further examine the cellular composition of the tunica media of distal PA, cells from the media of arteries with diameters ranging from 670 to 1,340 μm (mean 994 ± 25 μm; total of 30 arteries from 13 calves) were isolated and expanded in culture. In primary culture, as well as throughout several passages, the isolated cells appeared morphologically similar and exhibited characteristics for mesenchymal cell "spindle" morphology (Fig. 2*A*). Because of their distal location in the pulmonary arterial bed and spindle shape, we designated them here as distal-S-SMC (Table 2). Immunostaining assays demonstrated that, in culture, these cells expressed α-SM-actin and SM-MHC (Fig. 2*B*) as well as meta-vinculin (Fig. 2*C*), supporting in vivo observations and suggesting again that they are phenotypically well differentiated. The pattern of immunostaining of distal-S-SMC was similar to that of a specific population of well-differentiated SMC isolated from the outer media of the MPA but different from another, less-differentiated SMC population that can be isolated from the middle MPA media and obviously far different from the nonmuscle medial cells in the proximal vessels (Ref. 9 and Table 2).

Growth Characteristics Differ in SMC Cultured from Distal and Proximal PA

Proliferation in response to serum stimulation. We evaluated the growth capabilities of distal-S-SMC and compared them with different SMC populations obtained from the MPA of the same animal. Distal-S-SMC proliferated very slowly in serum-supplemented (10% CS) medium (Fig. 3) and did not replicate under serum-deprived (0.1% CS) conditions (not shown). Their proliferation rate in 10% CS was similar to that of well-differentiated SMC from the outer region of the MPA media but was much slower than all other MPA cell populations (Fig. 3, Table 2).

DNA synthesis in response to mitogens and hypoxia. The potential for DNA synthesis in response to purified peptide mitogens and/or hypoxia was determined in distal-S-SMC and compared with that of different

Fig. 1. The cellular composition of pulmonary arterial media gradually changes along the longitudinal proximal-to-distal axis from phenotypically heterogeneous to uniform. Immunostaining for 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*) is shown. Routine hematoxylin/eosin staining is presented (*left*: *A*, *D*, *G*, *J*, *M*). Shown from *top* to *bottom*: main pulmonary artery (MPA; *A–C*), intralobar pulmonary arteries (PA) with inner diameters of 3,000 μm (*D–F*), 1,500 μm (*G–I*), 500 μm (*J–L*), and 100 μm (*M–O*). Bars, 100 μm. *A–C*: media of MPA shows marked cellular heterogeneity. Cells within the subendothelial media are negative for all the SM markers tested (*). Cells in the middle media are negative for meta-vinculin (*B*, solid arrowheads) but positive, although heterogeneously, for SM-MHC-B (*C*, solid arrowheads). The outer media of the MPA is composed of cells positive for meta-vinculin and SM-MHC-B (arrows) as well as cells negative for all the SM markers tested (open arrowheads). *D–F*: media of proximal intralobar PA of a 3,000-μm inner diameter shows marked cellular heterogeneity, although no cellular layers similar to those of the MPA (subendothelial, middle, and outer) could be identified. The PA media is composed of cells either positive (arrows) or negative (arrowheads) for all SM markers tested (only meta-vinculin and SM-MHC-B are shown here). *G–O*: media of intralobar PA with inner diameters of 1,500 μm (*G–I*), 500 μm (*J–L*), and 100 μm (*M–O*) appeared phenotypically uniform; medial cells expressed all the SM markers tested (only meta-vinculin and SM-MHC-B are shown here).

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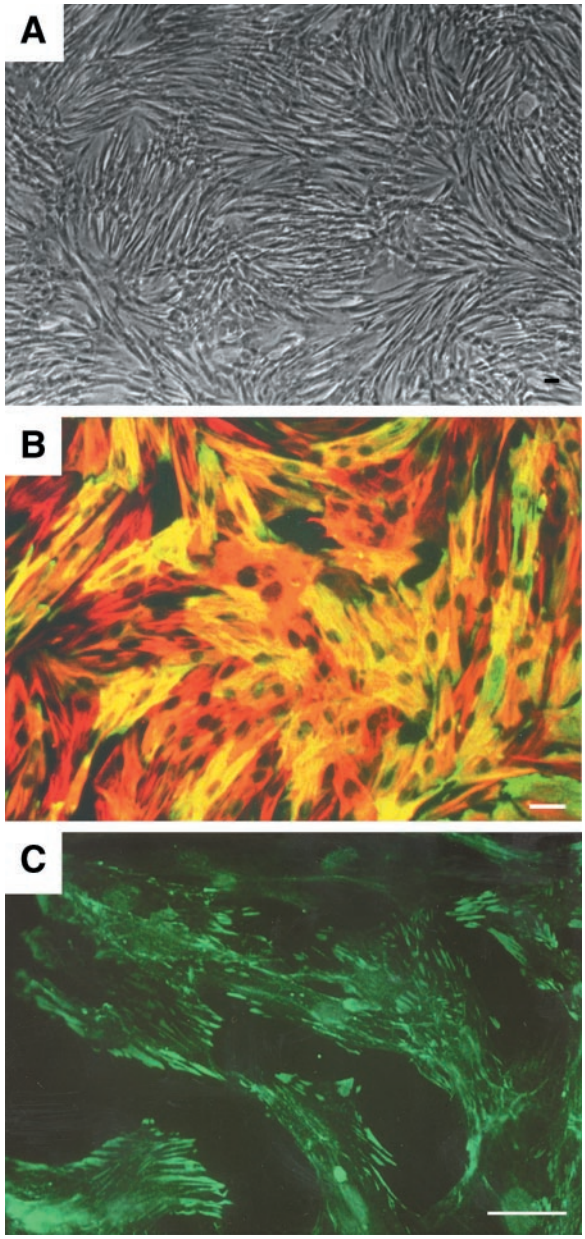

Fig. 2. Smooth muscle cells (SMC) cultured from the media of intralobar PA with a mean inner diameter of 994 ± 25 μm express SM markers of a "well-differentiated" SM phenotype (SM-myosin and meta-vinculin). *A*: the cells display a spindle-shaped morphology and the "hill-and-valley" pattern at confluence. *B*: double-label immunofluorescence staining for α-SM-actin (red) and SM-MHC (green). The superimposition of the 2 colors in cells expressing both proteins results in a yellow color. *C*: single-label immunofluorescence staining for the premembrane cytoskeletal muscle-specific protein meta-vinculin. Bars, 25 μm.

MPA-SMC populations obtained from the same animal. We chose to focus on quantitative comparison of distal-S-SMC with only those two MPA cell populations that expressed SM-related proteins (SMC from the middle and outer regions of the media). We chose not to include nonmuscle cell phenotypes from the MPA subendothelial and outer regions in the quantitative comparison with SMC populations because, as shown earlier, their replication rates far exceeded those of differentiated SMC populations.

Under normoxic conditions, relatively little DNA synthesis was detected in any of the three SMC types after 72 h of serum deprivation (0.1% CS). Yet, basal rates of DNA synthesis were significantly greater in MPA-"middle media"-SMC than in MPA-"outer media"-SMC or distal-S-SMC (Fig. 4*A*, 0.1% CS). Addition of 10% CS for 24 h increased DNA synthesis rates in all three SMC types, although the greatest increase was observed in MPA-middle media-SMC (Fig. 4*A*, 10% CS). PDGF-BB (10 ng/ml) and bFGF (30 ng/ml) also caused a significant (4- to 6-fold) increase in DNA synthesis rates, although it was less pronounced than that induced by 10% CS (Fig. 4*A*). IGF-I (100 ng/ml) initiated different responses in the three SMC types; no increase in DNA synthesis was noted in the MPA-outer media-SMC, whereas 2.2- and 3.6-fold increases in DNA synthesis were observed in the MPA-middle media-SMC and distal-S-SMC, respectively. Thus the rank order of DNA synthesis in response to mitogen stimulation was consistently MPA-middle media-SMC >> distal-S-SMC > MPA-outer media-SMC. Importantly, the specific profiles of mitogenic responses were also distinctly different in the three SMC types. Distal-S-SMC exhibited nearly equivalent responses (3.6-fold increase in DNA synthesis) to all three growth factors tested; MPA-outer media-SMC exhibited similar responses to PDGF and bFGF (5- and 4-fold increases, respectively) but did not respond to IGF-I; and MPA-middle media-SMC were progressively more sensitive to IGF-I (2.2-fold), bFGF (4.8-fold), and PDGF-BB (6.2-fold).

The effects of hypoxia (3% O_2) on DNA synthesis were then tested in the three SMC types in the presence and absence of serum stimulation. As has previously been shown, 3% O_2 is the O_2 concentration that maximally stimulates PA adventitial fibroblast and medial nonmuscle cell proliferation in neonatal bovine cells (6). In quiescent cells (72 h of serum deprivation), exposure to hypoxia (3% O_2) did not cause any significant changes in DNA synthesis compared with normoxia (21% O_2) in any of the SMC phenotypes studied (Fig. 4*B*, 0.1% CS). However, in SMC stimulated with serum, hypoxic exposure consistently attenuated DNA synthesis (Fig. 4*B*, 10% CS). Hypoxia had a greater suppressive effect on DNA synthesis in those SMC types that were more responsive to serum stimulation. Specifically, hypoxia decreased [^{3}H]thymidine incorporation more in MPA-middle media-SMC and distal-S-SMC (4- and 3-fold, respectively) than in MPA-outer media-SMC (1.2-fold decrease; Fig. 4*B*, 10% CS). Again, as for growth factor-induced response, each of the cell types responded differently to hypoxic exposure.

Changes in protein synthesis and cell size in response to serum stimulation. Because there were marked differences in DNA synthesis in the three analyzed SMC populations and because distal-S-SMC and MPA-outer media-SMC were relatively "growth resistant" even under serum-stimulated conditions, we investigated

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Table 2. *Qualitative in vitro characteristics of cells isolated from the media of the MPA and media of distal PA (≤1000-μm diameter)*

Site of Cell Isolation	Main Pulmonary Artery				Distal PA
Region of the media	Subendothelial media	Middle	Outer		Whole
Cell morphology	Rhomboid	Spindle	Epithelioid	Spindle	Spindle
Immunostaining					
α-SM-actin	−	+	−	+	+
SM-MHC	−	+	−	+	+
Meta-vinculin	−	−	−	+	+
Growth (10% CS)	++++	+++	++++	+	+
DNA synthesis					
Platelet-derived growth factor	++++	+++	++++	+	++
Basic fibroblast growth factor	+++	++	+++	+	+
IGF-I	++	+	++	−	+
Normoxia (10% CS)	++++	++++	++++	+/−	++
Hypoxia (10% CS)	++	−−−	++	−	−−
Protein synthesis	ND	+	ND	++++	+++
Cell size increase	ND	+/−	ND	++	++

Maximal positive values are ++++; little to no change is +/−; negative values are −, −−, or −−−. For immunostaining, data are from Fig. 2 (for distal PA SMC), from the previously published data for the MPA medial cells (Ref. 9), and from Fig. 3 (7 animals analyzed). For growth, data are as compared with *day 1* in culture. For DNA synthesis, data are from Fig. 4 (for SMC from the middle and outer regions of the MPA media and for distal SMC; 4 animals analyzed) and from the previously published data for subendothelial and outer media nonmuscle cells (Ref. 9). Also for DNA synthesis, data are as compared with serum-deprived (72 h in 0.1% CS) conditions. Hypoxia is shown as compared with normoxia (10% CS). Data are from Fig. 4 (4 animals analyzed). Protein synthesis is shown in 10% CS. Data are from Fig. 5 (4 animals analyzed). Cell size increase is shown in 10% CS as compared with serum-deprived (7 days in 0.1% CS) conditions. Data are from Fig. 5 (4 animals analyzed). CS, calf serum; ND, not determined.

changes in protein synthesis and cell size (as a measure of hypertrophy) in response to 10% serum (the condition that caused the greatest increase in cell growth). We found that the overall pattern of protein synthesis between the cell types under both serum-deprived (72 h in 0.1% CS) and serum-stimulated (10% CS) conditions was opposite to that of DNA synthesis and proliferation rates (compare Fig. 5*A* to Figs. 3 and 4). That is, MPA-middle media-SMC, which exhibited the highest rates of DNA synthesis in response to mitogenic stimulation and proliferated the fastest, demonstrated the lowest rates of protein synthesis per cell under all the conditions tested. Concurrently, MPA-outer media-SMC and distal-S-SMC, which showed low increases in DNA synthesis in response to mitogenic stimulation and grew slowly, consistently had higher rates of [^{35}S]methionine incorporation.

To analyze the change in cell size during the exponential growth (in 10% CS) compared with quiescence (72 h in 0.1% CS), we utilized flow cytometry analysis of the three SMC types (Fig. 5*B*). The faster replicating MPA-middle media-SMC showed a lesser increase in cell size after growth activation than did the more slowly growing MPA-outer media-SMC and distal-S-SMC (Fig. 5*B*, Table 2). Interestingly, the increase in cell size was the same for the distal-S-SMC and the MPA-outer media-SMC; however, [^{35}S]methionine incorporation was markedly higher in the MPA-outer media-SMC, suggesting different patterns of protein turnover for SMC from the distal PA vs. the MPA.

DISCUSSION

The present study demonstrates that the cellular composition of the mature bovine pulmonary arterial 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. The media of the proximal large PA, such as MPA, is composed of a mosaic of SMC and nonmuscle cells. At the other end of the pulmonary arterial bed, in distal PA with an inner diameter <1,500 μm (including arterioles as small as 100 μm), the arterial media is composed of a phenotypically uniform and apparently well-differentiated SMC phenotype (expressing all SM markers evaluated). Transition from the heterogeneous, more complex architecture at the proximal end to the uniform, simpler architecture in the distal lung is progressive from one arterial generation to the next, and this progressive simplification of architecture is accompanied by the increasing preponderance of well-differentiated SMC.

Our results provide a "cellular basis" for the different functional properties of vessels along the vascular tree. In his classic physiological analysis, Burton (3) stated that in the great arteries, large amounts of collagen are needed to provide tensile strength to withstand high wall tension, and large amounts of elastin are needed to provide distensibility to accommodate stroke volume, although the great arteries have relatively little SMC-dependent contractile functions. By contrast, small arteries are exposed to much less wall stress than large arteries, and pulse pressure is also less (4). In these distal arteries, contractile SMC are required to provide the tone essential for regulation of blood pressure and distribution of flow (3). Our observations that the media of the distal PA is composed of a uniform population of well-differentiated SMC support the notion that the function of small resistance arteries

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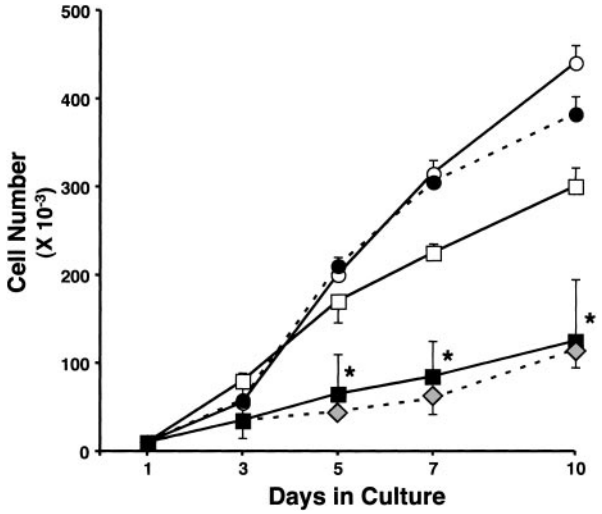

Fig. 3. Distal PA SMC and cell populations isolated from different medial layers (subendothelial, middle, and outer) of the MPA exhibit markedly different proliferative capabilities under 10% serum-stimulated conditions. ○, a nonmuscle cell population isolated from the subendothelial media of the MPA; ●, a nonmuscle cell population isolated from the outer media of the MPA; □, SMC isolated from the middle media of the MPA; ◆, SMC isolated from the outer media of the MPA; ■, SMC isolated from the distal PA (mean diameter 994 ± 25 μm). Distal PA-SMC grew slowly and plateaued at a lower density than all except one (the outer MPA media SMC, shown in ◆) MPA cell population. *Cell counts for distal PA-SMC and SMC from the outer MPA media were both different ($P < 0.05$) from all other cell populations, yet not significantly different from each other. Values are means ± SE; $n = 3$ replicate wells. Similar results were obtained in other experiments using cells from 7 other animals.

depends primarily on the contractile function of SMC. In addition, our findings of uniformity of SM phenotype in small PA, as opposed to large vessels, are consistent with reports by others showing that SMC in small resistance PA uniformly express a single type of voltage-gated K^+ channel, whereas multiple K^+ channels are expressed by SMC in large PA (21).

We observed uniform expression of the SM-MHC-B by all medial SMC in the distal pulmonary circulation. The SM-MHC-B isoform quite clearly affects the velocity of muscle shortening, which could contribute to regulation of tone as well as myogenic responses in resistance vessels (1, 16, 17). Interestingly, the SM-MHC-B isoform also may be an important contributor to maximal force generation in resistance pulmonary vessels. This finding in the lung vessels is consistent with SM-MHC-B expression pattern in small muscular arteries in other organs (7, 12, 13, 24). Thus our findings support the notion that SMC in the distal pulmonary circulation are well equipped to perform the primary function of regulating blood flow.

Recent studies have demonstrated that normal aortic media of the pig, apparently phenotypically uniform in vivo, yields cells of two distinct phenotypes in culture (11). Therefore, to explore the possibility that the distal PA media might contain cells of more than one functional phenotype not readily identifiable in vivo, we isolated in culture distal PA SMC and determined their phenotype on the basis of not only morphology and SM marker expression but also their growth capabilities. Our data, demonstrating that only one SMC

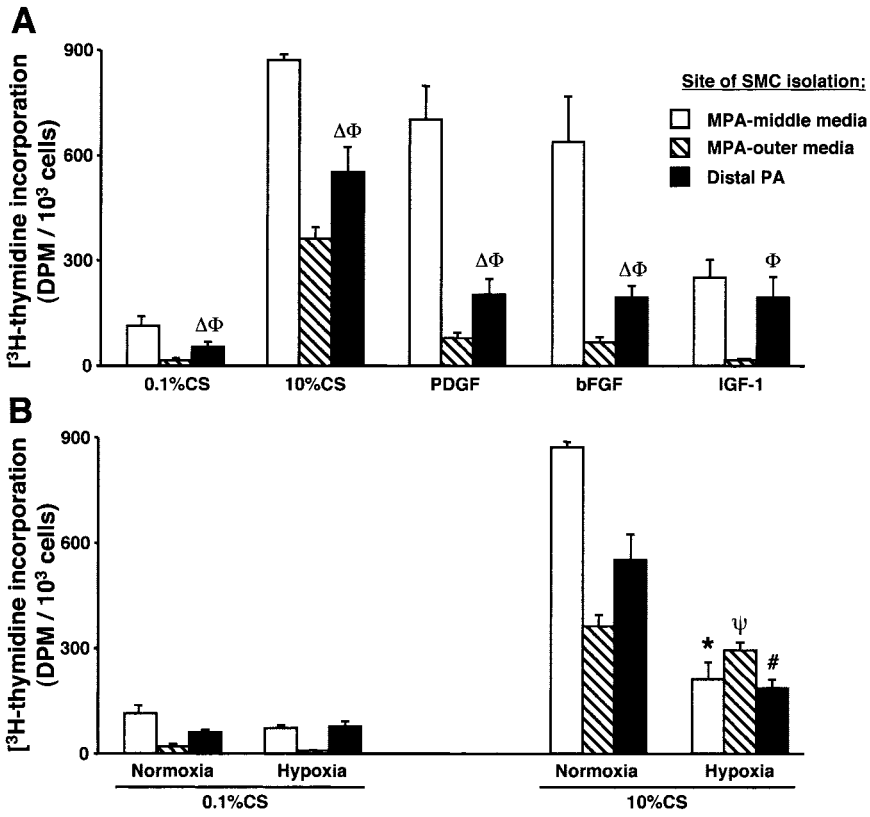

Fig. 4. SMC isolated from the distal PA (solid bars) and from the MPA middle media (open bars) and outer media (hatched bars) exhibit differences in DNA synthesis (measured by [^{3}H]thymidine incorporation) in response to mitogens (*A*) and hypoxia (*B*). *A*: quiescent [72 h in 0.1% calf serum (CS)] cells were stimulated with 10% CS and/or the following mitogens: platelet-derived growth factor-BB (PDGF-BB; 10 ng/ml), basic fibroblast growth factor (bFGF; 30 ng/ml), and IGF-I (100 ng/ml). $^{\Delta\Phi}$[^{3}H]thymidine incorporation in distal PA SMC differed significantly ($P < 0.05$) from that in SMC isolated from MPA-middle media and MPA-outer media, respectively. *B*: [^{3}H]thymidine incorporation in the 3 cell types is attenuated in response to hypoxia (3% O_2) compared with normoxia (21% O_2) under serum-stimulated (10% CS) conditions (*right*), yet it is not significantly affected by hypoxia under serum-deprived (0.1% CS, 72 h) conditions (*left*). *$^{\Psi\#}$Values for the 3 SMC types were significantly ($P < 0.05$) different from those (for each SMC type) under normoxia; $n = 4$ replicate wells. Similar results were reproduced in other experiments using matched cells from 4 other animals. DPM, disintegrations per minute.

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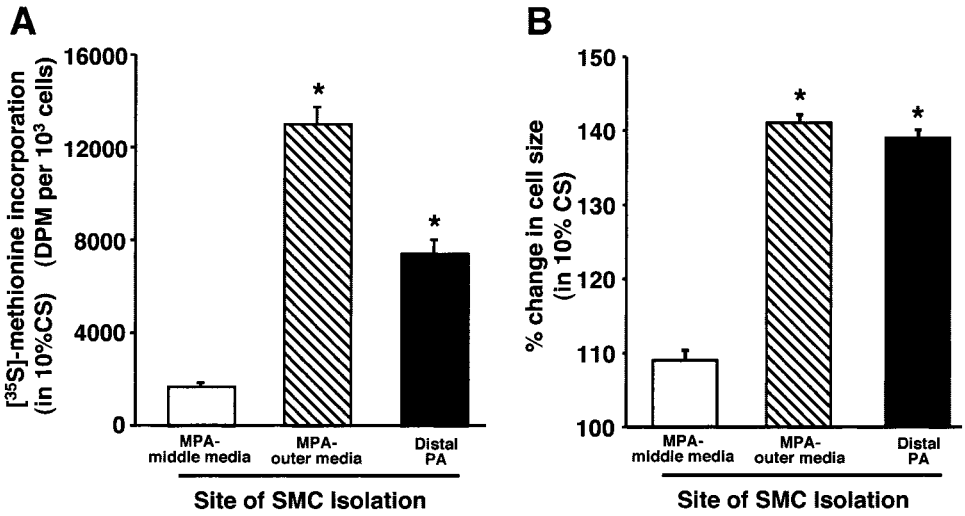

Fig. 5. SMC isolated from the proximal (MPA) and distal PA demonstrate distinct changes in cell size in response to serum stimulation (10% CS, 7 days) as percent control (0.1% CS). *A* and *B*: open bars, SMC isolated from the MPA-middle media; hatched bars, SMC isolated from the MPA-outer media; solid bars, SMC isolated from the distal PA media. *Values for both distal PA SMC and MPA-outer media-SMC were significantly ($P < 0.05$) different from those of the MPA-middle media-SMC. Similar results were obtained in other experiments using cells from 4 other animals.

phenotype was consistently obtained in culture, suggest that at least in the bovine species, the normal distal lung arterial media is composed of a phenotypically uniform population of SMC in terms of SM markers. Furthermore, these in vitro data support our in vivo observations that SMC, comprising the media of resistance PA, are of a well-differentiated SM phenotype and also demonstrate that they are highly proliferation resistant, as has recently been described for pig coronary artery SMC (5). It will be important to determine the degree to which the phenotype of these cells is controlled by such factors as extracellular matrix, physical forces, and such in vitro conditions as time in culture.

In the present study, the growth properties (proliferative and hypertrophic responses to mitogenic stimuli and hypoxia) of SMC isolated from distal PA (designated distal-S-SMC) were analyzed and compared with those of medial SMC populations from the proximal vessels (Table 2). Distal-S-SMC grew slowly under serum-stimulated conditions, and exposure to hypoxia further suppressed serum-stimulated replication. The increase in DNA synthesis that distal-S-SMC demonstrate in response to purified peptide mitogens was very modest, especially compared with that of the nonmuscle cell populations derived from proximal PA. Also noteworthy was the differential response to growth factors of cells from the different sources (Table 2). In addition, both the data presented here and our previous studies demonstrate that hypoxia exerts a suppressive effect on the growth of well-differentiated SMC isolated from the outer media of the bovine MPA and from the media of distal PA, whereas it stimulates proliferation of nonmuscle cell populations from the MPA (9). It remains to be determined whether hypoxia directly causes changes in cell phenotype, for example, in terms of SM markers in either the differentiated SMC populations or the nonmuscle medial cell populations.

Our data suggest that some subpopulation(s) of medial cells in bovine proximal vessels are "phenotypically predetermined" for high proliferative capabilities, whereas SMC in the distal circulation are not. In the media of proximal large vessels, we have previously described a preponderance of cells with high proliferative capabilities that appear to contribute selectively to the rapid increases in the medial thickness observed in hypoxia-induced pulmonary hypertension, although cell hypertrophy could also play a role (9, 25). These observations are consistent with others in a variety of species, including human, where selective contribution of resident cells with high proliferative capacity to the remodeling process has been proposed (11, 15). In contrast, medial SMC isolated from distal resistance PA had lower capacities for proliferation, observations compatible with previous in vivo data that have suggested hypertrophy as the predominate response to hypertensive stimuli of SMC in the distal pulmonary circulation (18). Thus current evidence suggests that the mechanisms by which the small vessels remodel in response to challenges such as hypertension are fundamentally different from those in large vessels.

In large vessels, a reservoir of phenotypically distinct SMC within the media itself can serve as a source of cells for both hypertrophy and hyperplasia as discussed earlier (3, 9, 20, 25). This raises the intriguing questions as to whether some cell subpopulation(s) contribute to the intimal thickening and the degree to which actual phenotypic switching occurs (19). Observations of cell heterogeneity and hypothesis regarding cell-specific contributions to remodeling do not exclude phenotype changes within a specific cell population. Remodeling in the distal vasculature, based on the current data, would appear to require, at least in part, recruitment of highly proliferative and/or synthetic cells from other vascular compartments. Thus the medial and even intimal changes observed in distal vessels could involve recruitment of adventitial fibroblasts or neighboring undifferentiated lung interstitial mesenchymal cells that are capable of differentiating into myofibroblasts and/or circulating peripheral blood hematopoetic or fibrocyte precursors, or even endothelial cells that can transdifferentiate into SM-like cells (12, 13, 18, 22). The capacity to recruit new cells, capable of

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rapidly differentiating toward a SM phenotype, may be a critical component of the distal vascular remodeling that occurs in hypertension (12, 13, 22, 23).

In conclusion, the present study provides analysis of the cellular composition of the normal arterial media at different locations within the pulmonary circulation of large mammalian species. Collectively, these experiments provide better understanding of the cellular composition of the normal arterial media at different locations within the pulmonary circulation and should serve as a basis for further analysis of the cellular changes that occur in the pulmonary arterial bed exposed to stress. The data raise the intriguing possibility that differences in the cellular composition of the media at different locations will dictate, at least in part, the cellular mechanisms utilized to achieve vessel wall thickening.

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