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Pulmonary Artery Adventitial Fibroblasts Cooperate with Vasa Vasorum Endothelial Cells to Regulate Vasa Vasorum Neovascularization

A Process Mediated by Hypoxia and Endothelin-1

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The precise cellular and molecular mechanisms regulating adventitial vasa vasorum neovascularization, which occurs in the pulmonary arterial circulation in response to hypoxia, remain unknown. Here, using a technique to isolate and culture adventitial fibroblasts (AdvFBs) and vasa vasorum endothelial cells (VVECs) from the adventitia of pulmonary arteries, we report that hypoxia-activated pulmonary artery AdvFBs exhibited pro-angiogenic properties and influenced the angiogenic phenotype of VVEC, in a process of cell-cell communication involving endothelin-1 (ET-1). We demonstrated that AdvFBs, either via co-culture or conditioned media, stimulated VVEC proliferation and augmented the self-assembly and integrity of cord-like networks that formed when VVECs were cultured on Matrigel. In addition, hypoxia-activated AdvFBs produced ET-1, suggesting a paracrine role for this pro-angiogenic molecule in these processes. When co-cultured on Matrigel, AdvFBs and VVECs self-assembled into heterotypic cord-like networks, a process augmented by hypoxia but attenuated by either selective endothelin receptor antagonists or oligonucleotides targeting prepro-ET-1 mRNA. From these observations, we propose that hypoxia-activated AdvFBs exhibit pro-angiogenic properties and, as such, communicate with VVECs, in a process involving ET-1, to regulate vasa vasorum neovascularization occurring in the adventitia of pulmonary ar-

teries in response to chronic hypoxia. (*Am J Pathol* 2006, 168:1793–1807; DOI: 10.2353/ajpath.2006.050754)

Previous work from our group demonstrated that vasa vasorum neovascularization occurs in the remodeled pulmonary artery adventitial compartment in a neonatal bovine model of hypoxia-induced pulmonary arterial hypertension (PAH).¹ These findings are in accordance with the well established paradigm that hypoxia is a potent stimulus for new vessel growth in a number of pathological settings.² Moreover, our data are consistent with the finding that adventitial neovascularization occurs in the medial and adventitial compartment of pulmonary arteries in patients with PAH.³ In the systemic circulation, the adventitial vasa vasorum microcirculation undergoes marked neovascularization in a number of vasculopathies characterized by vascular remodeling, including atherosclerosis, type 2 diabetes, metabolic syndrome, restenosis, and vasculitis.^{4–8} The precise consequence of vasa vasorum neovascularization in both pulmonary and systemic vasculopathies remains unknown, but it is likely to play a critical role in the development and progression of the disease process.

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This article is dedicated to Dr John “Jack” Reeves, who passed away on September 15, 2004.

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Despite the recently described importance of vasa vasorum neovascularization in both pulmonary and systemic vasculopathies, the precise cellular and molecular basis regulating expansion of this microcirculatory network remains unknown. Much attention regarding the cellular and molecular basis of new vessel growth has focused on the central role of endothelial cells in this process; however, an increased volume of data indicates that endothelium of de novo-formed microvessels receives and integrates "pro-angiogenic signals" from a number of nonendothelial cells, including fibroblast-like cells.^{9–12} Indeed, heterotypic cell-cell communication between endothelial cells and non-endothelial cells may represent a critical process in the initiation, stabilization, and maturation of new vessels.^{9–15} To the best of our knowledge, pulmonary artery fibroblasts, the principal cell type in the adventitial compartment, have not previously been described to exhibit pro-angiogenic properties; however, it is tenable to hypothesize as such, because they reside at the interface between pre-existing vasa vasorum and the surrounding tissue and are thus ideally positioned to take an active role in the process. To the best of our knowledge, there are no reports of vasa vasorum endothelial cells (VVECs) being isolated and cultured from the adventitial compartments of pulmonary arteries (or systemic vessels) to study mechanisms involved in vasa vasorum neovascularization.

Endothelin-1 (ET-1) together with its cognate receptors, ET_A and ET_B, have recently been added to the axis of pro-angiogenic molecular regulators of postnatal neovascularization in a number of pathological settings.¹⁶ In the systemic circulation ET-1 has been shown to regulate coronary artery vasa vasorum expansion in an experimental model of hypercholesterolemia.⁶ Whether the endothelin (ET) system plays a role in hypoxia-induced pulmonary artery adventitial vasa vasorum neovascularization has yet to be determined. It is plausible to speculate that it does, because ET-1 promoter activity is enhanced in response to hypoxia, and mature ET-1 peptide is released by a number of activated cells, including fibroblasts, endothelial cells, and lung macrophages, in response to injurious stimuli. Additionally, ET-1 has also been shown to stimulate proliferation and migration of endothelial cells,¹⁷ fibroblasts,¹⁸ and smooth muscle cells.¹⁹

The aim of the present study was to investigate further the cellular and molecular basis of adventitial vasa vasorum neovascularization in the setting of hypoxia-induced pulmonary arterial hypertension (PAH). We tested the hypothesis that hypoxia-activated pulmonary artery adventitial fibroblasts (AdvFBs) exhibit pro-angiogenic properties and, as such, communicate with VVECs, in a process involving ET-1, to regulate this adaptive process. To test this hypothesis, we first developed a technique to simultaneously isolate VVECs and AdvFBs from the same adventitial compartments of intralobar pulmonary arteries dissected from normoxic neonatal calves. Using cell culture techniques, together with a series of complementary biochemical assays, data from the present study indicate

that the pro-angiogenic properties of hypoxia-activated AdvFBs render them key regulators of adventitial vasa vasorum neovascularization occurring in the pulmonary arterial circulation in PAH.

Materials and Methods

Materials

Dulbecco's minimal essential Eagle's medium (DMEM) was purchased from Cellgro (Herndon, VA). Bovine calf serum (BCS) was from Gemini Bio-Products (Woodland, CA). Platelet endothelial cell adhesion molecule-1 (PECAM-1, 1:200), von Willebrand factor (1:100), and fetal liver kinase-1 (1:1000) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). α -Smooth muscle actin (1:600) was purchased from Sigma-Aldrich (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated *Lycopersicon esculentum* lectin (20 μ g/ml) and FITC-conjugated *Ulex europaeus* agglutinin 1 (20 μ g/ml) were both purchased from Vector (Burlingame, CA). FITC-conjugated anti-mouse or anti-rabbit IgG (1:300) secondary antibodies were purchased from Sigma. Cy3 (1:300) was from Jackson Laboratories (West Grove, PA). Hoechst 33342 (5 μ g/ml) was from Molecular Probes (Eugene, OR). PKH26 and PKH57 membrane dyes were purchased from Sigma-Aldrich. Matrigel was from BD Biosciences (San Jose, CA). Semiconductor nanocrystals QTracker 655 and QTracker 565 were purchased from the Quantum Dot Corp. (Hayward, CA). The ET-1 enzyme-linked immunosorbent assay (ELISA) was purchased from R&D Systems (Minneapolis, MN). ET-1 and the endothelin receptor antagonists BQ123 and BQ788 were from Sigma-Aldrich. Prepro-ET-1 phosphothioated antisense (5'-ATCATGGGGAAATAATCCAT-3') and scrambled (5'-ATCAAGCATAGTAATGATGC-3') oligonucleotides were from Oligos Etc. (Wilsonville, OR). ET_A and ET_B receptor antibodies (1:1000) for Western blots were purchased from Immuno-Biological Laboratories (Minneapolis, MN). The ET_B receptor antibody for immunohistochemistry (1:100) was purchased from Abcam (Cambridge, UK). The NuPAGE electrophoresis system was from Invitrogen (Carlsbad, CA). Hybond-P membrane was from Amersham Biosciences (Buckinghamshire, UK). Western Lightening detection system was from PerkinElmer Life Sciences (Boston, MA). The RNeasy Mini Kit was from Qiagen (Valencia, CA). The SuperScript II reverse transcriptase polymerase chain reaction kit and Platinum Supermix were from Invitrogen.

Cell Culture

Intralobar pulmonary arteries (8- to 10-mm external diameter) were dissected from lung tissue isolated from 2-week-old normoxic calves ($n = 5$). Adventitial tissues containing adventitial cells and vasa vasorum were dissected from these vessels, and explant cultures were established in 6-well plates in DMEM supplemented with

10% BCS under normoxic (21% O₂) conditions. Outgrowth populations of VVECs and AdvFBs were individually expanded using cloning rings and trypsinization techniques. Five separate VVEC isolates and five separate AdvFB isolates were obtained from five different normoxic calves. These isolates (VVECs, $n = 5$; AdvFBs, $n = 5$) were used, up to passage 7, in each of the experimental approaches detailed below. All animal procedures were undertaken using standard veterinary care and institutional guidelines at the Department of Physiology, School of Veterinary Medicine, Colorado State University.

Immunocytochemical Staining, Lectin Binding Assays, and Microscopy

VVECs and AdvFBs (isolated as described above) were fixed in either ice-cold methanol or 4% paraformaldehyde for 10 minutes, washed in phosphate-buffered saline, and incubated overnight in primary antibodies or lectins. Proteins were visualized using FITC-conjugated anti-mouse or anti-rabbit IgG or Cy3 secondary antibodies. The specificity of immunostaining (antibody control) was determined by omission of the primary antibody and incubation with appropriate nonimmune serum. Cell nuclei were counterstained with Hoechst 33342. Images were captured via epifluorescence microscopy using a microscope (Axiovert; Carl Zeiss Inc., Jena, Germany).

Cell Proliferation Assays

Three cell culture-based proliferation assays were used: 1) evaluation of [*methyl*-³H]thymidine (hereafter referred to as ³H-thymidine) incorporation into VVECs cultured over a 24-hour period as a measurement of DNA synthesis, 2) direct counting of viable VVECs cultured alone over a 5-day period using a hemocytometer and the trypan blue exclusion technique, and 3) direct counting of viable VVECs co-cultured with AdvFBs over a 5-day period using a hemocytometer and the trypan blue exclusion technique.

For the thymidine assays, VVECs (isolated as described above) were suspended in DMEM supplemented with 10% BCS, seeded at 2×10^4 cells/well in 24-well plates, cultured until 80% confluent, and "growth-arrested" by incubation in DMEM supplemented with 0.1% BCS for 72 hours. Growth-arrested VVECs were cultured for a further 24-hour period under normoxic (21% O₂) or hypoxic (7% O₂) conditions. In selective wells, VVECs were cultured in the presence of "hypoxia-conditioned media," ie, DMEM collected from hypoxia-activated AdvFBs cultured under hypoxic (7% O₂) conditions for 48 hours in the presence of 10% BCS. For each experimental condition, the culture media was supplemented with 0.25 μ Ci of ³H-thymidine. After 24 hours, the incorporation of ³H-thymidine into VVECs was evaluated.

For the direct counting of VVECs cultured alone, cells were suspended in DMEM supplemented with 10% BCS and seeded at 1.5×10^4 cells/well in 24-well plates. Cells were then allowed to adhere overnight, at which point the

cells were "growth-arrested" for 72 hours in DMEM supplemented with 0.1% BCS. VVECs were then cultured for a further 5-day period under normoxic or hypoxic (7% O₂) conditions in the presence or absence of ET-1 (10^{-7} mol/L), hypoxia-conditioned media, or prepro-ET-1 antisense oligonucleotides (7 μ mol/L). Fresh culture media (either DMEM plus 0.1% BCS, or "conditioned media"), including the reagents, was added each day of the 5-day period.

For the co-culture experiments, 1.5×10^4 VVECs (isolated as described above) were plated on top of Transwell inserts both with and without AdvFBs (2×10^5 cells/well) plated on the bottom of the 24-well plate as a feeder layer. Both cell types were suspended in DMEM supplemented with 10% BCS and allowed to adhere overnight before growth arrest (72 hours, DMEM plus 0.1% BCS). Co-cultures were maintained in DMEM supplemented with 0.1% BCS under both normoxic (21% O₂) and hypoxic (7% O₂) conditions. Fresh culture media was added every day for 5 days.

Establishment of Hypoxic Culture Conditions

Cells cultured under normoxic conditions were placed in a cell culture incubator set at 37°C and gassed with 21% O₂ and 5% CO₂. For hypoxic experiments, cells were placed in a customized BactronX environmental hood (Sheldon, Cornelius, OR) at 37°C gassed with 7% O₂ and 5% CO₂. Culture media was pre-gassed for 24 hours with 7% O₂ and 5% CO₂.

Cell Labeling Assays

To visualize cultured VVECs and AdvFBs using fluorescent time-lapse imaging, we used two techniques. First, we used an established technique for the labeling of cells with fluorescent cell membrane dyes.²⁰ Individual cell suspensions of VVECs and AdvFBs (isolated as described above) were labeled with the cell membrane dyes PKH67 or PKH26, according to the manufacturer's instructions. Second, we used emerging nanotechnology to label individual adherent cell cultures of VVECs and AdvFBs with semiconductor nanocrystals QTracker 565 and QTracker 655, according to the manufacturer's instructions.

Matrigel Assays

VVECs and AdvFBs (isolated as described above) were growth-arrested by incubating cells in DMEM supplemented with 0.1% BCS for 72 hours. VVECs (1×10^5 cells/well) and co-cultures (5×10^4 of both VVECs and AdvFBs) were suspended in fresh DMEM (supplemented with 0.1% BCS) and plated in 4-well chamber slides coated with the reconstituted basement membrane preparation Matrigel. Cultures were maintained under both normoxic and hypoxic (7% O₂) conditions for up to 48 hours in the absence or presence of ET-1 (10^{-7} mol/L); the endothelin receptor antagonist BQ123 (against ET_A, 10^{-7} mol/L) or BQ788 (against ET_B, 10^{-7} mol/L); prepro-

ET-1 antisense or scrambled phosphothioated oligonucleotide (7.5 $\mu\text{mol/L}$); or hypoxia-conditioned media. Fresh culture media (with or without reagents) was added every day.

Quantitative Morphometric Analysis

Time-lapse images of Matrigel cultures were acquired using transmitted light and epifluorescence microscopy using a microscope (Carl Zeiss) and archived into a personal computer as either JPEG or TIFF files. In each experiment, five randomly selected fields of view were captured in each of three wells per condition (ie, each condition performed in triplicate). The degree of self-assembly of VVECs and AdvFBs into cord-like networks under normoxic and hypoxic conditions, combined with the presence or absence of positive and negative regulators, was measured in archival images using two end-points: 1) surface area, ie, the area occupied by aggregates of cells that assembled into cord-like networks in a two-dimensional plane (as seen in photomicrographs), and 2) number of branch points in the cord-like networks, ie, the number of intersections between structures. Collapse of cord-like networks (an evaluation of network integrity) was determined as the retraction of branch-like structures into small star-shaped clusters. Additionally, network collapse was quantified as a reduction in surface area and number of branch points in the cord-like networks.

Quantitative morphometric analysis was performed on stored images using ImageJ morphometric analysis software (National Institutes of Health, <http://rsb.info.nih.gov/ij/>). For this, images were first opened using the ImageJ toolbar and converted to 8-bit grayscale (toolbar function: Image > Type > 8-bit). Grayscale images were calibrated by drawing a straight line over a 100- $\mu\text{mol/L}$ scale bar (that had previously been embedded into the image when captured by the microscope) (toolbar function: Analyze > Set Scale). Grayscale images were then converted to binary images with the threshold function set to auto (toolbar function: Image > Adjust > Threshold > Auto > Apply). Threshold pixels were set to foreground color, and remaining pixels were set to background color, with a black foreground and a white background. The two-dimensional surface area of cord-like networks was calculated as the total number of pixels in thresholded images (Process > Binary > Outline > Analyze particles). The minimum size of particles was set at 1 and the maximum set at 99999. The number of branch points in cord-like networks was counted manually by two investigators in five randomly selected fields of view in each of three wells per condition.

ET-1 ELISA

VVECs (1×10^5 cells/well), AdvFBs (1×10^5 cells/well), and co-cultures (5×10^4 cells of both cell types) were suspended in DMEM supplemented with 0.1% BCS in the absence or presence of antisense oligonucleotides targeting prepro-ET-1 mRNA. Cells were seeded in four-well

chamber slides precoated with the reconstituted basement membrane preparation Matrigel. After 12 hours, the conditioned cell culture medium was collected, and the concentration of ET-1 was measured by using a commercially available sandwich ELISA, according to the manufacturer's instructions.

Western Blotting

VVECs and AdvFBs (isolated as described above) were cultured to subconfluence (80%) under normoxic conditions, and protein homogenates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the NuPAGE electrophoresis system. Proteins were transferred to a Hybond-P membrane. Membranes were probed with primary antibodies overnight in Tris-buffered saline plus 0.05% (v/v) Tween 20 together with 5% (w/v) dried milk at 4°C. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Bands were visualized using the Western Lightning detection system.

RT-PCR Analysis

VVECs and AdvFBs were suspended in DMEM supplemented with 10% BCS, seeded (2×10^4 cells/well) in 24-well plates, cultured until 80% confluent, and incubated in DMEM supplemented with 0.1% BCS for 48 hours under normoxic conditions in the absence or presence of either antisense or scrambled oligonucleotides targeting prepro-ET-1 mRNA (7.5 $\mu\text{mol/L}$). Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) templates were synthesized from 3 μg of total RNA using the SuperScript II RT-PCR kit (Invitrogen). cDNA was mixed with 25 μl of Platinum Supermix (Invitrogen) and 1 μg of each oligonucleotide primer (prepro-ET-1 forward primer, 5'-ACATCTTTTCGTGTGCCAAGC-3', and reverse primer, 5'-CAAGTGGAAATCTCCTCTCCTCTCG-3') per reaction and subjected to 20–40 cycles of PCR. Bovine β -actin was amplified as an input control. The amplified PCR products were separated on a 1% agarose gel and stained with ethidium bromide. ImageJ was used on scanned films for quantification of amplified products.

Immunohistochemistry

Indirect immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections. Sections were dewaxed, placed in antigen-unmasking solution (Vector Laboratories, Burlingame, CA), microwaved for 10 minutes at 180°C, incubated in hydrogen peroxide for 30 minutes, and incubated with the primary antibodies overnight at 4°C. After a brief wash in phosphate-buffered saline, sections were incubated with appropriate secondary antibodies for 1 hour at room temperature. Antigenic sites were localized using an ABC kit (Vector), as appropriate. The specificity of immunostaining (antibody control) was demonstrated by omission of the pri-

Figure 1. Representative photomicrographs illustrating the isolation, culture expansion, and phenotypic characterization of VVECs and AdvFBs. **A:** The pulmonary artery adventitial compartment from a normoxic calf illustrating the adventitial (Adv) regions (blue circle), containing vasa vasorum and AdvFBs, that were dissected and cultured as explants (SM = smooth muscle layer). **B:** Two cell populations: VVECs (red circle) and AdvFBs (green circle) appeared from explants within 7 days. **C:** Following cloning ring expansion, individual populations of cultured VVECs exhibited a “cobblestone” morphology when cultures were confluent. **D:** In contrast, AdvFBs exhibited a more spindle-shaped morphology when cultures were confluent. **E–J:** Cultured VVECs expressed PECAM-1 (FITC-conjugated secondary antibody, green signal (**E**)), von Willebrand factor (Cy3-conjugated secondary antibody, red signal (**F**)), and flk-1 (Cy3-conjugated secondary antibody, red signal (**G**)), and bound the lectin *L. esculentum* (FITC-conjugated lectin, green signal, and rhodamine phalloidin F-actin, red signal (**H**)). Cultured AdvFBs expressed vimentin (Cy3-conjugated secondary antibody, red signal (**I**)) and α -smooth muscle actin (Cy3-conjugated secondary antibody, red signal (**J**)). In each panel the nuclei of cells are counterstained with Hoechst (blue signal). Scale bars = 600 μ m (**A**), 100 μ m (**B–D**), and 50 μ m (**E–J**).

mary antibody and incubation with appropriate nonimmune serum. Images of antigenic sites were captured using transmitted light using a Zeiss microscope linked to a personal computer.

Statistical Methods

Data are expressed as mean $\pm$ SD and were analyzed with Prism version 3.0 (GraphPad Software, San Diego, CA). Comparisons were made by using Student's *t*-test (two-tailed) or one-way analysis of variance with the Tukey posthoc test, as appropriate. A value of *P* < 0.05 indicates statistical significance.

Results

Isolation and Phenotypic Characterization of Cultured VVECs and AdvFBs

The objective of the present study was to begin to determine the cellular and molecular basis regulating pulmonary artery adventitial vasa vasorum neovascularization in the setting of hypoxic PAH. To this end, we developed a technique to simultaneously isolate and culture the cells of interest to us, namely VVECs and AdvFBs, from the

adventitial compartment of intralobar pulmonary arteries of normoxic neonatal calves.

Following the explant culture of tissue samples dissected from the adventitial compartments of pulmonary arteries from five normoxic calves (Figure 1A, blue circle), two outgrowth populations of cells with different morphologies appeared simultaneously within 7 days of culturing (Figure 1B). One cell population exhibited a ‘cobblestone’ morphology (Figure 1B, red circle), a characteristic feature of endothelial cells in culture, and was designated as ‘VVEC.’ The other cell population exhibited a more fibroblast-like morphology in culture (Figure 1B, green circle) and was designated as ‘AdvFB.’ Pure populations of VVECs and AdvFBs were then culture-expanded using cloning ring techniques (Figure 1, C and D, respectively). We successfully isolated VVECs (*n* = 5) and AdvFBs (*n* = 5) cells from all five calves studied, and these cell isolates were used in each of the experimental approaches detailed.

Immunocytochemistry revealed that cultured VVECs expressed markers consistent with an endothelial cell phenotype, including PECAM-1, von Willebrand factor, and flk-1 (Figure 1, E–G) and bound the lectin *L. esculentum* (Figure 1H). Cultured AdvFBs were negative for endothelial cell markers (data not shown) but did express vimentin (Figure 1I) and, to a variable extent, α -smooth

Figure 2. Cell proliferation data showing that hypoxia (7% O₂) significantly stimulated cultured VVEC DNA synthesis over a 24-hour period (A) and absolute cell number (B) over a 5-day period. The VVEC cell number was also significantly stimulated when VVECs were co-cultured with AdvFBs over a 5-day period, a process augmented by hypoxia (B). Additionally, VVEC DNA synthesis was significantly stimulated when VVECs were cultured in the presence of "hypoxia-conditioned media" (collected from hypoxia-activated AdvFBs cultured in the presence of 10% BCS) but not when cultured in conditioned media collected from AdvFBs cultured under normoxic conditions (C). The graphs are presented as mean values (mean ± SD) of five separate cell isolates (obtained from five different animals) measured in triplicate. **P* < 0.5; ** *P* < 0.01; and *** *P* < 0.001.

muscle actin, a marker of activated fibroblasts (Figure 1J).

Hypoxia and Factor(s) Released from AdvFBs Regulate the Proliferative Responses of Cultured VVECs

Using these cultured cells, we first sought to determine effects of hypoxia and AdvFBs (via co-culture and conditioned media) on the proliferative responses of cultured VVECs.

Proliferation assays demonstrated that DNA synthesis in cultured VVECs was significantly (*P* < 0.001) stimulated by hypoxia (7% O₂) (Figure 2A). In addition, hypoxia significantly (*P* < 0.05) increased the absolute VVEC number after 5 days in culture (Figure 2B). The VVEC number was also significantly increased (*P* < 0.01) when VVECs were co-cultured with AdvFBs under normoxic (21% O₂) conditions, a response augmented under hypoxic (7% O₂) conditions (Figure 2B). VVEC DNA synthesis was also significantly (*P* < 0.01) increased when cells, cultured under normoxic (21% O₂) and hypoxic (7% O₂) conditions, were incubated with "hypoxia-conditioned media" (collected from hypoxia-activated AdvFBs cultured in the presence of 10% BCS) (Figure 2C). In contrast, VVEC DNA synthesis was not significantly increased when cells, cultured under normoxic and hypoxic (7% O₂) conditions, were stimulated with conditioned media collected from AdvFBs cultured under normoxic conditions (Figure 2C).

VVECs Self-Assemble into Cord-Like Networks When Cultured on Matrigel: A Process Augmented by Hypoxia

To further investigate the effects of hypoxia on VVECs, we next cultured cells on polymerized Matrigel, a matrix that provides a physiologically relevant microenvironment for studies of cell morphology, biochemical function, and migration or invasion. Dissociated cell suspensions of VVECs (labeled with PKH26, red signal, Figure 3A) formed aggregates within 6 hours after being plated on Matrigel under normoxic conditions (Figure 3B). After 12 hours, aggregates began to elongate (Figure 3C) and by 20 hours had spread across

the Matrigel, forming cord-like networks (Figure 3D). Hypoxic exposure (7% O₂) for 20 hours markedly changed the morphology of cord-like networks formed by VVECs on Matrigel compared to normoxic cultures (Figure 3, E–H). Hypoxia-induced morphological changes to cord-like networks were associated with increases in both the two-dimensional surface area (Figure 3I) and number of branch points, compared to normoxic cultures (Figure 3J).

VVECs and AdvFBs Coalesced to Form Heterotypic Cord-Like Networks When Cultured on Matrigel: A Process Augmented by Hypoxia

In an attempt to mimic cell-cell interactions between VVECs and AdvFBs that occurs *in vivo*, we next co-cultured VVECs and AdvFBs on Matrigel. Admixed suspensions of VVECs (labeled with PKH26, red signal) and AdvFBs (labeled with PKH67, green signal) exhibited a dissociated pattern of distribution at the time of plating under normoxic conditions (Figure 4A). After 20 hours of culture under normoxic conditions on Matrigel, VVECs and AdvFBs coalesced and assembled into heterotypic cord-like networks (Figure 4B). Hypoxic exposure (7% O₂) for 20 hours markedly changed the morphology of heterotypic cord-like networks formed by VVECs and AdvFBs on Matrigel, compared to normoxic cultures (Figure 4C). Hypoxia-induced morphological changes to heterotypic cord-like networks in VVECs/AdvFBs co-cultures were associated with increases in both the two-dimensional surface area (Figure 4D) and number of branch points, compared to normoxic cultures (Figure 4E).

To determine the capabilities of VVECs and AdvFBs to move through a complex matrix, co-cultures were labeled with semiconductor nanocrystals (VVECs were labeled with QTracker 655, red signal, and AdvFBs were labeled with QTracker 565, green signal), and Matrigel invasion assays were performed. We report that quantum dot-labeled VVECs and AdvFBs coalesced and assembled into heterotypic cord-like structures within 20 hours when cultured under normoxic conditions (Figure 4F). Moreover, cells invaded the Matrigel forming three-dimensional heterotypic cord-like networks below the surface of the Matrigel (Figure 4G).

Figure 3. Time-lapse fluorescence microscopic images demonstrating that VVECs (PKH26-labeled, red fluorescence signal) self-assembled into cord-like networks when cultured on Matrigel under normoxic conditions for up to 20 hours (A–D), a process augmented when cultured were maintained under hypoxic (7% O₂) conditions (E–H). Quantitative image analysis (using ImageJ software) demonstrated that hypoxic exposure increased both surface area (I) and number of branch points (J) of cord-like structures in VVEC Matrigel cultures. Images are representative of at least four experiments. Scale bars = 100 μm. The images are representative of data obtained from five separate VVEC isolates. The graphs are presented as mean values ± SD of five separate cell isolates (obtained from five different animals) measured in triplicate. ****P* < 0.001.

Figure 4. A: Fluorescent microscopic image showing that admixed co-cultures of VVECs (PKH26-labeled, red signal) and AdvFBs (PKH67-labeled, green signal) exhibited a dissociated pattern of distribution when plated on Matrigel under normoxic conditions. B: VVECs and AdvFBs coalesced and self-assembled into heterotypic cord-like networks when cultured on Matrigel under normoxic conditions for up to 20 hours. C: The morphology of cord-like networks formed by VVECs (red) and AdvFBs (green) when co-cultured on Matrigel was markedly altered when cultures were exposed to 7% O₂ for 20 hours. D and E: Quantitative image analysis illustrating increases in both surface area (D) and number of branch points (E) of cord-like structures in VVEC/AdvFB Matrigel co-cultures in response to hypoxia. F: Quantum dot-labeled VVECs (QTracker 655, red signal) and AdvFBs (QTracker 565, green signal) coalesced and self-assembled into heterotypic cord-like networks when cultured on Matrigel under normoxic conditions. G: Quantum dot-labeled VVECs and AdvFBs moved through the Matrigel and formed heterotypic cord-like networks in three dimensions below the surface of the Matrigel (dashed line). The images are representative of data obtained from five separate VVEC isolates and five separate AdvFB isolates. The graphs are presented as mean values (± SD) of five VVEC isolates and five AdvFB isolates (obtained from five different animals) measured in triplicate. ***P* < 0.1; ****P* < 0.001.

ET-1 Is at Least One Pro-angiogenic Molecule That Regulates the Self-Assembly of VVECs and AdvFBs into Cord-Like Networks on Matrigel

We next sought to determine the role of the endothelin system in hypoxia-induced pulmonary artery adventitial vasa vasorum neovascularization.

Proliferation assays demonstrated that exogenous ET-1 (10^{-7} mol/L) significantly ($P < 0.05$) stimulated proliferation of cultured VVECs over a 5-day period (Figure 5A), an effect mimicked, to a similar extent, when cells were exposed to hypoxia (7% O_2) for 5 days (Figure 5A). To investigate the role of endogenous ET-1 in the regulation of hypoxia-induced VVEC proliferation, we cultured VVECs in the presence of antisense oligonucleotides (AS-ON) targeting prepro-ET-1 mRNA. Using this strategy, we were able to significantly ($P < 0.05$) attenuate hypoxia-induced VVEC proliferation (Figure 5A).

We next sought to determine the effects of exogenous ET-1 on the self-assembly of VVEC and AdvFB co-cultures into cord-like networks on Matrigel. The addition of exogenous ET-1 (10^{-7} mol/L) to normoxic co-cultures for 20 hours markedly altered the morphology of cord-like networks, compared to control cultures (Figure 5B) (control comparison shown in the inset). Quantitative morphometric analyses revealed that morphological changes to cord-like networks in Matrigel co-cultures in response to exogenous ET-1 were associated with increases in both the surface area (Figure 5C) and the number of branch points (Figure 5D).

We next questioned whether hypoxia increased endogenous ET-1 production in VVECs and AdvFBs cultured on Matrigel. To this end, we performed ELISAs on conditioned media collected from VVECs and AdvFBs cultured on Matrigel for 12 hours. Figure 5E shows that both individual cultures of VVECs and AdvFBs, together with co-cultures, release endogenous ET-1 peptide into culture medium over a 12-hour period. The release of endogenous ET-1 by these cells was significantly ($P < 0.5$) increased when cultures were exposed to hypoxia (7% O_2) for 12 hours (Figure 5E). Hypoxia-induced ET-1 release by VVECs and AdvFBs was attenuated by the addition of AS-ON prepro-ET-1 into the culture medium (Figure 5E). Interestingly, and rather unexpectedly, co-cultures did not release significantly more ET-1 than either VVECs or AdvFBs cultures when grown alone.

Having determined that both exogenous and endogenous ET-1 influence the self-assembly of VVECs and AdvFBs into cord-like networks on Matrigel, we next sought to establish which ET receptors were being expressed by these cells. Cultured VVECs and AdvFBs expressed both ET_A and ET_B receptors (Figure 5F).

We used RT-PCR, together with quantitative densitometry, to validate the selectivity of our prepro-ET-1 antisense oligonucleotide strategy. Prepro-ET-1 mRNA levels were significantly down-regulated in VVEC and AdvFB cultures incubated with AS-ON compared to control cultures (C) or cultures incubated with scrambled oligonucleotides (SCR-ON) (Figure 5, G and H).

ET Receptor Antagonism Attenuates the Self-Assembly of Cord-Like Networks in VVEC/AdvFB Matrigel Cultures

To investigate further the role of the endothelin system in the regulation of self-assembly of VVECs and AdvFBs into cord-like networks on Matrigel, we next took a pharmacological approach to block specific endothelin receptors. To this end, we cultured VVECs and AdvFBs under hypoxic conditions (7% O_2) both in the absence and presence of the selective receptor antagonist BQ123 (ET_A) or BQ788 (ET_B).

The cord-like networks that formed in hypoxic VVEC Matrigel cultures after 20 hours (Figure 6A) were markedly diminished when cells were incubated with either the ET_A receptor antagonist BQ123 (Figure 6B) or the ET_B receptor antagonist BQ788 (Figure 6C). In addition, the heterotypic cord-like networks that formed when VVECs and AdvFBs were co-cultured on Matrigel under hypoxic conditions (Figure 6D) were markedly diminished when cells were incubated in the presence of either the ET_A receptor antagonist BQ123 (Figure 6E) or the ET_B receptor antagonist BQ788 (Figure 6F). Quantitative morphometric analyses revealed that morphological changes to cord-like networks in VVEC cultures and co-cultures in response to ET receptor antagonism were associated with decreases in both the surface area (Figure 6G), and the number of branch points, compared to control (Figure 6H).

Antisense Oligonucleotides Targeting Prepro-ET-1 mRNA Attenuate the Self-Assembly of Cord-Like Networks in VVEC/AdvFB Matrigel Cultures

Having determined that endogenous ET-1 regulates the proliferative responses of VVECs, we next sought to determine the role of endogenous ET-1 in the assembly of cord-like networks in VVECs and co-cultures. Accordingly, we cultured unlabeled cells on Matrigel under hypoxic conditions (7% O_2) both in the absence and presence of prepro-ET-1 AS-ON or SCR-ON. After 20 hours without AS-ON, VVECs (Figure 7A) and co-cultures (Figure 7D) assembled into cord-like networks. In contrast, the assembly of cord-like networks was attenuated, in part, when VVECs (Figure 7B), and co-cultures (Figure 7E), maintained under identical conditions, were incubated with AS-ON. SCR-ON had no negative effect on the assembly or cord-like networks in either VVECs (Figure 7C) or co-cultures (Figure 7F). Quantitative morphometric analyses revealed that morphological changes to cord-like networks in VVEC cultures and co-cultures in response to AS-ON were associated with decreases in both the surface area (Figure 7G) and the number of branch points (Figure 7H), compared to control cultures.

Figure 5. A series of complementary experiments demonstrating a role for the endothelin system in vasa vasorum neovascularization. **A:** Cell proliferation data showing that exogenous and endogenous ET-1 regulate the proliferative properties of cultured VVEC proliferation over a 5-day period. **B:** Exogenous ET-1 (10⁻⁷ mol/L) markedly altered the morphology of heterotypic cord-like networks formed by VVEC/AdvFB co-cultures when cultured under normoxic conditions for 20 hours, compared to control cultures (inset). **C** and **D:** Quantitative image analysis demonstrating the stimulatory effects of exogenous ET-1 on both surface area (**C**) and number of branch points (**D**) in heterotypic cord-like structures in VVEC/AdvFB co-cultures on Matrigel. **E:** ELISA analysis showing that VVECs and AdvFBs cultured on Matrigel released ET-1 peptide into culture medium, a process significantly augmented by hypoxia. Hypoxia-induced ET-1 release from these cells was inhibited by incubating cells with AS-ON targeting prepro-ET-1 mRNA. **F:** Western blot analysis showing that VVECs and AdvFBs cultured under normoxic conditions expressed both ET_A and ET_B receptors. **G** and **H:** RT-PCR together with densitometry revealed that the level of prepro-ET-1 mRNA in VVECs and AdvFBs control cultures (**C**) was significantly down-regulated when cells were treated with prepro-ET-1 AS-ON for 20 hours, whereas scrambled oligonucleotides (SCR) had no significant effect. The graphs in **A**, **C**, and **D** are presented as mean values (± SD) of five VVEC isolates and five AdvFB isolates measured in triplicate. The image in **B** is representative of data obtained from five separate VVEC isolates and five separate AdvFB isolates (obtained from five different animals). The data in **E** are from one ELISA kit with mean values (± SD) from five VVEC isolates and five AdvFB isolates (obtained from five different animals) measured in duplicate. The immunoblot in **F** is representative of data obtained using five VVEC isolates and five AdvFB isolates (obtained from five different animals). The PCR reaction (**G** and **H**) is representative of data obtained using all VVEC and AdvFB isolates (obtained from five different animals). **P* < 0.5; ***P* < 0.01; and ****P* < 0.001.

Figure 6. Fluorescence microscopy showing that cord-like networks formed in hypoxic VVEC Matrigel cultures (**A**) were markedly attenuated when cells were incubated with either the ET_A receptor antagonist BQ123 (**B**) or the ET_B receptor antagonist BQ788 (**C**). In addition, heterotypic cord-like networks formed in hypoxic co-cultures of VVECs and AdvFBs on Matrigel cultures (**D**) were markedly attenuated when cells were incubated with either the ET_A receptor antagonist BQ123 (**E**) or the ET_B receptor antagonist BQ788 (**F**). **G** and **H:** Quantitative image analysis demonstrating the negative effects of selective ET receptor antagonism on both surface area (**G**) and number of branch points (**H**) of cord-like structures in VVECs and co-cultures. The images are representative of data obtained from five separate VVEC isolates and five separate AdvFB isolates. The graphs are presented as mean values (± SD) of five VVEC isolates and five AdvFB isolates (obtained from five different animals) measured in triplicate. ****P* < 0.001.

Figure 7. Phase-contrast photomicrographs showing that cord-like networks formed in hypoxic (unlabeled) VVEC Matrigel cultures (A) were markedly attenuated when cells were incubated with AS-ON targeting prepro-ET-1 mRNA (B). SCR-ON had no effect on cord-like network formation (C). D–F: Heterotypic cord-like networks formed in hypoxic (unlabeled) co-cultures of VVECs and AdvFBs on Matrigel cultures (D) were markedly attenuated when cells were incubated with AS-ON targeting prepro-ET-1 mRNA (E). SCR-ON had no effect on cord-like network formation in hypoxic co-cultures (F). G and H: Quantitative image analysis demonstrating the effects of AS-ON on both surface area (G) and number of branch points (H) in cord-like structures in VVECs and co-cultures. The images are representative of data obtained from five separate VVEC isolates and five separate AdvFB isolates. The graphs are presented as mean values ($\pm$ SD) of five VVEC isolates and five AdvFB isolates (obtained from five different animals) measured in triplicate. ** $P < 0.01$; *** $P < 0.001$.

Endogenous ET-1 and Paracrine Signaling from AdvFBs Influence the Self-Assembly of VVECs into Cord-Like Structures on Matrigel

Based on our findings that AdvFBs influence the proliferative responses of VVECs, together with the finding that hypoxia-activated AdvFBs release ET-1 peptide, we next sought to determine the effects of AdvFBs (via co-culture and conditioned media) on the ability of VVECs to self-assemble into cord-like networks when cultured on Matrigel.

In these experiments we performed a “dye swap,” and VVECs were labeled with PKH67 (green signal) to visualize cells (in previous experiments we labeled VVECs with PKH26, red signal).

The self-assembly of VVECs into cord-like networks when cultured on Matrigel under hypoxic conditions for up to 20 hours (Figure 8A) was markedly attenuated when VVECs were incubated with prepro-ET-1 antisense oligonucleotides (AS-ON) (Figure 8B). The addition of “hypoxia-conditioned media” (collected from hypoxia-activated AdvFBs) to cultures of VVECs treated with prepro-ET-1 antisense oligonucleotides restored the ability of VVECs to self-assemble into cord-like structures (Figure 8C). A similar effect on the restoration of cord-like formation was observed when AdvFBs (labeled with PKH26, red signal) were added to cultures of VVECs treated with prepro-ET-1 antisense oligonucleotides (Figure 8D). Quantitative morphometric analyses revealed that AS-ON

reduced both the surface area and number of branch points in cord-like networks formed in VVEC Matrigel cultures and that the addition of “hypoxia-conditioned media” and/or AdvFBs to these cultures reversed this process (Figure 8, E and F).

Hypoxia, Exogenous ET-1, and AdvFBs Enhance the Integrity of Cord-Like Structures Formed by VVECs on Matrigel

We next investigated conditions that would enhance the short-term integrity of VVEC cultures. Cord-like networks formed by VVECs when cultured under normoxic conditions for up to 20 hours (Figure 9A) typically began to collapse when cultured under normoxic conditions for 48 hours (Figure 9B). The process of collapse was attenuated, and the integrity of networks maintained, when VVECs were: 1) maintained under hypoxic conditions (7% O_2) for 48 hours (Figure 9C), 2) stimulated with exogenous ET-1 for 48 hours under normoxic conditions (Figure 9D), 3) co-cultured with AdvFBs for 48 hours under normoxic conditions (Figure 9E), or 4) cultured in the presence of “hypoxia-conditioned media” (Figure 9F). Quantitative morphometric analyses revealed that the enhanced integrity, observed when VVEC cultures were treated as above, was associated with increases in both the surface area and the number of branch points compared to untreated cultures (Figure 9, G and H).

Figure 8. Fluorescence microscopy showing that the self-assembly of VVECs (this time labeled with PKH67, green signal) into cord-like structures when cultured on Matrigel under hypoxic conditions (A) was markedly attenuated when VVECs were treated with prepro-ET-1 AS-ON (B). The addition of “hypoxia-conditioned media” (collected from hypoxia-activated AdvFBs) to cultures of VVECs treated with prepro-ET-1 AS-ON restored the ability of VVECs to self-assemble into cord-like networks (C). A similar effect was observed when AdvFBs (this time labeled with PKH26, red signal) were added to cultures of VVECs (PKH67-labeled) and treated with prepro-ET-1 AS-ON (D). **E** and **F**: Quantitative image analysis illustrating the effects of endogenous ET-1 inhibition, together with “hypoxia-conditioned media” (collected from hypoxia-activated AdvFBs), and co-culture with AdvFBs on both surface area (E) and number of branch points (F) of cord-like structures in hypoxic VVEC Matrigel cultures treated with and without prepro-ET-1 antisense oligonucleotides. The images are representative of data obtained from five separate VVEC isolates. The graphs are presented as mean values ($\pm$ SD) of five VVEC isolates and five AdvFB isolates (obtained from five different animals) measured in triplicate. * $P < 0.5$; ** $P < 0.01$; and *** $P < 0.001$.

Endothelin-B Receptor Expression Is Increased in Remodeled Pulmonary Arteries in a Neonatal Calf Model of Hypoxia-Induced PAH

Having established that the ET system regulates the self-assembly of VVECs and AdvFBs into cord-like structures in Matrigel cultures, we next set out to investigate the

expression pattern of components of the ET system in our neonatal bovine model of hypoxic PAH (which is associated with vasa vasorum neovascularization). Using archival lung tissue and immunohistochemistry, we report that in normoxic calves ET_B receptor expression localized to the endothelium of vasa vasorum and pulmonary arteries and adventitial compartment (Figure 10A). In lung tissue

Figure 9. Phase-contrast photomicrographs showing that cord-like networks formed by VVECs after 20 hours under normoxic conditions (A), typically began to collapse when cultured under normoxic conditions for up to 48 hours (B). However, the process of collapse was attenuated, and the integrity of networks was maintained, when VVECs were 1) maintained under hypoxic conditions (7% O₂) for 48 hours (C), 2) cultured under normoxic conditions in the presence of exogenous ET-1 (10^{-7} mol/L) (D), 3) co-cultured with AdvFBs under normoxic conditions (E), or 4) cultured under normoxic conditions in the presence of “hypoxia-conditioned media” (collected from hypoxia-activated AdvFBs) (F). **G** and **H**: Quantitative image analysis demonstrating the effects of hypoxia, exogenous ET-1, and AdvFBs on both surface area (F) and number of branch points (G) in cord-like structures in VVECs and co-cultures. The images are representative of data obtained from five separate VVEC isolates and five separate AdvFB isolates (obtained from five different animals). The graphs are presented as mean values ($\pm$ SD) from five VVEC isolates and five AdvFB isolates measured in triplicate. * $P < 0.5$; ** $P < 0.01$; and *** $P < 0.001$.

Figure 10. Histopathology and immunohistochemical analysis illustrating localization of endothelin B receptors (ETBr, brown signal) in the PA vessel wall in normoxic calf lung tissue (**A**). **B**: Immunohistochemical localization of endothelin B receptor (ETBr) expression (brown signal) in the PA vessel wall in hypoxic calf lung tissue. **C**: Representative photomicrograph illustrating negative antibody control immunostaining. The images were obtained from archival lung tissue from normoxic ($n = 5$) and hypoxic ($n = 5$) neonatal calves.

from hypoxic calves, ET_B expression was localized to the endothelium of vasa vasorum and pulmonary arteries, stromal cells contiguous to vasa, and in remodeled pulmonary arterial vessel walls (Figure 10B). Due to lack of antibody specificity, we were unable to evaluate either ET_A receptor or ET-1 peptide immunoreactivity.

Discussion

Once assigned a passive role in providing the outer vessel wall with nutrients and O₂, the vasa vasorum network is now recognized as a critical microcirculation that may contribute to the development and progression of vascular remodeling that occurs in a wide range of systemic vasculopathies.^{4–8} In accordance with these observations, we previously demonstrated that vasa vasorum neovascularization occurs throughout the longitudinal axis of the pulmonary arterial circulation in a neonatal bovine model of hypoxic PAH.¹ Moreover, we observed that this neovascularization process occurred principally in the vascular adventitial compartment. Although we detected increased expression of a number of key pro-angiogenic factors close to adventitial vasa vasorum,¹ the precise cellular and molecular mechanisms regulating expansion of the microvascular circulation in pulmonary arteries in response to chronic hypoxia remained unknown. In the present study, using a number of complementary experimental techniques, we provide compelling data indicating that hypoxia-activated AdvFBs exhibit pro-angiogenic properties and influence the angiogenic phenotype of VVECs. They accomplish this, in part, by their ability to produce ET-1. This is important new information given the emerging concept that the endothelium of de novo-formed microvessels receives and integrates “pro-angiogenic signals” from a number of nonendothelial cells, including fibroblast-like cells.^{9–12} Based on our observations, we propose that activated AdvFBs are critical regulators of adventitial vasa vasorum neovascularization that occurs in the adventitia of pulmonary arteries in response to chronic hypoxia.

The findings of the present study describing the pro-angiogenic properties of hypoxia-activated AdvFBs are in accordance with a number of previous studies that have shown that fibroblast-like cells in a number of tissues are rich sources of diffusible pro-angiogenic factors and that, when activated, influence the phenotype and

function of adjacent cell types in their tissue microenvironment.^{9–12} Previous studies have shown that fibroblast-like cells secrete cytokines and angiogenic growth factors that regulate the formation of capillary-like networks by human umbilical vein endothelial cells and systemically derived microvascular endothelial cells when cultured on extracellular matrix proteins.^{9,11,14,15} Additionally, other studies have shown that stromal cells, including fibroblast-like cells, not only provide initial stimuli for the angiogenic cascade but also provide a stabilizing force to newly formed vessels.^{9–15} Tissue fibroblasts are also pro-angiogenic at sites of wound healing and inflammation; these cells respond to chemotactic cytokines that are released by the tissue microenvironment and are frequently the first cell type to migrate to the wound site where they orchestrate reparative neovascularization by releasing angiogenic/growth factors capable of self-stimulation and activation of other cell types in the microenvironment and by depositing extracellular matrix proteins.¹⁰ Collectively, the findings from these studies, together with the findings of the present study, support the concept that “activated” AdvFBs regulate the angiogenic responses of endothelial cells in the setting of neovascular growth.

Our observation that moderate hypoxia augmented the pro-angiogenic properties of cultured AdvFBs is in accordance with a number of previous studies in which hypoxic conditions have been shown to induce angiogenic phenotypes in a number of stromal cell types.^{21–23} Moreover, our findings are consistent with the well established paradigm that hypoxia is a common feature of many of the pathological conditions in which neovascular growth is observed.² It is noteworthy that previous studies from our laboratory have established that AdvFBs exhibit the earliest, and most dramatic, proliferative responses to hypoxia exposure in vivo and in vitro.^{24,25}

In the present study, we successfully isolated VVECs (of aortic origin) from the adventitial compartment of pulmonary arteries. In culture, we show that moderate hypoxia stimulates the proliferation of VVECs. This finding is consistent with previous studies reporting the pro-proliferative effects of hypoxia on systemically derived endothelial cells.^{26,27} Interestingly, to our best knowledge, no previous studies have demonstrated the pro-proliferative effects of hypoxia on endothelial cells derived from the pulmonary arterial lumen; a paucity of information lends support to the concept that the endothelium of systemic

vessels responds differently to pro-angiogenic stimuli than the endothelium of pulmonary arteries.²⁸ It is noteworthy that, although there are reports that the pulmonary circulation is relatively resistant to new vessel growth,²⁹ a few contrasting reports challenge this notion.^{30,31} The concept of functional endothelial cell heterogeneity between vascular beds, and within the same vascular region, has received much attention in recent years, not the least of which are the important implications this has on our understanding of the molecular mechanisms underlying vascular disease.³²

It is well established that, in the vessel wall, endothelial cells and mural cells, including fibroblasts, pericytes, and smooth muscle cells, make physical cell-cell contacts, suggesting a role for intracellular communication in the regulation of vascular growth and function.³³ In an attempt to model cell-cell communication in the setting of the adventitial compartment, we co-cultured VVECs and AdvFBs on Matrigel. Although the use of endothelial/stromal co-culture systems have previously been used to study heterotypic cell-cell interactions,^{10–12,34} to our best knowledge this is the first time that VVECs have been isolated and then co-cultured with pulmonary artery AdvFBs as a model system to study the cellular and molecular basis of vasa vasorum neovascularization. We fully acknowledge, however, that cord-like network formation *in vitro* does not represent the complete process of new vessel formation. Limitations notwithstanding, we believe that our VVECs/AdvFBs/Matrigel co-culture system compares favorably with other angiogenic assay systems that have been used to study the mechanisms regulating new vessel growth. We used our co-culture system, in which VVECs and AdvFBs coalesced and self-assembled into complex cord-like networks, a process augmented by hypoxia, as a platform to study the molecular regulators of hypoxia-induced adventitial vasa vasorum neovascularization.

In the present study, several lines of evidence implicate the ET system in the regulation of hypoxia-induced pulmonary artery adventitial vasa vasorum neovascularization. We fully acknowledge, however, that it is likely that a number of additional pro-angiogenic molecular regulators are involved in the process. Indeed, we have previously shown that a number of pro-angiogenic molecules are increased in the adventitial and medial layers of pulmonary arteries in a neonatal bovine model of hypoxic PAH, including vascular endothelial growth factor, fibronectin, and thrombin.¹ It is likely, therefore, that ET-1 and vascular endothelial growth factor (and other pro-angiogenic factors) have complementary and coordinated roles during hypoxia-induced vasa vasorum neovascularization. Indeed, it is well established that vascular endothelial growth factor is a hypoxia-regulated gene that is involved in new vessel growth in a number of pathological settings.³⁵ Our decision to investigate the ET system was based on the emerging role for the ET system as a molecular regulator of postnatal neovascularization in a number of pathological settings.¹⁶ Additionally, although ET-1 has been implicated as a critical regulator in the pathogenesis of PAH, the precise biolog-

ical function of this peptide in the disease process remains unknown.

We demonstrated that ET-1 is released from both hypoxia-activated AdvFBs and VVECs, the former observation raising the possibility that ET-1 produced by AdvFBs regulated the proliferation of VVECs and facilitated the self-assembly of VVECs into cord-like networks. Although vascular adventitial fibroblasts isolated from the systemic circulation have previously been shown to synthesize and release ET-1 in response to angiotensin II,³⁶ our study is the first to report that pulmonary artery AdvFBs release ET-1, a process that was augmented when the cells were cultured under hypoxic conditions. Our data are consistent with the finding that ET-1 promoter activity and subsequent protein expression is enhanced in many cells, including fibroblasts, endothelial cells, and lung macrophages, in response to different stimuli, including hypoxia.³⁷

Using complementary strategies, we show that blocking the effects of endogenously produced ET-1 using ET receptor antagonists, or by targeting prepro-ET-1 mRNA using oligonucleotides, attenuates, in part, the assembly of cord-like networks formed in VVEC cultures and AdvFB/VVEC/Matrigel co-cultures. Our findings are in accordance with a number of previous studies. For example, ET-1 stimulates an angiogenic phenotype in cultured endothelial cells and regulates the assembly of capillary-like networks formed by aortic endothelial cells on Matrigel,³⁸ a process mediated by ET_B receptors in human umbilical vein endothelial cells cultured on fibrin.³⁹ In cardiovascular disease, ET receptor antagonism preserves biological integrity and reduces neovascularization. For example, chronic ET_A receptor antagonism prevents coronary artery vasa vasorum neovascularization in experimental hypercholesterolemia.⁶

Our data indicate that both ET_A and ET_B receptors are involved in the self-assembly of VVECs and AdvFBs into cord-like networks. These observations are consistent with studies investigating the role of the endothelin system in tumor neovascularization that have implicated both ET receptor subtypes in this process.⁴⁰ However, the precise role of each ET receptor subtype in the self-assembly of VVECs and AdvFBs into cord-like networks remains unknown. Based on observations in the tumor literature, it is tenable to speculate that the ET_B receptor subtype predominantly regulates the early proliferative function of endothelial cells in the angiogenic cascade, whereas the ET_A receptor subtype is involved in stromal cell migration/recruitment and maturation of new vessels.⁴¹ Finally, whether the ET system is involved in adventitial neovascularization, which occurs in some forms of human PAH,³ remains to be established.

In conclusion, this study provides new information by describing the pro-angiogenic properties of hypoxia-activated AdvFBs. Our observations raise the possibility that hypoxia-activated AdvFBs cooperate with VVECs in a process involving ET-1 to regulate pulmonary artery adventitial neovascularization in the setting of hypoxic PAH. These findings have important implications in our further understanding of the complex nature of vascular remodeling that occurs in PAH and may have important

implications for unraveling the cellular and molecular basis of vasa vasorum neovascularization that occurs in the systemic circulation in response to injurious stimuli. Although the precise consequence of adventitial vasa vasorum neovascularization remains unknown, we speculate that this vascular network serves as a custom-delivery system for the trafficking and recruitment of inflammatory cells, circulating progenitor cells, and detrimental metabolic substrates to the outer vessel wall. Targeting the neovascularization process represents a potential therapeutic strategy aimed at inhibiting or reversing vascular remodeling in a number of pathological settings. Ongoing work in our laboratory is aimed at investigating the specific role of ET receptor subtypes in hypoxia-induced vasa vasorum neovascularization using our co-culture system, together with animal models of hypoxic PAH.

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