SHORT COMMUNICATION

Endothelial to Mesenchymal Transition Contributes to Endothelial Dysfunction in Pulmonary Arterial Hypertension

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Pulmonary arterial hypertension (PAH) is a progressive disease characterized by lung endothelial cell dysfunction and vascular remodeling. Normally, the endothelium forms an integral cellular barrier to regulate vascular homeostasis. During embryogenesis endothelial cells exhibit substantial plasticity that contribute to cardiac development by undergoing endothelial-to-mesenchymal transition (EndoMT). We determined the presence of EndoMT in the pulmonary vasculature *in vivo* and the functional effects on pulmonary artery endothelial cells (PAECs) undergoing EndoMT *in vitro*. Histologic assessment of patients with systemic sclerosis-associated PAH and the hypoxia/SU5416 mouse model identified the presence of von Willebrand factor/ α -smooth muscle actin—positive endothelial cells in up to 5% of pulmonary vessels. Induced EndoMT in PAECs by inflammatory cytokines IL-1 β , tumor necrosis factor α , and transforming growth factor β led to actin cytoskeleton reorganization and the development of a mesenchymal morphology. Induced EndoMT cells exhibited up-regulation of mesenchymal markers, including collagen type I and α -smooth muscle actin, and a reduction in endothelial cell and junctional proteins, including von Willebrand factor, CD31, occludin, and vascular endothelial-cadherin. Induced EndoMT monolayers failed to form viable biological barriers and induced enhanced leak in co-culture with PAECs. Induced EndoMT cells secreted significantly elevated proinflammatory cytokines, including IL-6, IL-8, and tumor necrosis factor α , and supported higher immune transendothelial migration compared with PAECs. These findings suggest that EndoMT may contribute to the development of PAH. (*Am J Pathol* 2015, 185: 1850–1858; <http://dx.doi.org/10.1016/j.ajpath.2015.03.019>)

A healthy pulmonary endothelial barrier is integral to regulating the extravasation of cells and cytokines from the blood and to maintaining vascular homeostasis.¹ Dysfunction of the endothelial barrier can occur in response to inflammatory mediators such as IL-6 and tumor necrosis factor α (TNF α), as well as pathogens.^{2–4} A significant number of studies have highlighted the contribution of endothelial cell dysfunction in human pathologies, including the development and progression of pulmonary arterial hypertension (PAH).^{5–7} Loss of endothelial barrier integrity, disordered endothelial proliferation, and enhanced inflammatory cell infiltration are common features believed to contribute to the pathologic vascular remodeling in PAH.^{5–7} Studies suggest exposure of endothelial cells to chronic stresses and inflammatory factors can promote

endothelial cells to undergo a process termed endothelial-to-mesenchymal transition (EndoMT).^{8–10}

A number of studies support the capacity of endothelial cells to contribute to vascular smooth muscle cells (SMCs) and cardiac fibroblast populations during embryogenesis through the process of EndoMT.^{11–13} Frid et al⁸ reported the presence in mature adult vascular endothelium of transitional cells co-expressing endothelial markers and α -smooth muscle actin (α -SMA) and estimated EndoMT to be a comparatively rare

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phenomena under normal circumstances. More recently EndoMT was reported in a number of adult pathologies, including the intestinal microvascular endothelial cells in inflammatory bowel disease,^{9,14} diabetic renal fibrosis,¹⁵ cardiac fibrosis,¹⁰ and portal hypertension.¹⁶ Lineage tracking in pre-clinical fibrosis models suggests that as many as 16% of lung fibroblasts that express α -SMA and collagen type I are derived from endothelial cells.¹⁷ The expansion of pulmonary artery SMCs (PASMCs) and fibroblasts that contribute to vascular remodeling in all forms of PAH, including PAH associated with systemic sclerosis (SSc), in part result from pulmonary artery endothelial cells (PAECs) undergoing EndoMT.^{18,19}

Endothelial cells undergoing EndoMT lose their endothelial characteristics and undergo a transition toward a more mesenchymal-like phenotype.^{9,16–18,20} In addition to inflammatory cytokine induction of EndoMT, an imbalance in the transforming growth factor β (TGF- β)/bone morphogenic protein axis was also implicated.^{10,16} We and others have reported a critical role for inflammation and TGF- β in the development of PAH.^{6,7,21–23} However, the contribution to vascular remodeling in PAH and the cellular effects of EndoMT on human PAECs remains poorly understood.

Here, we determined the presence of transitional EndoMT cells in an experimental model of PAH and SSc-PAH patients. We observed the co-expression of von Willebrand factor (vWF) and α -SMA in a small proportion of pulmonary endothelial cells in vessels from experimental PAH models and SSc-PAH patients but not control vessels. With the assessment of the functional effect of EndoMT, TNF α , IL-1 β , and TGF- β in combination promoted PAECs to undergo actin cytoskeletal rearrangement and to develop a mesenchymal cell structural characteristic similar to that of pulmonary fibroblasts, a process we termed induced EndoMT (I-EndoMT). I-EndoMT cells exhibited a reduction of endothelial and junctional protein expression and gained expression of mesenchymal markers, including *de novo* collagen type I synthesis. Consistent with EndoMT contributing to endothelial dysfunction, I-EndoMT cells secreted elevated proinflammatory cytokines compared with PAECs and failed to establish an integral biological barrier. Furthermore, PAECs barriers that comprised 10% I-EndoMT cells led to significantly enhanced permeability. Our data suggest that EndoMT may promote dysfunction of the endothelium and may contribute to the development of PAH.

Materials and Methods

Reagents

Endothelial basal EGM-2 media (CC-3156) was supplemented with bullet kit growth factors (CC-4176; Lonza, Verviers, Belgium) to form growth media. Primary antibodies were α -SMA—cyanine 3 (fluorescent staining; C6198; Sigma Chemical, Poole, UK); vWF, CD31, and α -SMA (A008202, M082329, and M0851, respectively; Dako, Cambridge, UK); vascular endothelial-cadherin, calponin, occludin, and β -tubulin (ab33168, ab46794, ab31721, and

ab179513, respectively; Abcam, Cambridge, UK); collagen type I (Millipore, Billerica, MA). Fluorescent secondary antibodies were anti-mouse, anti-rabbit, and anti-goat (A-11005, A-21070, and A-11078, respectively; Invitrogen, Paisley, UK). Horseradish peroxidase-conjugated secondary antibodies were anti-rabbit, anti-mouse (7074 and 7046, respectively; Cell Signaling, Hitchin, UK), and anti-goat (P044901; Dako). Species-matched IgG controls were anti-mouse, anti-rabbit, and anti-goat (X013, X0720, and I5000, respectively; Vector, Peterborough, UK). Texas-Red phalloidin (T7471; Invitrogen) and DAPI mounting media (H-1200; Vector) were used. TNF α , TGF- β , and IL-1 β (300-01A, 100-21C, and 200-01B, respectively; PeproTech, London, UK) were used, and all other reagents were purchased from Sigma Chemical.

Preclinical and Clinical PAH Samples

The hypoxia/SU5416 murine model of PAH was performed in accordance with Home Office regulations under the UK Home Office Animals (Scientific Procedures) Act as previously published.²⁴ After 21 days hemodynamic measurements and histologic analysis confirmed that mice had significant elevated right ventricular systolic pressures, remodeled pulmonary arteries, and right heart hypertrophy. Human tissues were obtained from SSc patients with clinically defined PAH (SSc-PAH) with mean pulmonary artery pressure ≥ 25 mm Hg and healthy control (HC) donors with full consent according to the UK National Health Service guidelines to meet the requirements of the Human Tissue Act 2004. Experiments were approved by the local ethics committee.

Histology, Immunohistochemistry, Immunocytochemistry, and Fluorescence

Lung tissues from hypoxia/SU5416 mice were stained with hematoxylin and eosin or double stained with α -SMA and vWF (dilution 1:200; Dako). Fluorescent staining was performed on hypoxia/SU5416 mice and human donors after sections were deparaffinized and underwent antigen retrieval for 30 minutes in citrate buffer. Sections were stained for α -SMA (dilution 1:100; Sigma Chemical) and vWF (dilution 1:100; Dako) with appropriate fluorescent secondary antibodies and DAPI mounting medium (Vector). We assessed 20 pulmonary arteries 10 to 100 μ m in diameter per mouse ($n = 4$ per group) and 20 pulmonary arteries 40 to 200 μ m diameter per human section ($n = 4$ per group), and the frequency of colocalization was determined with a Zeiss Axioskop 2 fluorescent microscope (Carl Zeiss, Jena, Germany) and Axiovision software version 4.8.2.0 (Carl Zeiss) to Z-stack images through multiple layers of tissue sections. For immunocytochemistry, PAECs and I-EndoMT cells were seeded at 5×10^3 per well in 8 well chamber slides, precoated in attachment factor (Invitrogen). After 24 hours, cells were fixed and permeabilized in ice-cold acetone/methanol (1:1 ratio), or 4% paraformaldehyde/Triton X-100 (phalloidin), for 10 minutes then blocked in 10% serum of appropriate species. Cells were incubated with primary

antibodies (dilution 1:500) overnight at 4°C before incubating with appropriate secondary antibodies (dilution 1:1000) for 1 hour at room temperature.

Cell Cultures

PAECs (Invitrogen) were grown in basal media (EGM-2 media that contained 2% fetal calf serum, FCS; Lonza) and growth factor bullet kit (Lonza). I-EndoMT cells were induced by the addition of 5 ng/mL TNF α , 5 ng/mL TGF- β , and 0.1 ng/mL IL-1 β (PeproTech) to PAEC growth media. PSMCs (Lonza) and human lung fibroblasts (HLFs) from HC donors and SSc-PAH patients were established from lung biopsies and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Cell lines were used between passages four to eight.

Western Blot Analysis

Cell monolayers were lysed in RIPA buffer (Sigma Chemical), and protein levels were determined by bicinchoninic acid assay (Pierce, Rockford, IL). Expression of vWF, CD31, vascular endothelial-cadherin, occludin, calponin, α -SMA, and collagen type I by PAECs and I-EndoMT cells at day 2, 4, and 6 was compared with HLFs and PSMCs by Western blot analysis, and expression was normalized to β -tubulin. All antibodies were used at 1:1000 dilution. β -Tubulin was used at 1:10,000 dilution.

Proinflammatory Cytokine Levels in Supernatant Fluids

PAECs, I-EndoMT, HC-HLFs, and SSc-PAH HLFs were cultured to confluence, and media were exchanged for basal EGM-2 media. After 24 hours conditioned media were collected, and cytokine secretion was quantified with human proinflammatory 10plex Meso Scale Diagnostics plates (MSD; Rockville, MD) according to the manufacturer's instructions. Cytokine concentrations were normalized to cell number as determined by crystal violet and expressed as pg/mL/10⁴ cells.

Ammonium Sulfate Precipitation

Conditioned media was collected from monolayers of PAECs, I-EndoMT, and SSc-PAH HLFs after 24 hours and mixed with an equal volume of saturated ammonium sulfate solution and incubated overnight at 4°C to precipitate proteins. Media was centrifuged, the precipitated protein pellet was resuspended in protein lysis buffer, and collagen type I secretion was determined by Western blot analysis.

Migration Assay

Confluent monolayers of PAECs, I-EndoMT cells, and SSc-PAH HLFs in 96-well plates were scratched (V&P Scientific, San Diego, CA), and monolayers were treated with basal EGM-2 media that contained 1.5 μ g/mL mitomycin-C (Sigma Chemical) supplemented with either 2% or 10% FCS.

Migration was assessed after 24 hours (Axiovision software) and quantified with WimScratch image analysis software (Wimasis Image Analysis, Munich, Germany).

Cell Number

Cell numbers were determined with the triphenylmethane dye, crystal violet (4-[(4-dimethylaminophenyl)-phenyl-methyl]-*N,N*-dimethyl-aniline). Briefly, 5 $\times$ 10³ PAECs or I-EndoMT cells were seeded per well in 96-well plates. Cells were treated with basal EGM-2 media that contained 2% or 10% FCS, and cell numbers were assessed at 1, 4, and 7 days after treatment. Media were removed, and monolayers were washed and incubated for 10 minutes with crystal violet. Monolayers were washed in water, and the crystal violet was solubilized with 33% citric acid. Crystal violet was quantified with a Mithras plate reader (560 nm absorbance), and cell numbers were determined from a standard curve.

Endothelial Barrier Function Assays

Barrier function of PAECs, I-EndoMT cells, and mixed cultures of I-EndoMT/PAECs (1:10 ratio) were quantified with electrical cell-substrate impedance sensing (ECIS; Applied BioPhysics, Troy, NY) as previously described.²⁵ Cells were seeded at 5 $\times$ 10⁴ cells/0.8 cm² in eight-well slides, each containing 10 gold electrodes (8W10E). Impedance, resistance, and capacitance was measured at 11 different frequencies across the monolayers over 36 hours. Mathematical modeling by using the ECIS Z θ software version 1.2.193.0 (Applied BioPhysics) was used to quantify cell-cell junction strength as a measure of paracellular permeability and barrier function and cell membrane capacitance. Elevated cell membrane capacitance correlates with elevated cell membrane invaginations, representing the potential transcellular route of permeability.^{25–33} Permeability was also assessed with the albumin-flux assay. Confluent monolayers were established with 1 $\times$ 10⁵ cells per well of a 24-well insert of 3- μ m pore (VWR International, Lutterworth, UK). After 24 hours media were removed, and basal EGM-2 was added with or without 10 ng/mL TNF α . After 24 hours 0.5% w/v fluorescein isothiocyanate (FITC)-albumin was added to the inserts, and FITC-albumin in the bottom chamber was quantified after 1 hour by using a Mithras plate reader (MSD) at 485 nm/535 nm.

Immune Cell Transendothelial Migration

Immune cell transendothelial migration was performed as previously published.⁶ Confluent monolayers of PAECs, I-EndoMT, and mixed I-EndoMT/PAECs (dilution 1:10) cultures were established in Transwell inserts (as described in the previous paragraph). Freshly isolated peripheral blood mononuclear cells (PBMCs; 1 $\times$ 10⁶) from HC donors were added to the upper chamber. After 2 hours the number of transendothelial migrated PBMCs was determined.

Statistical Analysis

Data are presented as means $\pm$ SEM, and n represents the number of donor samples or cell lines. Statistical analysis was performed by one-way analysis of variance or Mann-Whitney

tests between groups. Two-way analysis of variance was used to calculate significance for repeated measures, followed by Bonferroni post hoc test. $P \leq 0.05$ was considered significant.

Results

EndoMT in Pulmonary Vessels of Experimental Models of PAH and SSc-PAH Patients

The hypoxia/SU5416 preclinical murine PAH model exhibits many of the pathologic vascular changes observed clinically (Supplemental Figure S1). We sought to determine *in vivo* the presence of transitional EndoMT cells in the hypoxia/SU5416 PAH model and lung biopsies from SSc-PAH patients. Lungs were isolated from hypoxia/SU5416 mice, and the frequency of transitional EndoMT cells was assessed by colocalization of vWF and α -SMA. We observed transitional EndoMT cells to be present at low levels in control tissues (approximately 1% of vessels), with significantly higher levels within the hypoxia/SU5416 treatment group, at a frequency of $6\% \pm 1\%$ of vessels (Figure 1, A and C). We next assessed the presence of transitional EndoMT cells in the pulmonary endothelium of SSc-PAH patients. We observed colocalized vWF and α -SMA in the endothelium in up to $4\% \pm 1\%$ of pulmonary arterioles from SSc-PAH patients but not from HCs (Figure 1, B and C).

Inflammatory Cytokines Induce EndoMT in PAECs *in Vitro*

Previous studies have found that intestinal microvascular endothelial cells exposed to inflammatory cytokines *in vitro* lose their endothelial appearance and gain mesenchymal properties.⁹ We have previously reported elevated circulatory levels of inflammatory cytokines, including IL-1 β and TNF α , in PAH patients and the hypoxia/SU5416 experimental PAH model.^{24,34–37} With the use of the inflammatory cytokines TNF α , IL-1 β in combination with TGF- β but

Figure 1 EndoMT is detected in the hypoxia/SU5416 preclinical model of PAH and SSc-PAH patients. **A:** Immunohistochemical analysis of lung tissue from the murine hypoxia/SU5416 model of PAH. Sections are stained with vWF (green), α -SMA (red), and DAPI (blue). Colocalization (EndoMT; white arrows) was observed in approximately 6% of vessels 10 to 100 μ m in diameter in the hypoxia/SU5416 group, whereas colocalization in control groups was detected in $<1\%$ of vessels. **B:** Immunohistochemical analysis of lung sections from human biopsies of HC (control) and SSc-PAH lungs. EndoMT was detected in approximately 4% of SSc-PAH vessels between 40 and 200 μ m in diameter examined. Colocalization is absent in HC vessels. Colocalization is indicated by white arrows. **C:** Quantification of vessels exhibiting luminal colocalization in the hypoxia/SU5416 preclinical model and in human HC and SSc-PAH lungs. Values are expressed as means $\pm$ SEM. $n = 4$ (A and B). $*P \leq 0.05$, determined by analysis of variance and Mann-Whitney tests for mouse and human samples, respectively. Scale bars: 20 μ m (A and B). EndoMT, endothelial to mesenchymal transition; HC, healthy control; PAH, pulmonary arterial hypertension; α -SMA, α -smooth muscle actin; SSc, systemic sclerosis; SU, SU5416; Veh, vehicle; vWF, von Willebrand factor.

Figure 2 Proinflammatory cytokines induce PAECs to undergo I-EndoMT *in vitro*. **A:** PAECs treated with TNF α , IL-1 β , and TGF- β (+) in combination or vehicle (–). After 6 days endothelial cobblestone structural characteristic is lost, and cells reorganize actin filaments to gain a spindle-like appearance, similar to HLFs isolated from SSc-PAH patients. Indicated by light microscopy and phalloidin staining. **B:** Analysis of endothelial and mesenchymal cell surface markers by Western blot analysis indicates PAECs undergo I-EndoMT *in vitro*, lose their endothelial markers, and gain mesenchymal markers. Mesenchymal-positive controls were SSc-PAH HLFs and PSMCs. **White lines** denote samples were resolved on different gels, and equal protein loading was confirmed in all cases using β -tubulin. **C:** Collagen type I secretion was assessed by ammonium sulfate precipitation of conditioned media from PAECs, I-EndoMT cells, and SSc-PAH HLFs. **D:** Immunocytochemical analysis of endothelial marker CD31 and mesenchymal markers collagen type I and calponin. **E:** EndoMT cells positively co-express vWF (green) and α -SMA (red). Nuclei are stained with DAPI (blue). $n = 3$ (**A** and **C**); $n = 4$ (**B** and **E**). HLF, human lung fibroblast; I-EndoMT, induced-endothelial to mesenchymal transition; PAEC, pulmonary artery endothelial cell; PAH, pulmonary arterial hypertension; PSMC, pulmonary artery smooth muscle cell; α -SMA, α -smooth muscle actin; SSc, systemic sclerosis; TGF- β , transforming growth factor β ; TNF α , tumor necrosis factor α ; VE, vascular endothelial; vWF, von Willebrand factor.

not alone (data not shown), PAECs underwent I-EndoMT *in vitro* (Figure 2). After 6 days PAECs lost their endothelial cobblestone structural characteristic and underwent actin filament reorganization, forming spindle-like cells that resembled fibroblasts (Figure 2A). Withdrawal of TNF α , IL-1 β , and TGF- β after 6 days failed to promote I-EndoMT cells to re-establish a PAEC structural characteristic (data

not shown). I-EndoMT cells exhibited a significant reduction in endothelial cell markers, CD31 and vWF, and tight and adherens junction proteins, occludin and vascular endothelial-cadherin. Conversely, I-EndoMT cells acquired calponin, α -SMA, and collagen type I expression, similar to that observed on PSMCs and SSc-PAH HLFs (Figure 2, B, D, and E).

Table 1 Secretomic Profile of PAECs, I-EndoMT Cells, HC-HLFs, and SSc-PAH HLFs

Secreted cytokines	PAECs (pg/mL/10 ⁴ cells)	I-EndoMT (pg/mL/10 ⁴ cells)	HC-HLFs (pg/mL/10 ⁴ cells)	SSc-PAH HLFs (pg/mL/10 ⁴ cells)
IFN- γ	4.0 (4)	1.4 (1.4)	<LLOQ	4.1 (2.1)
IL-10	<LLOQ	2.2 (0.5)	<LLOQ	3.0 (1.2)
IL-12p70	<LLOQ	<LLOQ	<LLOQ	<LLOQ
IL-13	3.5 (3.4)	18.3 (3)*	2.3 (1.4)	28.1 (8.9)*
IL-1 β	<LLOQ	<LLOQ	<LLOQ	<LLOQ
IL-2	<LLOQ	2.0 (0.8)	0.8 (0.2)	4.0 (0.9)*
IL-4	<LLOQ	1.3 (0.3)	0.5 (0.2)	1.2 (0.1)
IL-6	12 (6.6)	474 (95)**	260 (120)	595 (18)***
IL-8	28 (6.5)	620 (71)***	58 (21)	503 (38)**
TNF- α	0.3 (0.2)	6.1 (1.6)*	0.4 (0.2)	2.2 (0.5)*

Conditioned media from PAECs, I-EndoMT cells, HC-HLFs, and SSc-PAH HLFs were analyzed and quantified by MSD 10-plex cytokine array. Values below the LLOQ were excluded. $n = 4$. Values are expressed as means $\pm$ SEM.

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

HC, healthy control; HLF, human lung fibroblast; I-EndoMT, induced-endothelial to mesenchymal transition; IFN, interferon; LLOQ, lowest level of quantification; PAEC, pulmonary artery endothelial cell; PAH, pulmonary arterial hypertension; SSc, systemic sclerosis; TNF- α , tumor necrosis factor α .

I-EndoMT Cells Exhibit a Proinflammatory Phenotype

We sought to quantify the secretion of inflammatory cytokines from I-EndoMT cells. I-EndoMT cells secreted significantly higher levels of the following inflammatory cytokines: IL-4, IL-13, IL-6, IL-8, and TNF α than that of PAECs. Consistent with PAECs transitioning toward a more mesenchymal phenotype, secreted cytokine levels from I-EndoMT cells were similar to that secreted from HLFs established from SSc patients with PAH (Table 1). Furthermore, I-EndoMT cells secreted collagen type I, in contrast to PAECs (Figure 2C).

I-EndoMT Cells Fail to Form Effective Barriers and Exhibit Altered Cellular Processes

Under normal circumstances the endothelium forms an integral barrier that regulates a low and selective permeable barrier and immune surveillance.⁶ We demonstrate in Figure 2 that PAECs exposed to inflammatory cytokines undergo cellular changes. Characterization of the functional consequences of I-EndoMT demonstrated a significantly enhanced basal cellular migratory response compared with that of PAECs (Figure 3, A and B). The growth of PAECs and I-EndoMT cells was monitored at days 1, 4, and 7 after seeding equal numbers of cells. PAECs exhibited a significant increase in cell number compared with I-EndoMT cells at days 1, 4, and 7 at 2% and 10% FCS (Figure 3C).

Loss of an integral endothelial barrier is believed to contribute to the activation and proliferation of the underlying vascular SMCs and fibroblasts, thus contributing to vascular remodeling in PAH.⁷ We assessed the capacity of PAECs and I-EndoMT cells alone or in coculture with PAECs to form biological barriers and to support immune cell transendothelial migration (Figure 3, D–G). Assessment of endothelial permeability by using FITC-albumin found that monolayers of I-EndoMT cells exhibited a fivefold increase in leak compared

with PAECs alone. TNF α was previously found to promote endothelial cell permeability.³⁸ Consistently, TNF α induced a fourfold increase in PAECs leak similar to that observed in unstimulated I-EndoMT cells. In contrast I-EndoMT cells did not significantly respond to TNF α treatment. Extending these studies we sought to mimic the *in vivo* situation¹⁷ and assessed the capacity of mixed I-EndoMT/PAECs cultures to form barriers. Basal permeability of mixed cultures was significantly elevated, whereas treatment with TNF α induced a similar fold increase in permeability in all monolayers (Figure 3D).

We sought to confirm the reduction in barrier integrity observed in I-EndoMT cells by using ECIS technology.^{25,27} Resistance, impedance, and capacitance were determined across cell monolayers. Consistent with the enhanced leak observed with transwell assays, I-EndoMT and mixed monolayers exhibited a significant reduction in cell–cell junction strength values, indicative of increased paracellular permeability compared with PAECs alone (Figure 3E). Elevated cell membrane capacitance values, indicative of increased cell membrane invaginations and a greater potential for a transcellular permeability, was detected across I-EndoMT monolayers compared with PAECs (Figure 3F). Thus, I-EndoMT cells form less-integral biological barriers.^{25–33}

Leukocyte infiltration and retention in the pulmonary vasculature is a key feature of PAH.^{39,40} To investigate the role played by EndoMT in leukocyte infiltration, immune cell transendothelial migration was quantified across monolayers of PAECs, I-EndoMT cells, or mixed 1:10 cultures. Monolayers of I-EndoMT cells supported a significant increase in basal PBMC transendothelial migration compared with PAECs alone (Figure 3G). Unstimulated mixed cultures of I-EndoMT/PAECs failed to reach significance, whereas treatment with TNF α significantly increased PBMCs transendothelial migration in all conditions.

Figure 3 Migration, growth rate, and barrier function of I-EndoMT cells. **A** and **B**: I-EndoMT cells have a higher rate of migration than PAECs, similar to the migration properties of SSc-PAH human lung fibroblasts in *in vitro* scratch wound migration assays. **C**: Crystal violet assays were used to quantify I-EndoMT and PAEC numbers after 1, 4, and 7 days in the presence of 2% or 10% FCS. **D**: Barrier function of homogenous and mixed I-EndoMT/PAECs cultures was quantified in Transwell permeability assays. Monolayers were stimulated $\pm$ TNF α ; FITC-albumin movement across monolayers was quantified as a measure of permeability after 1 hour. **E**: Quantification of Rb value as a measure of paracellular permeability and barrier function of cell monolayers. **F**: Cm of cell monolayers. Cm values correlate to the number of invaginations in the luminal and abluminal cell membranes, representing the potential for a transcellular route of permeability. **G**: Immune cell transendothelial migration across homogenous and mixed cultures stimulated $\pm$ TNF α was quantified by cell counting. $n = 3$ (**B–D**); $n = 4$ (**E–G**). $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$, determined by one-way analysis of variance between groups or two-way analysis of variance for repeated measures. Cm, cell membrane capacitance; I-EndoMT, induced-endothelial to mesenchymal transition; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; PAEC, pulmonary artery endothelial cell; PAH, pulmonary arterial hypertension; Rb, cell–cell junction strength; SSc, systemic sclerosis; TNF- α , tumor necrosis factor α ; Veh, vehicle.

Discussion

Endothelial dysfunction is a contributing factor to vascular remodeling in PAH, including SSc-PAH, which is characterized by the cellular expansion and accumulation of α -SMA and collagen type I-positive cells. These cells were proposed to originate from activated medial PASMCs and lung fibroblasts^{6,19,41}; however, it was hypothesized that endothelial cells undergoing EndoMT may contribute to endothelial dysfunction and the population of activated mesenchymal cells in fibrotic and chronic inflammatory disorders, including inflammatory bowel disease, cardiac fibrosis, and portal hypertension.^{9,10,16,19} Here, we demonstrate the presence of transitional EndoMT cells in the pulmonary vasculature of

patients with SSc-PAH and in the hypoxia/SU5416 model of PAH in a small proportion of vessels. In contrast EndoMT was only observed at negligible levels in control groups. Our data support the notion that endothelial cells may undergo EndoMT *in vivo*; however, this may need further confirmation by using lineage tracking in preclinical models.

Inflammatory cytokines such as TNF α and IL-1 β were found to be elevated in the serum of PAH and SSc patients.^{22,35} Previously, it was found that microvascular endothelial cells exposed to proinflammatory cytokines induce EndoMT *in vitro*.⁹ We demonstrate that human PAECs can undergo a transition toward a mesenchymal cell phenotype in response to TNF α , IL-1 β , and TGF- β in combination. I-EndoMT cells lose their endothelial

cobblestone appearance and adapt a structural characteristic that resembles HLFs. Consistent with the structural characteristic changes observed in I-EndoMT cells, we observed a reduction in expression of endothelial cell markers and a composite up-regulation of mesenchymal surface markers, including α -SMA and collagen type I.

We have previously found that the elevated serum levels of inflammatory cytokines, including IL-6 and IL-8, are associated with reduced survival in PAH patients.^{22,35,37} A number of studies have also reported elevated inflammatory cytokines to be associated with the development of organ-based complication in SSc, including IL-6 and IL-13.^{22,39,41} Whereas PAECs secreted relatively low levels of proinflammatory cytokines, I-EndoMT cells secreted significantly elevated levels of IL-6, IL-8, and TNF α , similar to that by SSc-PAH fibroblasts. Consistent with this, I-EndoMT monolayers supported higher immune trans-endothelial migration compared with PAECs alone.

Damage to the endothelium requires the proliferation and migration of endothelial cells to restore the integrity of the endothelial barrier and vascular homeostasis. We have previously found that failure of PAECs to form an effective exclusion barrier contributes to vascular remodeling in experimental models of PAH.⁶ We assessed a number of cellular responses key to maintaining an effective biological barrier. I-EndoMT cells exhibited a significant increase in motility compared with PAECs, whereas growth rate was significantly lower than PAECs. Furthermore, I-EndoMT cells exhibited a significant reduction in tight junctional protein expression and a significant elevation in a number of proinflammatory cytokines. Interestingly, these cytokines were previously found to enhance permeability, immune cell adhesion, and transendothelial migration of endothelial monolayers.^{2,3,42–46} Consistently, the presence of I-EndoMT cells in cellular barriers resulted in significantly elevated paracellular and transcellular permeability, as well as supporting enhanced PBMC transendothelial migration compared with PAECs alone.

Collectively our data support the notion that EndoMT is a process that occurs in the pulmonary endothelium of PAH patients. I-EndoMT cells exhibit elevated secretion of proinflammatory proteins and collagen type I, similar to fibroblasts isolated from SSc-PAH patients. Our data suggest that, although EndoMT may be an infrequent event in the pulmonary vasculature in PAH, it is an important pathologic event that may exacerbate vascular leak, inflammatory infiltration, and vascular remodeling.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.03.019>.

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