

Classification and Functional Characterization of Vasa Vasorum-Associated Perivascular Progenitor Cells in Human Aorta

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SUMMARY

In the microcirculation, pericytes are believed to function as mesenchymal stromal cells (MSCs). We hypothesized that the vasa vasorum harbor progenitor cells within the adventitia of human aorta. Pericytes, endothelial progenitor cells, and other cell subpopulations were detected among freshly isolated adventitial cells using flow cytometry. Purified cultured pericytes were enriched for the MSC markers CD105 and CD73 and depleted of the endothelial markers von Willebrand factor and CD31. Cultured pericytes were capable of smooth muscle lineage progression including inducible expression of smooth muscle myosin heavy chain, calponin, and α -smooth muscle actin, and adopted a spindle shape. Pericytes formed spheroids when cultured on Matrigel substrates and peripherally localized with branching endothelial cells in vitro. Our results indicate that the vasa vasorum form a progenitor cell niche distinct from other previously described progenitor populations in human adventitia. These findings could have important implications for understanding the complex pathophysiology of human aortic disease.

INTRODUCTION

Just as the circulatory system provides nourishment to support tissue and organ function, the larger blood vessels themselves require access to a blood supply to maintain vascular health. The vasa vasorum (Latin: “vessels of the vessels”) serve this vital role in large blood vessel homeostasis. In the ascending thoracic aorta, the microvascular network of vasa vasorum is particularly rich (Heistad et al., 1981). Here, the vasa vasorum originate from the coronary and brachiocephalic arteries and represent a continuum of the epicardium (Heistad et al., 1981). Vasa vasorum penetrate the adventitial layer and provide nourishment to the media of large blood vessels (>0.5 mm thick) with greater than 29 elastic lamellae at birth such as the thoracic aorta, pulmonary artery, and saphenous vein of humans, sheep, and canines (Wolinsky and Glagov, 1967). The vasa vasorum deliver oxygen and nutrients to and remove waste from the outer two-thirds of the medial layer while the inner media is fed from the lumen. First-order, larger (>100 μ m) vessels run longitudinally along the host vessel and branch into second-order, smaller (<100 μ m) vessels that penetrate the adventitia and into the medial layer (Kwon et al., 1998). Since the first realization of this dynamic microvascular network by Koester over 150 years ago (Koester, 1876), the role of the vasa vasorum in large-vessel homeostasis and disease states has been relatively undefined. While the adventitia provides the majority of

biomechanical support to the vessel, new roles for the adventitia and vasa vasorum in vascular homeostasis, injury response, and disease are actively emerging (Havelka and Kibbe, 2011; Majesky, 2015).

Cells isolated from the microvasculature of vascularized tissues such as skeletal muscle, fat, placenta, and pancreas have been shown to be capable of multi-lineage differentiation, localized to small arterioles within these tissues, and characterized as pericytes (Chen et al., 2013; Corselli et al., 2011; Crisan et al., 2012, 2008a, 2008b; Park et al., 2011; Traktuev et al., 2008; Zimmerlin et al., 2010). Analogous to these small blood vessels that feed tissues is the perivascular network of the vasa vasorum associated with large blood vessels. While multi-potential progenitors have been isolated from the adventitia of human pulmonary artery (Hoshino et al., 2008), human internal thoracic artery (Klein et al., 2011; Zengin et al., 2006), human fetal aorta (Invernici et al., 2007), human saphenous vein (Campagnolo et al., 2010), and human adult ascending aorta (Pasquinelli et al., 2007, 2010), these cells uniformly exist within a “zone of vasculogenesis” (Zengin et al., 2006), and are distinct from the vasa vasorum. To our knowledge, no prior study has investigated a progenitor cell population associated with the vasa vasorum specifically in the human adult aorta as a progenitor cell repository. This question has been addressed only in animal models, where cells reportedly isolated from explants of vasa vasorum in the aorta of the adult rat exhibited differentiation toward the

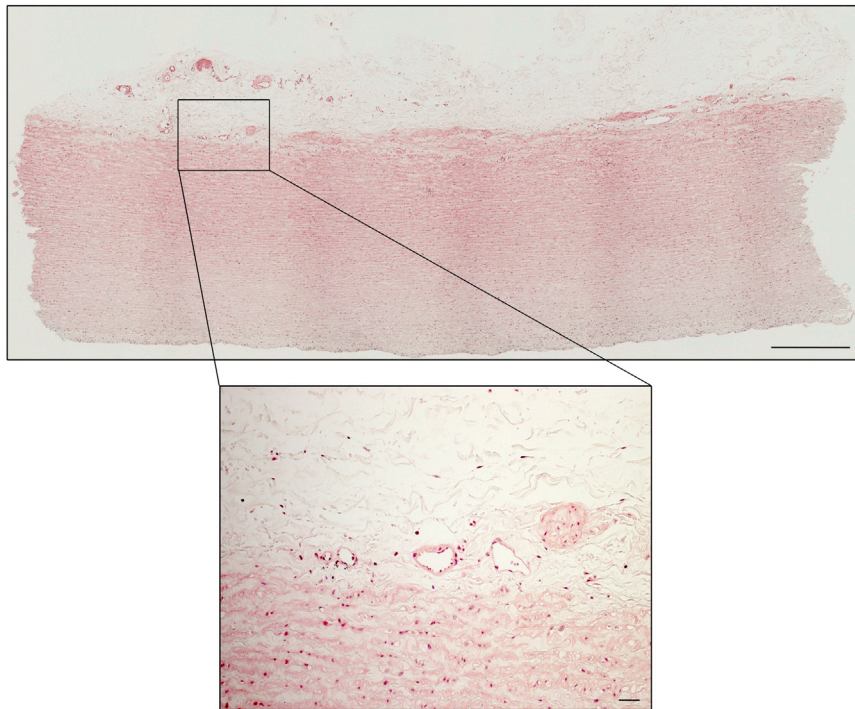


Figure 1. Representative H&E-Stained Cross-Section of Human Ascending Aorta
All three layers of the aortic wall: adventitia (adv), media (med), and intima (int). Dashed line indicates adv/med border. Inset shows a higher magnification of the adventitial area where vasa vasorum are located (arrowheads). Scale bar, 250 μm .

neuronal lineage (Montiel-Eulefi et al., 2012), and the post-natal rat aorta was shown to contain pericyte progenitor cells (Howson et al., 2005). We hypothesized that the vasa vasorum harbor progenitor cells within the adventitia of human aorta. In the present study, we documented the presence of several subpopulations of perivascular progenitor cells. We report successful purification and expansion of functional pericytes *ex vivo* that can differentiate toward the smooth muscle lineage. Our findings of vasa vasorum-associated progenitor cell subsets in human thoracic aorta could have important implications in the study and treatment of large-vessel diseases involving vasa vasorum in humans.

RESULTS

Vasa Vasorum-Associated Cells in Human Adult Thoracic Aorta Natively Express Markers of Progenitor Cells

We sought the *in situ* location of cells expressing markers of progenitor, smooth muscle, pericyte, and endothelial cells in human aortic adventitia. Vasa vasorum of all sizes in the adventitia were evaluated (Figure 1). As expected, cells expressing a pericyte marker profile were found to be localized to the vasa vasorum in the adventitia of human aorta. Immunohistochemical analysis of transverse full-thickness sections of human adult ascending thoracic aorta revealed a population of pericytes ($\text{CD146}^+/\alpha\text{-SMA}^\pm/\text{NG2}^+/\text{CD34}^-$

vWF^-) surrounding endothelial cells ($\alpha\text{-SMA}^-/\text{CD34}^+/\text{CD31}^+/\text{vWF}^+$) associated with smaller vessels of the vasa vasorum ($<25 \mu\text{m}$) (Figure 2). A subset of CD146^+ cells co-expressed α -smooth muscle actin ($\alpha\text{-SMA}$) (Figure 2A) in a perivascular location adjacent to vWF^+ (von Willebrand factor) (Figures 2B and 2C) and CD34^+ endothelial cells (Figure 2D). Subsets of pericytes ($\alpha\text{-SMA}^+/\text{CD31}^-$) expressed CD34 (Figures 2E–2G) and a subset of CD31^+ cells also co-expressed CD34 in the endothelium (Figure 2G). NG2 was expressed by SMA^+ pericytic cells (Figure 2H). Expression of CD105 , a marker found on mesenchymal stromal cells (MSCs), was noted in pericytes and endothelial cells of vasa vasorum (Figure 2I). Another noted population of interest included $\text{CD34}^+/\alpha\text{-SMA}^-$ cells that were situated superficially to vasa vasorum, which we have deemed “supra-vasa” cells (Figure 2J). $\text{CD34}^+/\alpha\text{-SMA}^-$ supra-vasa cells were found to lack CD146 (Figure 2K) and co-express CD90 (Figure 2L). These findings are representative of five patient specimens analyzed.

Flow-Cytometry Characterization of Perivascular Progenitor Cell Surface Proteomes in Human Adult Thoracic Aortic Adventitia

Analytical flow cytometry of freshly isolated adventitial cells from seven human ascending thoracic aortas (Figure 3A) confirmed the presence of multiple distinct subpopulations of cells from all live events (Figure 3B). Non-hematopoietic (NH) cells were gated on the basis of

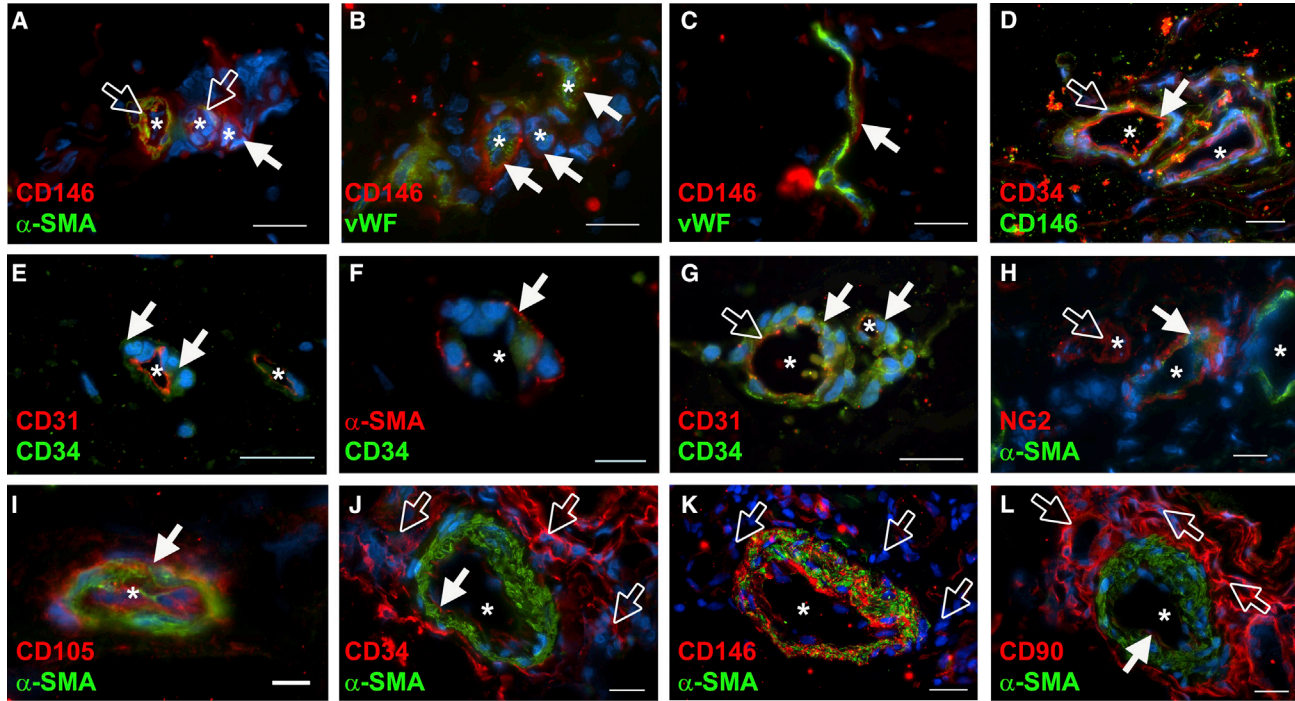


Figure 2. Immunodetection of Vasa Vasorum-Associated Cell Populations in Human Aorta

Sections of human aorta were immunolabeled using markers of progenitor cells and markers of the smooth muscle and endothelial lineages. An asterisk denotes the lumen of described vessels, and nuclei appear in blue in all panels. All representative vessels in all panels were selected above the adventitial/media border within respective sections.

- (A) Transverse sections of small-diameter vessels (<25 μm) of the vasa vasorum in the adventitia displayed co-expression of CD146⁺ (red) with the smooth muscle marker α -SMA (green, open arrows) amid CD146⁺ cells lacking α -SMA expression (arrow).
- (B) CD146⁺ cells (red, arrows) surrounded vWF⁺ endothelial cells (green) lining in small-diameter vessels (<25 μm).
- (C) Longitudinal view of CD146⁺ pericytes (red, arrow) wrapping vWF⁺ endothelial cells (green).
- (D) CD146⁺ pericytes (green, open arrow) surrounded CD34⁺ endothelial cells (red, arrow) of the vasa vasorum.
- (E) A small vasa vasorum (<25 μm) revealing expression of CD34 (green, arrows) in CD31⁻ peri-endothelial cells. Endothelial cells are CD31⁺ (red).
- (F) Serial section adjacent to the section in (E) shows co-expression of CD34⁺ (green) with SMA (red, arrow) in pericytes surrounding α -SMA⁻ endothelial cells.
- (G) CD34 (green) was expressed in subsets of CD31⁺ (red, open arrow) endothelial cells and in CD31⁻ pericytes (arrows).
- (H) NG2 was expressed in α -SMA⁻ cells (red, open arrow) and co-expressed in α -SMA⁺ cells (green, arrow) in vasa vasorum vessels <25 μm .
- (I) CD105 immunolabeling (red, arrow) revealed co-expression α -SMA⁺ pericytes and in endothelial cells.
- (J) CD34⁺/SMA⁻ cells were located in the endothelium (red, arrow) and surrounding α -SMA⁺ cells (green) in the periphery of vasa vasorum (open arrows).
- (K) A serial section of the image shown in (J) revealed SMA⁻ cells (open arrows) in the same supra-vasa location as CD34⁺ cells shown in (J) surrounding CD146⁺/SMA⁺ cells of the vasa vasorum.
- (L) CD90 (red, open arrows) was also detected in α -SMA⁻ cells located in the periphery of vasa vasorum α -SMA⁺ cells (green) and in endothelial cells (arrow).

Results shown are one representative of five independent patient specimens examined. Scale bars, 25 μm (A–H and J–L) and 10 μm (I).

absent CD45 expression amid absence of CD56 (Figure 3C, middle panel) and further analyzed on the basis of CD34 and CD31 expression. Freshly isolated adventitial cells from adult human aorta included a population of cells that exhibited a pericyte profile of CD146⁺/CD34⁻/CD31⁻ and were found to comprise 3.83% \pm 1.23% of all NH cells in the adventitia (Figure 3C [lower left panel]

and Table 1). Most of these pericytes expressed CD90 (74.78% \pm 8.17%). When a gating strategy indiscriminant of CD34 was applied to CD56⁻/CD45⁻/CD31⁻ cells (as displayed in Table 1), we found that an appreciable number of CD146⁺/CD31⁻ cells co-expressed CD34 (15.4% \pm 5.87%) while another, slightly smaller subset co-expressed CD90 and CD34 (10.59% \pm 4.2%).

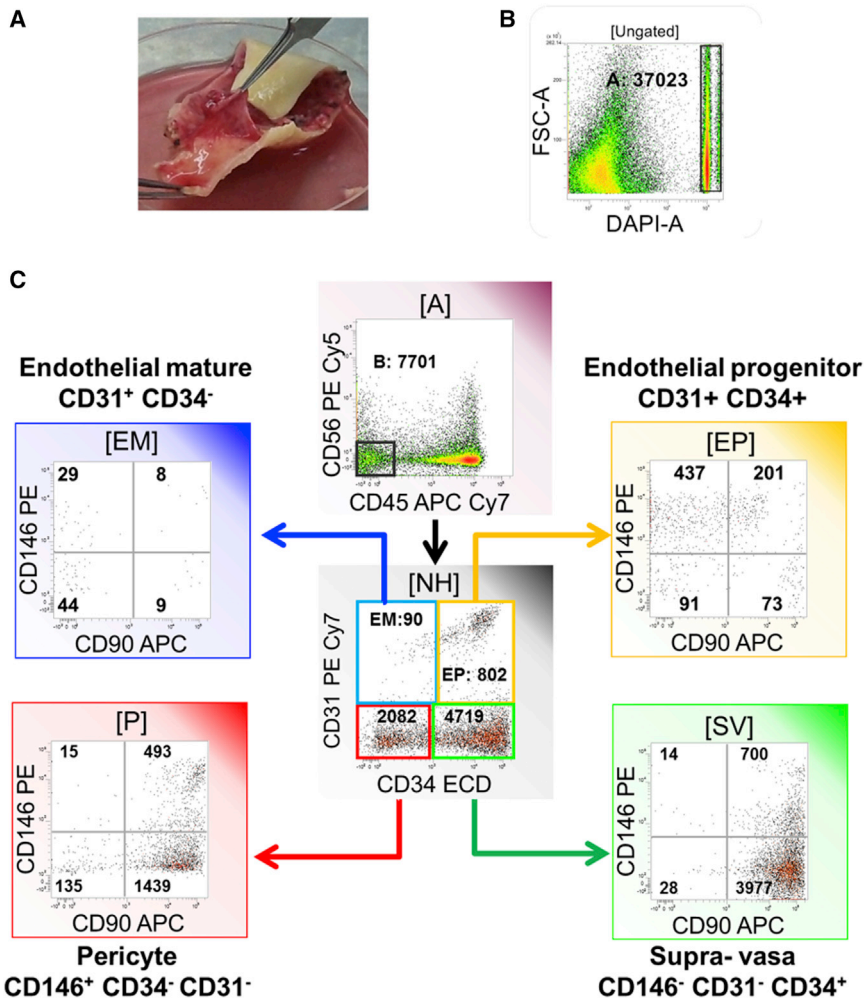


Figure 3. Classification of Adventitial Cell Populations in a Representative Patient Specimen via Multi-dimensional Analytical Flow Cytometry

(A) The adventitia (top layer) was delaminated from the aortic media (lower layer) and processed for isolation of primary cells. (B) Detection of all events and all nucleated cells (denoted as A) using forward scatter (FSC) and DAPI in fixed and gently permeabilized cells to detect events with $\geq 2N$. (C) Classification of nucleated events identified in (B) into subpopulations. First, a non-hematopoietic population ([NH]) of $CD45^-/CD56^-$ cells was established from the parent gate ([A]) (top middle panel). Next, the [NH] gate (center panel) was utilized to delineate four distinct cell subpopulations (four flanking panels): endothelial mature ([EM]) were classified as $CD31^+/CD34^-$ (top left panel); endothelial progenitor ([EP]) were classified as $CD31^+/CD34^+$ (top right panel); pericytes ([P]) were classified as $CD146^+/CD34^-/CD31^-$ (bottom left panel); and supra-vasa ([SV]) were classified as $CD146^-/CD31^-/CD34^+$ (bottom right panel). Solid-line boxes depict selected gates, and numbers within each profile represent counts within each noted gate. Each panel depicts a representative light-scatter profile for the candidate population from one representative patient specimen of seven independent patient specimens analyzed. Prevalence of each subpopulation was quantified from seven independent patient specimens was characterized further. See also [Table 1](#).

$CD34^+$ cells characterized by the surface marker profile $CD146^-/CD56^-/CD45^-/CD34^+/CD31^-$ were found to be more abundant than $CD146^+/CD31^-$ pericytes and comprised $32.75\% \pm 6.82\%$ of NH adventitial cells ([Table 1](#)). These cells were considered to be non-endothelial cells since they lacked $CD31$ expression and were consistent with cells localized in native adventitia in the periphery of vasa vasorum, the so-called supra-vasa cells described above in aortic tissue sections. Most of these supra-vasa cells also co-expressed $CD90$ ($71.24\% \pm 9.28\%$). Mature endothelial cells expressing the profile of $CD56^-/CD45^-/CD34^-/CD31^+$ comprised $1.69\% \pm 0.99\%$ of NH cells in the adventitia ([Table 1](#)). We considered these cells to be mature endothelial cells due to their lack of $CD34$ expression. About one-fifth of these mature endothelial cells expressed $CD146$ ($19.79\% \pm 13.14\%$) whereas only about one-tenth expressed $CD90$ ($9.20\% \pm 3.40\%$). Cells lacking $CD34$, $CD146$, and $CD90$

represented a majority ($74.60\% \pm 12.39\%$) of $CD31^+$ endothelial cells ([Table 1](#)). A cell subset was considered committed to endothelial lineage or in a progenitor state on the basis of $CD31$ expression alone or $CD31$ co-expression with $CD34$, respectively. These $CD34^+/CD31^+$ endothelial progenitor cells (EPCs) were fairly abundant in aortic adventitia and, on average, comprised $8.29\% \pm 3.49\%$ of all NH cells ([Table 1](#)). About half of the EPCs expressed $CD146$ ($53.45\% \pm 4.94\%$) while about one-third co-expressed $CD90$ ($32.08\% \pm 5.37\%$), which was appreciably more than the proportion of $CD90^+$ cells in the endothelial mature ($CD34^-/CD31^+$) fraction ($9.20\% \pm 8.99\%$).

Profile of Ex Vivo Expanded Perivascular Subsets

The ex vivo phenotype of cultured cells displaying the pericyte profile of $CD146^+/CD90^+/CD56^-/CD45^-/CD34^-/CD31^-$ were evaluated from human adventitia of three



Table 1. Prevalence of Adventitial Cell Subpopulations

	Pericytes CD56 ⁻ /CD45 ⁻ / CD146 ⁺ /CD31 ⁻			Supra-Vasa CD56 ⁻ /CD45 ⁻ /CD146 ⁻ / CD34 ⁺ /CD31 ⁻			Endothelial Mature CD56 ⁻ /CD45 ⁻ / CD34 ⁻ /CD31 ⁺			Endothelial Progenitor CD56 ⁻ /CD45 ⁻ /CD34 ⁺ / CD31 ⁺		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Nucleated cells (%)	0.65	0.81	0.31	3.86	2.32	0.88	0.23	0.35	0.13	0.90	0.97	0.37
Non-heme (%)	3.83	3.25	1.23	32.75	18.04	6.82	1.69	2.62	0.99	8.29	9.23	3.49
CD146 ⁺ (%)	100	-	-	0	-	-	19.79	34.76	13.14	53.45	13.08	4.94
CD34 ⁺ (%)	15.40	15.52	5.87	100	-	-	0	-	-	100	-	-
CD90 ⁺ (%)	74.78	21.63	8.17	71.24	24.56	9.28	9.20	8.99	3.40	32.08	14.22	5.37
CD90 ⁺ /CD34 ⁺ (%)	10.59	11.12	4.2	71.24	24.56	9.28	0	-	-	32.08	14.22	5.37
CD146 ⁻ /CD90 ⁻ (%)	0	-	-	28.76	24.56	9.28	74.60	32.78	12.39	32.65	11.71	4.43

independent patients using fluorescence-activated cell sorting (FACS) (Figure 4A). Sorted pericytes exhibited uniform elongated cell morphology (Figure 4B). Not surprisingly, the adventitia of human aorta was noted to be a highly heterogeneous microenvironment with many cell types, as evidenced by detection of gene transcripts from several cell types including smooth muscle cells (SMCs) (CD146 and CNN), endothelial cells (CD31 and VWF), pericytes (CD146), and MSCs (CD73, CD90, and CD105) (Figures 4C–4J). To confirm the phenotypic profile of FACS-purified adventitial cells sorted on the pericyte surface proteome of CD146⁺/CD90⁺/CD56⁻/CD45⁻/CD34⁻/CD31⁻, qPCR analysis revealed enriched expression of the pericyte markers CD146, the mesenchymal stem cell markers CD73 and CD105, and depleted expression of the hematopoietic marker CD45 and endothelial markers CD31 and vWF when compared with native adventitia, as expected. There were similar levels of CD90 and CNN gene expression noted in both the parent adventitia specimen and sorted pericytes.

FACS-purified pericytes maintained in their basal culture medium displayed minimal and heterogeneous expression of CNN and α-SMA with minimal co-expression of these SMC markers. Smooth muscle myosin heavy chain (SM-MHC) was not detected in pericytes under basal growth conditions (Figure 4K). Under transforming growth factor β1 (TGF-β1) and platelet-derived growth factor BB (PDGF-BB) stimulation in growth medium, most sorted pericytes acquired SM-MHC expression, revealed an increased number of cells expressing SM-MHC or α-SMA with CNN, and adopted a spindle-like morphology when compared with cells cultured in the absence of TGF-β1 and PDGF-BB (Figure 4K). However, the proportion of pericytes co-expressing CNN with either α-SMA or SM-MHC did not appear to change under TGF-β1 and PDGF-BB stimulation (Figure 4K).

Functional Evaluation of Sorted Pericytes In Vitro

To assess the ability of primary cultured pericytes to participate in vasculogenesis, we employed an in vitro endothelial branching assay using the culture substrate GFR-Matrigel. After 24 hr of culture, endothelial cells from human pulmonary artery formed branched networks on Matrigel (Figure 5A). Within 1–2 hr of initial seeding on Matrigel-coated tissue culture surfaces, sorted pericytes briefly exhibited extensions and then spontaneously assembled into spheroid colonies within 6 hr (Figure 5B and Movie S1). When pericytes were co-cultured with endothelial cells, they localized on the periphery of branching endothelial cells (Figure 5C, arrows). Spheroid cultures of pericytes sprouted cellular offshoots that continued to expand over 5 days and extend cell processes between cell clusters (Figures 5D and 5E).

DISCUSSION

The adventitia of ascending thoracic aorta is a rich bed of vasa vasorum and represents a specialized perivascular niche. Here, we classified adventitial cells within human adult thoracic aorta as progenitor cell subsets using their antigenic profile, function, and native location associated with the microvascular network of the vasa vasorum, and characterized their potential for smooth muscle lineage progression.

It has been shown that vasa vasorum of human arteries contain pericyte-like cells (Andreeva et al., 1998), and we demonstrate that vasa vasorum-associated pericytes from the adventitia of human adult aorta (CD146⁺/CD90⁺/CD34⁻/CD31⁻) were similar in marker profile to pericytes characterized in small arterioles of several other vascularized tissues (Crisan et al., 2008c). Our findings in human vasa vasorum are consistent with the prior work of others who identified that CD146 co-expressed on the surface of

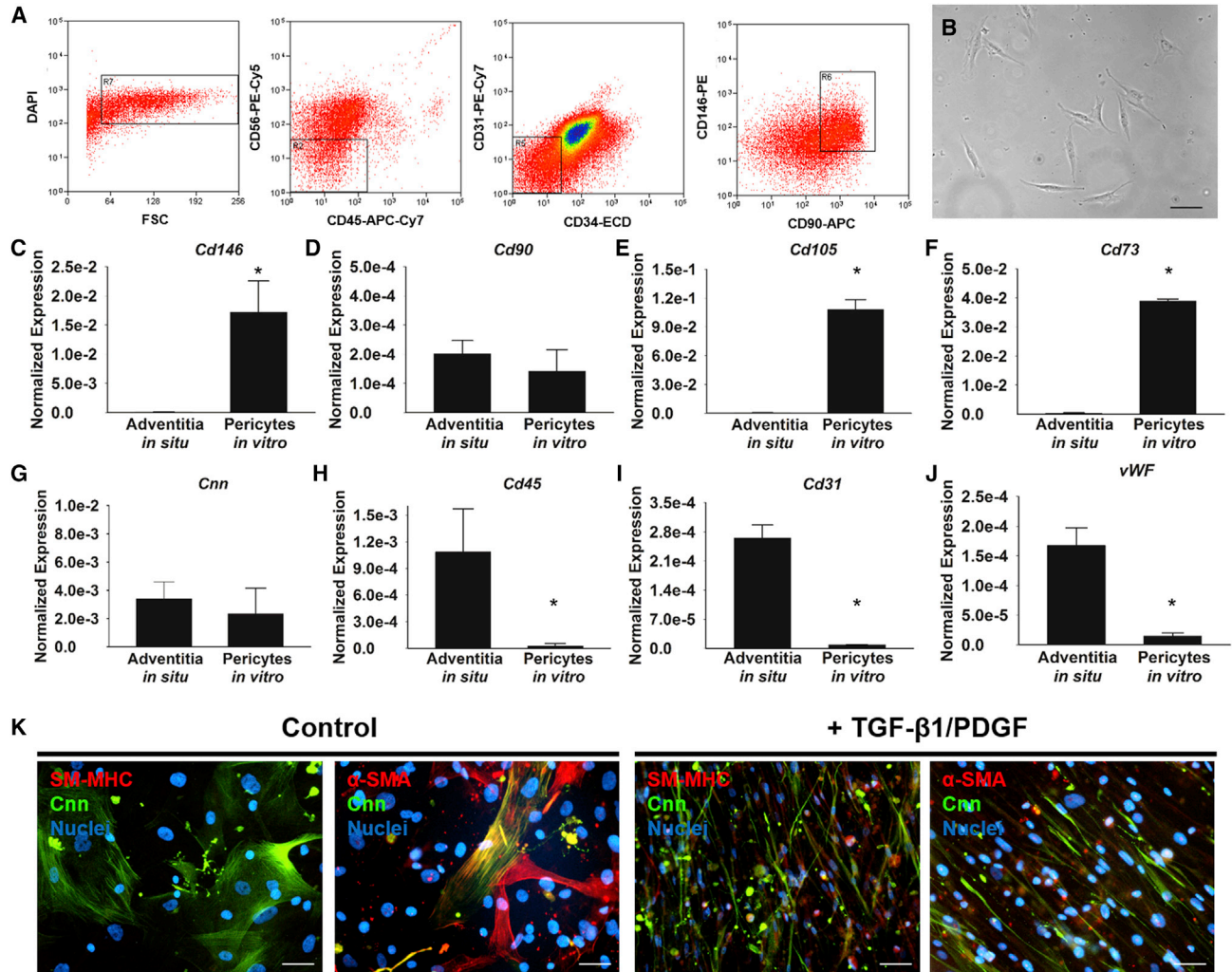


Figure 4. Characterization of FACS-Isolated Pericyte Populations

(A) Pericytes were isolated using multi-dimensional FACS on the basis of a surface proteome of CD146⁺/CD90⁺/CD56⁻/CD45⁻/CD34⁻/CD31⁻. DAPI was used to discriminate live from dead/apoptotic cells (far left panel). Cells were first gated for exclusion of CD45 and CD56 (second panel), followed by exclusion of CD31 and CD34 (third panel), and finally for expression of CD146 and CD90 (far right panel).

(B) Bright-field micrograph of sorted pericytes in culture. Scale bar, 100 μ m.

(C–J) Pooled real-time qPCR analysis in total RNA isolated from adventitia tissue (n = 5 independent patient specimens) and in vitro cultivated sorted pericytes (n = 3 independent patient specimens) revealed that sorted pericytes were enriched for transcript expression of the pericyte marker *CD146* (C), had equivalent expression of the mesenchymal stem cell markers *CD90* (D), and increased expression of *CD105* (E) and *CD73* (F). Sorted pericytes expressed smooth muscle marker *CNN*, similar to that of all native adventitial cells (G), and were depleted of expression for the hematopoietic lineage marker *CD45* (H) and endothelial lineage markers *CD31* (I) and *VWF* (J). Error bars represent mean \pm SEM. *p < 0.05.

(K) Sorted pericytes cultured in the presence of TGF- β 1 and PDGF (right panels) adopted a spindle-shaped morphology, and the number of cells positive for markers of the smooth muscle lineages including expression of SM-MHC, α -SMA, and Cnn was increased. Control cells (left panels) maintained in their normal growth medium had a rhomboid morphology, lacked appreciable SM-MHC α -SMA and Cnn expression. Scale bar, 50 μ m.

See also [Table S1](#).

NG2⁺ pericytes (Crisan et al., 2008c; Sacchetti et al., 2007) and with studies revealing expression of the progenitor markers nestin and Wilms' tumor protein on small (<50 μ m) vasa vasorum in healthy human abdominal aorta

and iliac, femoral, and renal arteries (Vasuri et al., 2012). CD146 has been detected in the endothelium of vessels in tissues (Duda et al., 2006), and using multi-parameter analytical flow cytometry, we detected co-expression of

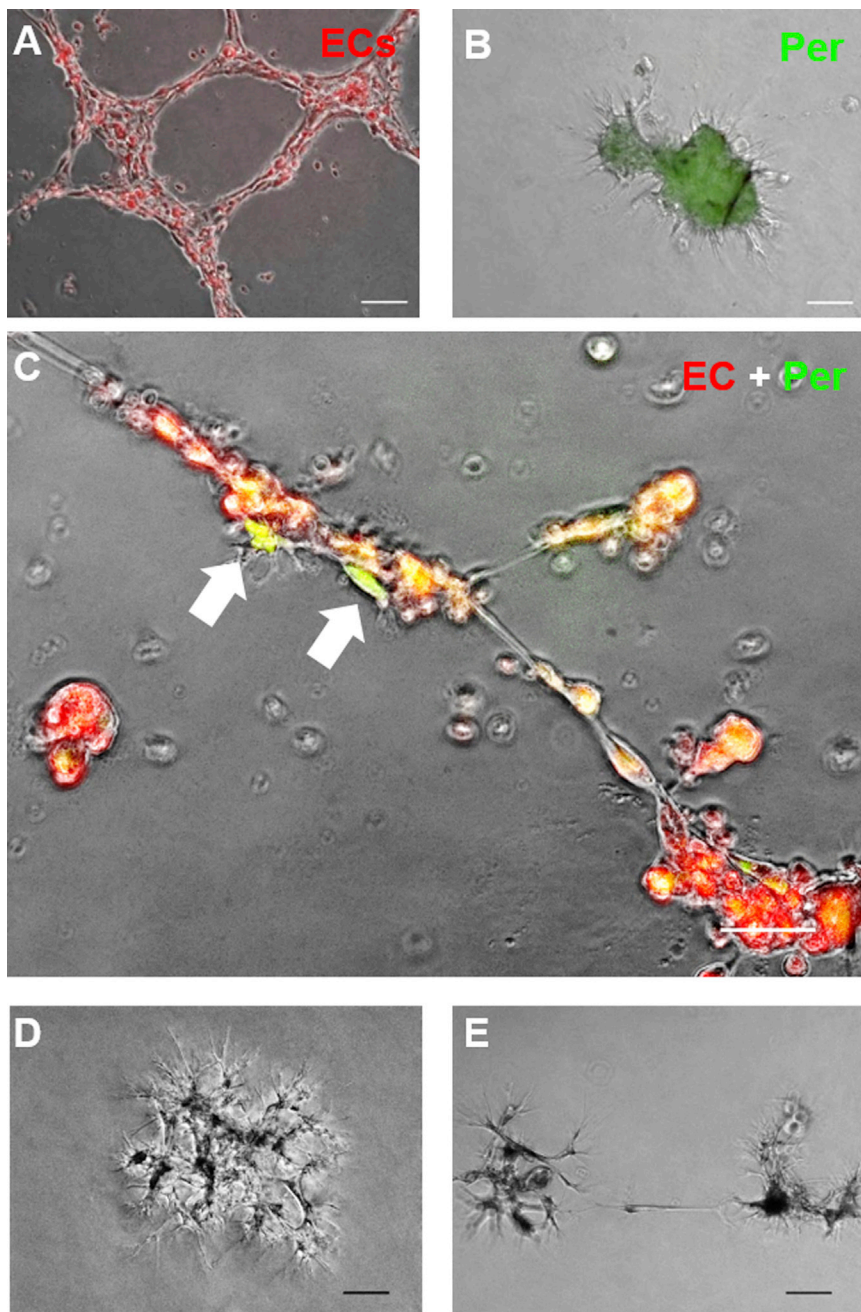


Figure 5. Functional Analysis of Sorted Pericytes and Endothelial Cells from Human Aortic Adventitia

(A) Human endothelial cells from pulmonary artery form branching networks on the surface of Matrigel substrates. Scale bar, 100 μ m.

(B) Sorted human adventitia-derived pericytes cultured on the surface of Matrigel spontaneously form spheroid cultures within 4–6 hr. Scale bar, 100 μ m.

(C) Sorted pericytes (green, arrows) associated in the periphery of branching endothelial cells (red). Scale bar, 100 μ m.

(D) Spheroids of pericytes in culture alone on Matrigel expanded over 5 days. Scale bar, 50 μ m.

(E) Anastomoses between cell clusters expanding from spheroids within 5 days. Scale bar, 100 μ m.

Results shown are representative of three independent experiments. See also [Movie S1](#).

CD146 on CD31⁺ cells, especially in cells that were also positive for CD34, which have been described as EPCs (Zimmerlin et al., 2010). While our ex vivo cultivated pericytes were sorted exclusively of CD34⁺ cells, others have characterized cells co-expressing CD146 and CD34 as pericytes (Traktuev et al., 2008) and have suggested that these cells may be transitory between CD146⁻/CD34⁺ adventitial cells (Zimmerlin et al., 2010) and CD146⁺/CD34⁻ pericytes, since CD34⁺ adventitial cells have been shown to be progenitors of pericytes (Corselli et al., 2011). Confirming

this prior finding was our detection of CD34 expression in α -SMA⁺ pericytes of small-diameter (<25 μ m) vasa vasorum, and we noted their prevalence in human aortic adventitia to be, on average, ~15% of non-endothelial cells defined by the surface marker profile of CD146⁺/CD56⁻/CD45⁻/CD31⁻. Thus, CD34⁺ pericytes in human adventitial vasa vasorum are not as prevalent as those described for the stromal vascular fraction in adipose tissue, where others have reported prevalence of CD34⁺ pericytes of up to 32% (Zimmerlin et al., 2010). CD34⁺ cells within adipose

**Table 2. Respective Differentiation Potential of Progenitor Cell Populations in Human Adult Tissues: Examples from the Literature**

Subpopulation	Surface Proteome	Differentiation Potential
Pericyte	CD146 ⁺ /CD34 ⁻ /CD31 ⁻	muscle (Crisan et al., 2008c; Park et al., 2011), bone (Crisan et al., 2008c), cartilage (Crisan et al., 2008c), fat (Zimmerlin et al., 2010), myocardium (Chen et al., 2013)
Adventitial cells/supra-vasa	CD34 ⁺ /CD31 ⁻	bone (Pasquinelli et al., 2010), cartilage (Pasquinelli et al., 2010), fat (Pasquinelli et al., 2010; Zimmerlin et al., 2010), smooth muscle/pericyte (Campagnolo et al., 2010; Corselli et al., 2011), endothelial (Pasquinelli et al., 2007; Zengin et al., 2006)
Endothelial progenitor	CD34 ⁺ /CD31 ⁺	endothelial (Aicher et al., 2007; Kirton and Xu, 2010), fat (Zimmerlin et al., 2010)
Endothelial mature	CD34 ⁻ /CD31 ⁺	endMT (Medici et al., 2010), fat (Zimmerlin et al., 2010)

endMT, endothelial to mesenchymal transition.

tissue have been described as pericytes, since they exhibited both a surface marker profile and in vivo location indicative of pericytes as well as functionality to co-localize with branching endothelial cells in vitro (Traktuev et al., 2008). Hence, there appears to be heterogeneity in both the surface proteome and prevalence of pericytes across tissues. We also noted CD34⁺ stromal cells, which were non-endothelial (CD31⁻) and non-pericytic (CD146⁻), and were so named due to their location in the periphery of vasa vasorum arterioles and their similarity in marker profile and location to other perivascular cells in white adipose tissue (Corselli et al., 2011; Zimmerlin et al., 2010). Our present findings corroborate the work of West et al. (2016), who characterized the stromal vascular fraction in a large cohort of human adipose tissue collected from multiple centers using flow cytometry, and reported that pericytes (CD45⁻/CD146⁺/CD34⁻) and adventitial cells (CD45⁻/CD146⁻/CD34⁺) have a prevalence of 8% and 33%, respectively, and together represent subpopulations of perivascular stem cells (West et al., 2016).

The FACS-purified and cultivated pericytes described in the present study along with those described by others who documented MSC-like behavior of pericytes do not appear to be the same as mesodermal progenitor cells (MPCs) (Caplan, 2008; Crisan et al., 2008c), a subset of MSCs in human bone marrow-derived populations that were shown to exhibit angiogenic potential (Petrini et al., 2009). Localization of our ex vivo cultured pericytes alongside branching endothelial cells in vitro is characteristic of other pericytic populations (Mayo and Bearden, 2015) and distinguishes our aortic-derived pericytes from MPCs. Furthermore, the capacity of these aortic adventitial-derived pericytes to form spontaneous spheroids and subsequent sprouting has not been previously reported for similar cell types. A lack of CD34 and CD31 expression on our sorted adventitia-derived pericytes defines them as a population altogether distinct from MPCs, MSCs derived

from CD34⁺ adventitial cells (Corselli et al., 2011; West et al., 2016; Zimmerlin et al., 2010), and CD34⁺ supra-vasa cells described herein by our group.

Several vascularized tissues of fetal and adult origin such as white adipose, lung, pancreas, muscle, and placenta were all found to harbor cells capable of multi-lineage differentiation (Caplan, 2008; Corselli et al., 2011; Crisan et al., 2008c); and a recent study challenges the notion of a universal MSC by conversely showing that populations of cells in multiple tissues and organs with similar surface marker profiles differ broadly in their in vivo potential (Sacchetti et al., 2016). Since the aortic adventitia is associated with peri-adventitial adipose and human pericytes isolated from stromal vascular fraction of white adipose tissue (Zimmerlin et al., 2010) both exhibited high adipogenic potential, one could reasonably question whether the cells described in our study are adipose stem cells (ASCs). ASCs exhibit basal and TGF-β1-inducible expression of α-SMA (Lee et al., 2006), and pericytes may represent progeny of a subset of ASCs (Baer, 2014). In contrast to our sorted pericytes, ASCs have also been described in situ as CD34-expressing cells that lack expression of α-SMA, CD146, and CD105 (Baer, 2014). Therefore, the CD34⁺/CD146⁻ cells we classified as supra-vasa are the more likely candidates to be considered ASCs, and populations from the stromal vascular fraction with identical reported surface proteomes were previously shown to be adipogenic, albeit not as potently as the pericyte fraction (Zimmerlin et al., 2010). Indeed, as summarized in Table 2, similar populations in human adult specimens of blood vessels and other vascularized tissues have been shown to have multi-lineage potential, like MSCs. Several highly analogous cell populations in other human adult blood vessels have been shown to be SMC/pericyte progenitors and exhibited propensity for adipogenic, chondrogenic, and osteogenic lineage progression (Klein et al., 2011). Furthermore, several studies of human fetal tissues (Invernici et al., 2007) and



in large (Zaniboni et al., 2014) and small animal models (Howson et al., 2005; Hu et al., 2004) have contributed important knowledge regarding the identity, location, and function of MSCs and other perivascular cell subsets. Curiously, recent fate-mapping interrogations in mouse could not find any appreciable contribution of pericytes and SMCs to other lineages during aging or induced disease states (Guimarães-Camboa et al., 2017). On the contrary, others revealed that adventitial cells co-expressing Sca-1, CD34, and Gli1 contribute to atherosclerotic lesions in the intima in ApoE^{-/-} mice (Hu et al., 2004). Likewise, Kraumann et al. (2016) showed through inducible lineage tracing that CD34⁺/Sca1⁺/Gli1⁺ adventitial cells differentiate to mature SMCs, migrate to intimal atherosclerotic lesions, and localize in close proximity to regions of vascular calcification in vivo. Since Gli1⁺ cells co-express CD34 and lack CD31, they appear to be distinct from our pericytes, but isolated cells may include a subpopulation of pericytes or other non-endothelial adventitial cells that partially share these markers. Future in vivo studies investigating the fate of our pericytic population in physiological and pathological settings are required to better appreciate the potential contribution of vasa vasorum-associated pericytes to other lineages.

The adventitia of large vessels is highly vascularized. As such, we are not surprised that our multi-parameter analytical flow cytometric analysis revealed a relatively high prevalence of cells expressing surface markers consistent with the endothelial lineage, namely CD31. We also detected CD34 expression in subsets of CD31⁺ endothelial cells of adventitial vasa vasorum, which we classified as EPCs. Still, it is possible that the EPCs from human aortic adventitia described here may have been derived from the circulation. The presence of EPCs in the adventitia raises the question of their potential for neovascularization and their functionality in the setting of cardiovascular disease. In the human aorta, cells expressing stem cell markers STRO-1, CD34, and c-kit have been localized to the medial layer, even more so in the adventitia, and were noted to be more prevalent in both layers in cases of aortic aneurysm and dissection than in normal healthy aortas (Shen et al., 2012). A purported role for vascular wall stem cells in vascular disease is currently an area of intense interest (Nguyen et al., 2013; Tang et al., 2012). Our current finding of a discrete progenitor cell niche associated with the adventitial vasa vasorum provides additional support that these unique cell populations may play a distinct and important role in thoracic aortic disease. Although we detected pericytes capable of smooth muscle lineage progression in both normal and aneurysmal aorta, we did not compare functional differences on the basis of disease, and additional work will help to discern potential differences in surface proteome

and functionality of vascular wall progenitors in aortic disease.

In conclusion, the vasa vasorum and surrounding connective tissue in human adult thoracic aortic adventitia is a niche for progenitor cell populations. Importantly, their presence and the demonstrated function of pericytes as smooth muscle progenitors and potential for multi-lineage progression adds to the evolving dichotomy related to MSCs' angiogenic potential in vascularized tissues as either progenitors of endothelial cells or local cells that secrete angiogenic growth factors in addition to being recruited to support neovessel formation. The pericyte cultures are distinct from other prior reports investigating progenitor cells in the media and adventitia of human thoracic aorta (Invernici et al., 2007; Pasquinelli et al., 2007, 2010; Shen et al., 2012) because of their association with the vasa vasorum. Pericytes, adventitial cells, and other perivascular progenitor cells such as those in the endothelial lineage associated with the vasa vasorum may influence medial biology. The function and purpose of these progenitor cells in the vasa vasorum microenvironment may prove to be pivotal in the homeostasis and/or remodeling of the aortic wall in normal or pathologic states.

EXPERIMENTAL PROCEDURES

Tissue Collection and Processing

Human ascending thoracic aorta specimens (n = 34 patients) were collected during elective aortic valve replacement due to valvulopathy, from ascending aortic replacement operations due to aneurysm (average aortic diameter was 51.6 ± 5.87 mm [mean ± SD]), or during heart transplants, with informed patient consent and approval of the Institutional Review Board at the University of Pittsburgh under protocol # PRO07020120 and via the Center for Organ Recovery and Education (Pittsburgh, PA). Upon excision, tissue specimens were placed on ice and transported to the laboratory. Specimens were collected from 28 males and 6 females whose average age was 51.5 ± 14.0 years (mean ± SD).

Histology and Immunohistochemistry

Portions (~0.5 cm²) of human ascending thoracic aorta specimens were submerged in OCT medium, frozen over liquid nitrogen, and stored at -80°C prior to cyrosectioning (6 μm). Blocked slides (5% goat serum, 1 hr) were incubated in primary unconjugated antibodies: mouse anti-human CD146 (1:50, BD Biosciences, #550314) or CD34 (1:50, BD Biosciences, #347660), α-SMA (1:200, DAKO, #M0851 or pre-diluted, Abcam, #15267), CD31 (pre-diluted, DAKO, #M082301-2), CD105 (1:50, Invitrogen, #MHCD105000), NG2 (1:50, BD Biosciences, #554275), CD90 (1:50, Abcam, #134361) in blocking solution overnight at 4°C, or in conjugated primary antibodies (CD146-Alexa Fluor488, 1:25, Fisher Scientific, #50-174-931) or vWF-fluorescein isothiocyanate (FITC) (1:100, US Biological, #V2700-01C) in blocking buffer for 2 hr at room temperature. Secondary labeling was accomplished



with biotinylated goat anti-mouse (1:80, Vector, #BA-9200), biotinylated goat anti-rabbit (1:80, Vector, #BA-1000), donkey anti-mouse-Alexa488 (1:250, Jackson ImmunoResearch, #115-545-003), or donkey anti-rabbit-Alexa488 (1:250, Invitrogen, #A-11008) for 1 hr at room temperature. When necessary, tertiary labeling with streptavidin-Cy3 (1:250, Sigma, #S6402) was performed. Nuclei were further labeled with DAPI (1:500, Invitrogen #D1306) for 30 min at room temperature. Washed slides were mounted with coverslips using Prolong Gold (Invitrogen, #P36934) and allowed to dry overnight. Slides were visualized using a Nikon TE-2000-E2 inverted microscope using bright-field or epifluorescence microscopy and captured using a CoolSNAP ES2 Monochrome 1,394 × 1,040 High-Resolution Camera (Photometrics) and NIS Elements Software 3.2 (Nikon).

Primary Cell Isolation

Primary cells were isolated from 13 independent patient specimens. Upon specimen acquisition in the laboratory within 1–2 hr of harvest, the adventitia was immediately stripped away from the medial layer and rinsed twice in ice-cold PBS with 1% penicillin-streptomycin and 1% fungizone (Invitrogen). Tissue was then finely minced using safety scalpels and rinsed in PBS and processed further, similar to what we have previously described (Zimmerlin et al., 2010) (see Supplemental Experimental Procedures). Isolated primary adventitial cells that were placed into tissue culture were propagated in basal growth medium (DMEM, 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin [all from Invitrogen]). Gentamycin (Invitrogen) (250 µg/mL) was added for 24–48 hr. Cells were maintained in a humidified incubation chamber at 37°C and 5% CO₂ until sufficient numbers were reached to facilitate FACS (~2 passages).

Flow Cytometry

Fresh isolates of primary aortic adventitial cells were prepared as above and their surface marker proteome was subsequently characterized using analytical flow cytometry without ex vivo culture, similarly to previously described methods (Zimmerlin et al., 2010) (see also Supplemental Experimental Procedures).

Freshly isolated primary adventitial cells from eight patients were separately expanded and subcultured to ≤70% confluence for two passages in either basal growth medium (DMEM, 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin) or endothelial growth medium in a 37°C humidified chamber with 5% CO₂ until a population of 0.5–1 × 10⁷ cells was reached. Cells were immunolabeled as described above for analytical flow cytometry. DAPI was added to unfixed and unpermeabilized cell suspension just prior to sorting to discriminate live from dead/apoptotic cells. Cells were sorted using three of a five-laser MoFlo Astrios high-speed cell sorter (Beckman Coulter, University of Pittsburgh Cancer Institute Flow Cytometry Core Facility) enclosed in a Class II biosafety cabinet. Cells that had been subcultured in basal growth medium were sorted on the basis of the pericyte surface marker profile of (CD146⁺/CD90⁺/CD56⁻/CD45⁻/CD34⁻/CD31⁻) and were plated into 6-well plates with basal growth medium supplemented with gentamicin and expanded. Medium was replenished every second day.

In Vitro Pericyte Expansion

Sorted pericytes were propagated for one to two passages with medium replenishment every second day and then cryopreserved in growth medium containing 10% DMSO. Pericytes were then subcultured in basal growth conditions to confluence. Cells were cultured in the presence of TGF-β1 (2 ng/mL) and PDGF-BB (50 ng/mL) to encourage smooth muscle lineage progression for 14 days in commercial smooth muscle cell basal growth medium (Cell Applications). All growth factors were purchased from R&D Systems. Cells were cultured in basal growth medium as a negative control. Medium was replenished every second day.

RNA Isolation and Real-Time qPCR

Portions of human aortic adventitial specimens were placed in RNAlater solution (Life Technologies) and stored at –20°C until use. Specimens (15–20 mg) were homogenized in TRIzol reagent using the gentleMACS Tissue Dissociator (Miltenyi). Cells expressing the pericyte profile of CD146⁺/CD90⁺/CD34⁻/CD31⁻ under the parent gate of CD56⁻/CD45⁻ were expanded to P7 and kept at <70% confluence. Gene expression of *CNN*, *CD146*, *CD90*, *CD31*, *VWF*, *CD73*, and *CD105*, were quantified from total RNA (see also Supplemental Experimental Procedures).

Immunocytochemistry

Sorted pericytes (CD146⁺/CD90⁺/CD56⁻/CD45⁻/CD34⁻/CD31⁻) (P7) were cultured on coverglass under conditions stimulating progression of the smooth muscle lineage as described above. Cells were then fixed in 4% paraformaldehyde and analyzed for phenotypic markers via immunocytochemical detection of α-SMA, Cnn, and SM-MHC (see also Supplemental Experimental Procedures).

Endothelial Branching Assay

Sorted pericytes (CD146⁺/CD90⁺/CD56⁻/CD45⁻/CD34⁻/CD31⁻) (passage <8) were maintained in basal growth medium and kept at <70% confluence. Cells were then seeded at a density of 1.2 × 10⁴ cells/cm² on growth factor reduced (GFR)-Matrigel (Corning)-coated 24-well plates in endothelial growth medium on GFR-Matrigel. Pericytes were also separately seeded on Matrigel substrates in co-culture in a 1:10 ratio with primary human pulmonary artery endothelial cells (hPAECs, P8) (Lonza). For co-culture experiments, populations were labeled with Cell Tracker Green (pericytes) or Red (hPAECs) (Invitrogen). Cells were cultured at 37°C and 5% CO₂ in a humidified microscope stage-top incubation chamber (Tokai Hit). Cells on Matrigel were visualized using phase-contrast and epifluorescence microscopy on a Nikon TE-2000-E2 inverted microscope equipped with FITC and tetramethylrhodamine isothiocyanate filter cubes and a 10× objective, and images were captured every 10 min for up to 50 hr using a CoolSNAP ES2 Monochrome 1,394 × 1,040 High Resolution Camera and NIS Elements Software 3.2. Images were also captured after 1, 3, and 5 days of culture.

Statistical Analyses

Data displayed represent a minimum of three independent experiments performed. Differences in gene expression were assessed using a two-tailed, unpaired Student's t test. A p value of <0.05 was considered statistically significant.



SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one table, and one movie and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2017.04.028>.

AUTHOR CONTRIBUTIONS

J.A.P. conceived the research design and developed the concept of the study together with M.B., V.S.D., and T.G.G. T.G.G. provided human aortic tissue specimens. J.A.P. and B.W.E. performed experiments with contributions from J.C.H. and T.D.R. M.B. and J.A.P. performed analysis and interpreted the results with contributions from V.S.D., B.W.E., J.C.H., T.D.R., and T.G.G. E.M.M. performed flow cytometry data acquisition. V.S.D. and A.D.D. performed flow cytometry analysis. J.A.P. and M.B. wrote the manuscript with contributions from V.S.D. and T.G.G.

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