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 brachiophelial 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 µm) vessels run longitudinally along the host vessel and branch into second-order, smaller (<100 µm) 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 report-edly isolated from explants of vasa vasorum in the aorta of the adult rat exhibited differentiation toward the...
neuronal lineage (Montiel-Eulefi et al., 2012), and the postnatal 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 (CD146+/α-SMA−/NG2+/CD34−/vWF−) surrounding endothelial cells (α-SMA+/CD34+/CD31+/vWF+) associated with smaller vessels of the vasa vasorum (<25 μm) (Figure 2). A subset of CD146+ cells co-expressed α-smooth muscle actin (α-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 (α-SMA+/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 CD34+/α-SMA− cells that were situated superficially to vasa vasorum, which we have deemed “supra-vasa” cells (Figure 2J). CD34+/α-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...
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% ± 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% ± 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% ± 5.87%) while another, slightly smaller subset co-expressed CD90 and CD34 (10.59% ± 4.2%).
CD34+ cells characterized by the surface marker profile CD146+/CD90+/CD56+/CD45-/CD34+/CD31- were found to be more abundant than CD146+/CD90-/CD56- cells and comprised 32.75% ± 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 vaso-rum, the so-called supra-vasa cells described above in aortic tissue sections. Most of these supra-vasa cells also co-expressed CD90 (71.24% ± 9.28%). Mature endothelial cells expressing the profile of CD56-/CD45-/CD34+/CD31- comprised 1.69% ± 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% ± 13.14%) whereas only about one-tenth expressed CD90 (9.20% ± 3.40%). Cells lacking CD34, CD146, and CD90 represented a majority (74.60% ± 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% ± 3.49% of all NH cells (Table 1). About half of the EPCs expressed CD146 (53.45% ± 4.94%) while about one-third co-expressed CD90 (32.08% ± 5.37%), which was appreciably more than the proportion of CD90+ cells in the endothelial mature (CD34-/CD31+) fraction (9.20% ± 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

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### 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 ≥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.
Table 1. Prevalence of Adventitial Cell Subpopulations

|                      | Pericytes CD146+/CD31+/CD45+/C0 | Supra-Vasa CD146+/CD31+/CD45+/C0 | Endothelial Mature CD31+/C0/CD45+/CD34+/C0 | Endothelial Progenitor CD34+/CD45+/CD56+ | Mean  | SD    | SEM  | Mean  | SD    | SEM  | Mean  | SD    | SEM  |
|----------------------|---------------------------------|---------------------------------|-------------------------------------------|------------------------------------------|-------|-------|------|-------|-------|------|-------|-------|------|
| Nucleated cells (%)  | 0.65                            | 0.81                            | 0.31                                      | 0.23                                     | 0.35  | 0.13  | 0.90 | 0.97  | 0.37  |      |       |       |      |
| Non-heme (%)         | 3.83                            | 3.25                            | 1.23                                      | 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    | –     | 100   |      |       |       |      |
| CD146+/CD90− (%)     | 0                               | –                               | 28.76                                     | 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).

**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 pericycle-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...
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 μm) 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...
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 μm) 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

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 μm.
(B) Sorted human adventitia-derived pericytes cultured on the surface of Matrigel spontaneously form spheroid cultures within 4–6 hr. Scale bar, 100 μm.
(C) Sorted pericytes (green, arrows) associated in the periphery of branching endothelial cells (red). Scale bar, 100 μm.
(D) Spheroids of pericytes in culture alone on Matrigel expanded over 5 days. Scale bar, 50 μm.
(E) Anastomoses between cell clusters expanding from spheroids within 5 days. Scale bar, 100 μm.
Results shown are representative of three independent experiments. See also Movie S1.
Endothelial mature CD34+/CD31−/C0
Adventitial cells/supra-vasa CD34+/CD31− endMT, endothelial to mesenchymal transition.

The 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+/CD31−) 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 (Crisan 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

### 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), 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.
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, Kramann 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 cryosectioning (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, #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 until a population of 0.5–1 × 10⁷ cells was reached. 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).

**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 −80°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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REFERENCES

Aicher, A., Rentsch, M., Sasaki, K., Ellwart, J.W., Fandrich, F., Siebert, R., Cooke, J.P., Dimmeler, S., and Heeschen, C. (2007). Non-bone marrow-derived circulating progenitor cells contribute to postnatal neovascularization following tissue ischemia. Circ. Res. 100, 581–589.
Andreeva, E.R., Pugach, I.M., Gordon, D., and Orekhov, A.N. (1998). Continuous subendothelial network formed by pericyte-like cells in human vascular bed. Tissue Cell 30, 127–135.
Baer, P.C. (2014). Adipose-derived mesenchymal stromal/stem cells: an update on their phenotype in vivo and in vitro. World J. Stem Cells 6, 256–265.
Campagnolo, P, Cesselli, D., Al Haj Zen, A., Beltrami, A.P., Kranck, N., Katare, R., Angelini, G., Emanuelli, C., and Madeddu, P. (2010). Human adult vena saphena contains perivascular progenitor cells endowed with clonogenic and proangiogenic potential. Circulation 121, 1735–1745.
Caplan, A.I. (2008). All MSCs are pericytes? Cell Stem Cell 3, 229–230.
Chen, C.W., Okada, M., Proto, J.D., Gao, X., Sekiya, N., Beckman, S.A., Corselli, M., Crisan, M., Saparov, A., Tobita, K., et al. (2013). Human pericytes for ischemic heart repair. Stem Cells 31, 305–316.
Crisan, M., Chen, C.W., Sun, B., Yap, S., Rubin, J., and Peault, B. (2011). The tunica adventitia of human arteries and veins as a source of mesenchymal stem cells. Stem Cells Dev. 21, 1299–1308.
Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., and Peault, B. (2008a). Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. Methods Cell Biol. 86, 295–309.
Crisan, M., Huard, J., Zheng, B., Sun, B., Yap, S., Logar, A., Giacobino, J.P., Castella, L., and Peault, B. (2008b). Purification and culture of human blood vessel-associated progenitor cells. Curr. Protoc. Stem Cell Biol. Chapter 2, Unit 2B.2.1–2B.2.13.
Crisan, M., Yap, S., Castella, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008c). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3, 301–313.
Crisan, M., Corselli, M., Chen, W.C., and Peault, B. (2012). Perivascular cells for regenerative medicine. J. Cell Mol. Med. 16, 2851–2860.
Duda, D.G., Cohen, K.S., di Tomaso, E., Au, P., Klein, R.J., Scadden, D.T., Willett, C.G., and Jain, R.K. (2006). Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J. Clin. Oncol. 24, 1449–1453.
Guimarães-Camboa, N., Cattaneo, P., Sun, Y., Moore-Morris, T., Gu, Y., Dalton, N.D., Rockenstein, E., Maslia, E., Peterson, K.L., Stallcup, W.B., et al. (2017). Pericytes of multiple organs do not behave as mesenchymal stem cells in vivo. Cell Stem Cell 20, 345–359.e5.
Havelka, G.E., and Kibbe, M.R. (2011). The vascular adventitia: its role in the arterial injury response. Vasc. Endovascular Surg. 45, 381–390.
Heistad, D.D., Marcus, M.L., Larsen, G.E., and Armstrong, M.L. (1981). Role of vasa vasorum in nourishment of the aortic wall. Am. J. Physiol. 240, H781–H787.
Hoshino, A., Chiba, H., Nagai, K., Ishii, G., and Ochiai, A. (2008). Human vascular adventitial fibroblasts contain mesenchymal stem/progenitor cells. Biochem. Biophys. Res. Commun. 368, 305–310.
Howson, K.M., Aplin, A.C., Gelati, M., Alessandri, G., Parati, E.A., and Nicosia, R.F. (2005). The postnatal rat aorta contains pericyte progenitor cells that form spheroidal colonies in suspension culture. Am. J. Physiol. Cell Physiol. 289, C1396–C1407.
contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J. Clin. Invest. 113, 1258–1265.

Invernici, G., Emanuelli, C., Madeddu, P., Cristina, S., Gadau, S., Benetti, A., Ciusani, E., Stassi, G., Siragusa, M., Nicosia, R., et al. (2007). Human fetal aorta contains vascular progenitor cells capable of inducing vasculogenesis, angiogenesis, and myogenesis in vitro and in a murine model of peripheral ischemia. Am. J. Pathol. 170, 1879–1892.

Kirton, J.P., and Xu, Q. (2010). Endothelial precursors in vascular repair. Microvasc. Res. 79, 193–199.

Klein, D., Weishardt, P., Kleff, V., Jastrow, H., Jakob, H.G., and Ergun, S. (2011). Vascular wall-resident CD44+ multipotent stem cells give rise to pericytes and smooth muscle cells and contribute to new vessel maturation. PLoS One 6, e20540.

Koester, W. (1876). Enerarteritis and arteritis. Berl Klin Wochenschr 13, 454–455.

Kramann, R., Goettsch, C., Wongboonsin, J., Iwata, H., Schneider, R.K., Kuppe, C., Kaesler, N., Chang-Panesso, M., Machado, E.G., Gratwohl, S., et al. (2016). Adipitional MSC-like cells are progenitors of vascular smooth muscle cells and drive vascular calcification in chronic kidney disease. Cell Stem Cell 19, 628–642.

Kwon, H.M., Sangiorgi, G., Ritman, E.L., McKenna, C., Holmes, D.R., Jr., Schwartz, R.S., and Lerman, A. (1998). Enhanced coronary vasospasm in apoE-deficient mice. Circ. Res. 83, 409–421.

Lee, W.C., Rubin, J.P., and Marra, K.G. (2006). Regulation of alpha-smooth muscle actin protein expression in adipose-derived stem cells. Cells Tissues Organs 183, 80–86.

Majesky, M.W. (2015). Adventitia and perivascular cells. Arterioscler. Thromb. Vasc. Biol. 35, e31–e35.

Mayo, J.N., and Bearden, S.E. (2015). Driving the hypoxia-inducible pathway in human pericytes promotes vascular density in an exosome-dependent manner. Microcirculation 22, 711–723.

Medici, D., Shore, E.M., Lounie, V.Y., Kaplan, F.S., Kalluri, R., and Glagov, S. (1967). Nature of species differences in the media of human arteries and arterioles. J. Physiol. 193, 193–199.

Nguyen, A.T., Gomez, D., Bell, R.D., Campbell, J.H., Clowes, A.W., Gabbiani, G., Giachelli, C.M., Parmacek, M.S., Raines, E.W., Rusch, N.J., et al. (2013). Smooth muscle cell plasticity: fact or fiction? Circ. Res. 112, 17–22.

Park, T.S., Gavina, M., Chen, C.W., Sun, B., Teng, P.N., Huard, J., Deasy, B.M., Zimmerlin, L., and Peault, B. (2011). Placental perivascular cells for human muscle regeneration. Stem Cells Dev. 20, 451–463.

Pasquinelli, G., Tazzari, P.L., Vaselli, C., Foroni, L., Buzzi, M., Storci, G., Alviano, E., Ricci, F., Bonafo, M., Orrico, C., et al. (2007). Thoracic aortas from multiorgan donors are suitable for obtaining resident angiogenic mesenchymal stromal cells. Stem Cells 25, 1627–1634.

Pasquinelli, G., Pacilli, A., Alviano, F., Foroni, L., Ricci, F., Valente, S., Orrico, C., Lanzoni, G., Buzzi, M., Luigi Tazzari, P., et al. (2010). Multidistrict human mesenchymal vascular cells: pluripotency and stemness characteristics. Cytotherapy 12, 275–287.

Petrini, M., Pacini, S., Trombi, L., Fazzi, R., Montali, M., Ikehara, S., and Abraham, N.G. (2009). Identification and purification of mesodermal progenitor cells from human adult bone marrow. Stem Cells Dev. 18, 857–866.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M., and Bianco, P. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131, 324–336.

Sacchetti, B., Funari, A., Remoli, C., Giannicola, G., Kogler, G., Liedtke, S., Cosso, G., Serafini, M., Sampaoloesi, M., Tagliafico, E., et al. (2016). No identical “mesenchymal stem cells” at different times and sites: human committed progenitors of distinct origin and differentiation potential are incorporated as adventitial cells in microvessels. Stem Cell Rep. 6, 897–913.

Shen, Y.H., Hu, X., Zou, S., Wu, D., Coselli, J.S., and LeMaire, S.A. (2012). Stem cells in thoracic aortic aneurysms and dissections: potential contributors to aortic repair. Ann. Thorac. Surg. 93, 1524–1533.

Tang, Z., Wang, A., Yuan, F., Yan, Z., Liu, B., Chu, J.S., Helms, J.A., and Li, S. (2012). Differentiation of multipotent vascular stem cells contribute to vascular diseases. Nat. Commun. 3, 875.

Traktuev, D.O., Merfeld-Claus, S., Li, J., Kolonin, M., Arap, W., Pasqualini, R., Johnstone, B.H., and March, K.L. (2008). A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ. Res. 102, 77–85.

Vasuri, F., Fittipaldi, S., Buzzi, M., Degiovanni, A., Stella, A., D’Errico-Grigioni, A., and Pasquinelli, G. (2012). Nestin and WT1 expression in small-sized vasa vasorum from human normal arteries. Histol. Histopathol. 27, 1195–1202.

West, C.C., Hardy, W.R., Murray, I.R., James, A.W., Corselli, M., Pang, S., Black, C., Lobo, S.E., Sukhija, K., Liang, P., et al. (2016). Prospective purification of perivascular presumptive mesenchymal stem cells from human adipose tissue: process optimization and cell population metrics across a large cohort of diverse demographics. Stem Cell Res. Ther. 7, 47.

Wolinsky, H., and Glagov, S. (1967). Nature of species differences in the medial distribution of aortic vasa vasorum in mammals. Circ. Res. 20, 409–421.

Zaniboni, A., Bernardini, C., Alessandri, M., Mangano, C., Zanoni, A., Bianchi, F., Sarli, G., Calza, L., Bacci, M.L., and Forni, M. (2014). Cells derived from porcine aorta tunica media show the pericyte and mesenchymal stromal-like cell properties in in vitro culture. Stem Cell Reports 10, 61–71.
Supplemental Information

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

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Primary cell isolation

The minced tissue in PBS was strained through a 70 µm molecular sieve. The pass-through was collected and held at 37°C while remaining tissue was enzymatically digested in RPMI media containing 0.4% collagenase type IV (Worthington) and 350 KU/mL DNase I (Sigma) for 30 min at 37°C with gentle agitation. The digestion medium and tissue was passed through a 70 µm sieve and tissue was returned to fresh digestion medium for another 30 min at 37°C with gentle agitation. Following a final straining through a 70 µm sieve and wash with PBS, all filtrates were pooled and centrifuged at 400 x g for 10 min at 4°C. At this point, cells were labeled for analytical flow cytometry or directly placed into tissue culture for propagation prior to fluorescence-activated cell sorting. For analytical flow cytometry, red blood cells were lysed from pellets re-suspended in 10X lysis buffer (1.5 M ammonium chloride, 100 mM potassium bicarbonate, 1.3 mM EDTA, pH 7.2) for 10 min at room temperature with gentle rocking. After centrifugation, the remaining pellet was washed in PBS, re-suspended in 2 mL FBS and kept on ice and stained for analytical flow cytometry.

Flow cytometry

Briefly, freshly isolated cells were pelleted (~1-4 x 10^5 cells), incubated in 1 µL neat mouse serum (Sigma) on ice, protected from light, and labeled with the following fluorochrome-conjugated monoclonal mouse anti-human antibodies (2 µL per antibody): CD31-PE-Cy7 (Biolegend, #303117), CD45-APC-Cy7 (BD Biosciences, #348805 ), CD90-APC (BD Biosciences, #559869) and from Beckman Coulter: CD34-ECD (#BD2709U), CD56-PE-Cy5 (#IM2654), and CD146-PE (#A07483). Cells (minimum of 10^4) were separately labeled with antibodies to classifier molecules only (CD56, CD45, CD34 and CD31) to facilitate gating of the
outcome measures for CD146 and CD90. Following labeling, cells were washed in staining buffer (4% calf serum in 15 mM sodium azide in PBS-A: 4 M sodium chloride, 80 mM potassium chloride, 240 mM monosodium phosphate and 44 mM potassium dihydrogen phosphate, pH 7.2), fixed in 0.5% methanol-free formaldehyde in PBS and held at 4°C, protected from light. Prior to analysis on the cytometer, cells were fixed in 4% formaldehyde in PBS-A for 15 min at RT and washed in 0.5% BSA in PBS-A with 0.5% sodium azide. Following centrifugation at 400 \( x \) \( g \) and removal of the supernatant, cells were permeabilized with 0.5% BSA in PBS-A with 0.5% saponin for precisely 10 min. Upon a second wash in 0.5% BSA in PBS-A with 0.5% sodium azide, cells were centrifuged and the pellet was re-suspended in staining buffer and incubated with 7.7 µg/mL 4,6-diamidino-2-phenylindole (DAPI, Invitrogen). Cells were protected from light and kept on ice prior to flow cytometry. Data were acquired on a four-laser LSR Fortessa cytometer (BD Biosciences, UPCI Cytometry Facility). Cells were gated for pericyte, adventitial and endothelial progenitor and endothelial mature subpopulations as previously established (Zimmerlin et al., 2010). The instrument was calibrated and BD Calibrite™ beads and single antibody-stained IgG capture beads (BD Biosciences) were used to determine a compensation matrix for all fluorochromes (Zimmerlin et al., 2010). Compensation of fluorochromes and data analysis was conducted offline using VenturiOne software package (Applied Cytometry, Sheffield, UK) and high-throughput parallel processing of all specimens.

RNA isolation and qPCR analysis

Total RNA was isolated from adventitial tissue extracts and cell lysates using the RNeasy Plus Mini kit (QIAgen) according to the manufacturer’s instructions. Gene expression of CNN, CD146, CD90, CD31, VWF, CD73 and CD105, were quantified from 1 µL of template RNA using inventoried Taqman® Gene Expression Assays (Table S1) and 1-step RNA-to-CT™ kit (Life Technologies). Thermocycling conditions were as follows: RNA was reverse-transcribed at 48°C for 15 min followed by Taq activation at 95°C for 10 min and 40 cycles of denaturation
for 15 seconds at 95°C and primer and probe annealing at 60°C for 1 min. All target gene probes were labeled with FAM as the 5’ reporter dye and TAMRA as the 3’ quencher dye. Assays were carried out in triplicate on an ABI Prism 7900HT sequence detection system in the Genomics and Proteomics Core Laboratories of the University of Pittsburgh. Data was analyzed using SDS 2.2.2 Software (Invitrogen). Relative gene expression levels were calculated using the $2^{\Delta \Delta CT}$ method and reported as relative gene expression normalized to 18S as the endogenous control.

Immunocytochemistry

Briefly, cells were fixed in 4% paraformaldehyde for 10 min, washed two times with wash buffer (1X PBS containing 0.05% Tween 20) and permeabilized with 0.1% Triton X-100. After incubation in a blocking solution consisting of wash buffer with 5% goat serum (Sigma), cells were incubated in the primary antibodies: αSMA (1:200, #M0851, Dako), CNN (1:200, #ab46794, Abcam) and SM-MHC (1:200, #ab82255, Abcam) for 2 h at room temperature. After three washes, secondary (biotinylated anti-mouse (1:500, #BA-9200, Vector) or anti-rabbit (1:500, #BA-1000, Vector), anti-rabbit-FITC (1:500, #111-095-003, Jackson ImmunoResearch), anti-rabbit-488 (1:500, #A11008, Invitrogen) were applied for 1 h and tertiary labels (streptavidin-conjugated FITC (#S3762, Sigma) or cyanine 3 (#S6402, Sigma), 1:500, 30 min) were applied at room temperature when necessary. Nuclei were detected using labeling with DAPI (1:1000, Invitrogen, 5 min). After a final three washes, cells were imaged in 1X PBS using epi-fluorescence on a Nikon TE-2000-E2 inverted microscope and images were captured using a CoolSNAP ES2 Monochrome 1394x1040 High Resolution Camera (Photometrics) and NIS Elements Software 3.2.