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CD90 Identifies Adventitial Mesenchymal Progenitor Cells in Adult Human Medium- and Large-Sized Arteries

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SUMMARY

Mesenchymal stem cells (MSCs) reportedly exist in a vascular niche occupying the outer adventitial layer. However, these cells have not been well characterized *in vivo* in medium- and large-sized arteries in humans, and their potential pathological role is unknown. To address this, healthy and diseased arterial tissues were obtained as surplus surgical specimens and freshly processed. We identified that CD90 marks a rare adventitial population that co-expresses MSC markers including PDGFRα, CD44, CD73, and CD105. However, unlike CD90, these additional markers were widely expressed by other cells. Human adventitial CD90+ cells fulfilled standard MSC criteria, including plastic adherence, spindle morphology, passage ability, colony formation, and differentiation into adipocytes, osteoblasts, and chondrocytes. Phenotypic and transcriptomic profiling, as well as adoptive transfer experiments, revealed a potential role in vascular disease pathogenesis, with the transcriptomic disease signature of these cells being represented in an aortic regulatory gene network that is operative in atherosclerosis.

INTRODUCTION

Vascular remodeling is essential to the maintenance of homeostasis, the normal response to injury, and the development of disease (Carmeliet, 2003; Psaltis and Simari, 2015). These processes are thought to be dependent on the action of progenitor cells that reside within the vascular wall. Research to characterize resident vascular progenitor cells is ongoing, with the majority of experimentation conducted thus far having used animal cells and models, or largely *ex vivo* human studies (Kovacic et al., 2008; Kovacic and Boehm, 2009; Psaltis et al., 2011; Michelis et al., 2014; Psaltis and Simari, 2015).

Several important findings have arisen from this research. For example, comprehensive studies of the microvasculature of skeletal muscle and adipose have provided important insights into the stem cell populations of these vascularized tissues (Zimmerlin et al., 2010; Corselli et al., 2012; Chen et al., 2013). Additional studies, largely conducted in small animals, have suggested that the outermost layer of the vascular wall, the tunica adventitia, is a complex and dynamic environment hosting an important niche for adventitial mesenchymal stem cells (MSCs). In murine models, adventitial MSCs have been shown to drive pathways contributing to vascular disease (Kramann et al., 2016). However, in humans, almost all investigations of resident vascular MSCs from medium- and large-sized vessels have focused on characterizing cells under *ex vivo* culture conditions (Psaltis and Simari, 2015). For example, Pasquinelli et al. (2007) digested segments of human thoracic aortas and then studied cells that remained in culture after three to five passages. In another study, human adventitial fibroblasts were derived from cultured digests of the entire adventitial layer of pulmonary arteries (Hoshino et al., 2008). In a separate series of investigations, CD44+ cells isolated from human internal thoracic arteries using immunoselection beads were cultured prior to experimentation, including analysis of RNA and HOX gene expression (Klein et al., 2011, 2013). Similarly, Campagnolo et al. (2010) investigated *ex-vivo*-cultured CD34+ CD31–cells from human saphenous veins.

Certain knowledge with regard to the *in situ* and *in vivo* characteristics of human adventitial MSCs has begun to emerge, but important areas to improve our understanding remain. Chong et al. (2013b) used essentially only immunofluorescence (IF) staining to investigate platelet-derived growth factor receptor α (PDGFRα)-expressing cells in the adult human vasculature. In another study, Corselli et al. (2012) undertook IF, flow cytometric, and *ex vivo* culture studies of human white adipose tissue. While they

suggested that CD34 identifies MSC-like cells that reside in the adventitia of vessels in adipose samples, the vessels described do not appear to have been greater in size than large arterioles (up to approximately 200 μm in diameter). Importantly, the existence of these MSC-like cells in non-adipose vessels was not studied, and they did not study medium- and large-sized vessels that are critical for important human diseases such as atherosclerosis. Billaud et al. (2017) recently studied the vaso-vasorum (microvessels that provide blood supply to larger vessels) of the adult human aorta, and suggested that a CD34– population of CD146+ pericytes exhibits MSC-like characteristics. However, Billaud et al. did not report on other potential MSC populations that exist beyond the microvasculature of the vaso-vasorum, nor did they explore links to clinically relevant disease states such as atherosclerosis. Considering these studies, what has been lacking is a detailed characterization of adult human adventitial MSCs in their *in situ* and *in vivo* native state, without *ex vivo* expansion or manipulation prior to characterization. Furthermore, an important next step is to use advanced techniques such as high-throughput transcriptional profiling to begin to relate these cells to clinically relevant disease states.

We therefore sought to systematically define resident adventitial MSCs in medium- and large-sized arteries in the adult human, *in situ* and *in vivo*, using the previously described criteria for MSCs (Dominici et al., 2006). Having identified and characterized these cells, we then sought to understand the changes that occur in these MSCs with the development of atherosclerosis and thoracic aortic aneurysm (TAA), compared with healthy vessels, and how these cellular and molecular changes are mechanistically linked to disease progression.

RESULTS

CD90 Marks, *In Situ* and *In Vivo*, an Adventitial Cell Population in the Human Aorta and Internal Thoracic Artery that Co-expresses MSC Markers

The clinical characteristics of all patients enrolled in this study are provided in Table S2. A total of 67 subjects participated and donated vascular tissues to this study. There was no overlap among any of these groups (each subject only donated tissues to either the internal thoracic artery [ITA], the healthy aorta, or the diseased aorta groups).

To identify and define putative resident adventitial MSCs in adult humans we first performed IF staining and flow cytometry to characterize these cells in surplus samples of ITA, ascending aortic aneurysm ("diseased aorta"), and healthy aorta isolated from donor hearts undergoing implantation at cardiac transplant. Prior work, the majority of which reflects *ex vivo* experiments in cultured cells, suggested that postnatal MSC-like vascular progenitor cells express PDGFRα, CD44, and CD90, while being negative for hematopoietic and endothelial markers (Covas et al., 2005; Pasquinelli et al., 2007; Campagnolo et al., 2010; Klein et al., 2011; Chong et al., 2013b). Therefore, we stained ITA and aorta sections for these putative vascular MSC markers.

We found that by IF staining, PDGFRα (Figures 1A and 1B) and CD44 (Figures 1C and 1D) mark a subset of adventitial cells. However, these markers were also widely expressed by α-smooth muscle actin (αSMA)+ medial smooth muscle cells (SMCs) (Figures 1A–1D). Therefore, we concluded that PDGFRα and CD44 were non-specific markers for vascular MSCs in human vessels. In contrast, CD90 was not expressed by medial αSMA+ cells. Interestingly, CD90+ was expressed by discrete clusters of adventitial cells in both the ITA (Figure 1E) and aorta (Figure 1F). Furthermore, adventitial CD90+ cells co-expressed PDGFRα (Figures 1G and 1H). CD90+ adventitial cells also largely co-expressed PDGRFβ and CD34 (Figures S1A–S1D), although there was also widespread expression of PDGFRβ by medial SMCs. Notably, adventitial CD90+ cells did not express the SMC markers calponin or smoothelin (Figures S2A–S2D). Together, these data suggested that, among these markers, CD90 was the most reasonable candidate for specifically identifying adventitial MSCs in adult human vessels. While CD34 also appeared to be identify a distinct population of adventitial cells, and others have also claimed this identifies a population of vascular progenitor cells (Corselli et al., 2012), CD34 is not considered to be a typical MSC marker (Dominici et al., 2006).

To explore the possibility that CD90 identifies adventitial MSCs in the adult human, we performed flow cytometry of freshly isolated cells from the healthy and diseased aorta. For this analysis, freshly isolated cells underwent immediate fixation and permeabilization to permit detection of both surface and internal markers and we then gated on DAPI+ cells to selectively identify whole nucleated cells (Figure 2). Flow cytometry on ITA samples was not feasible due the smaller amounts of tissue and low cell numbers after sample digestion and processing. Consistent with our IF staining, of all nucleated cells isolated from the healthy aorta, a high proportion expressed traditional MSC markers, including PDGFRα (67.6% ± 6.1%), CD44 (72.6% ± 3.0%), CD73 (42.0% ± 3.0%), and CD105 (56.3% ± 1.5%), whereas a much smaller subset expressed CD90 (9.5% ± 0.7% of all nucleated cells) (Figures 2A–2C). On further flow cytometry analysis of the CD90+ cell population from the healthy aorta, a majority of these cells co-expressed PDGFRα, CD44, CD73, and CD105 (Figure 2D). Most healthy aorta CD90+ cells were negative for αSMA (67.7% ± 5.5%) and the endothelial marker CD31 (95.3% ± 2.0%) (Figure 2E).

Similar results were obtained from flow cytometry analysis of diseased aorta cells, where, among all freshly

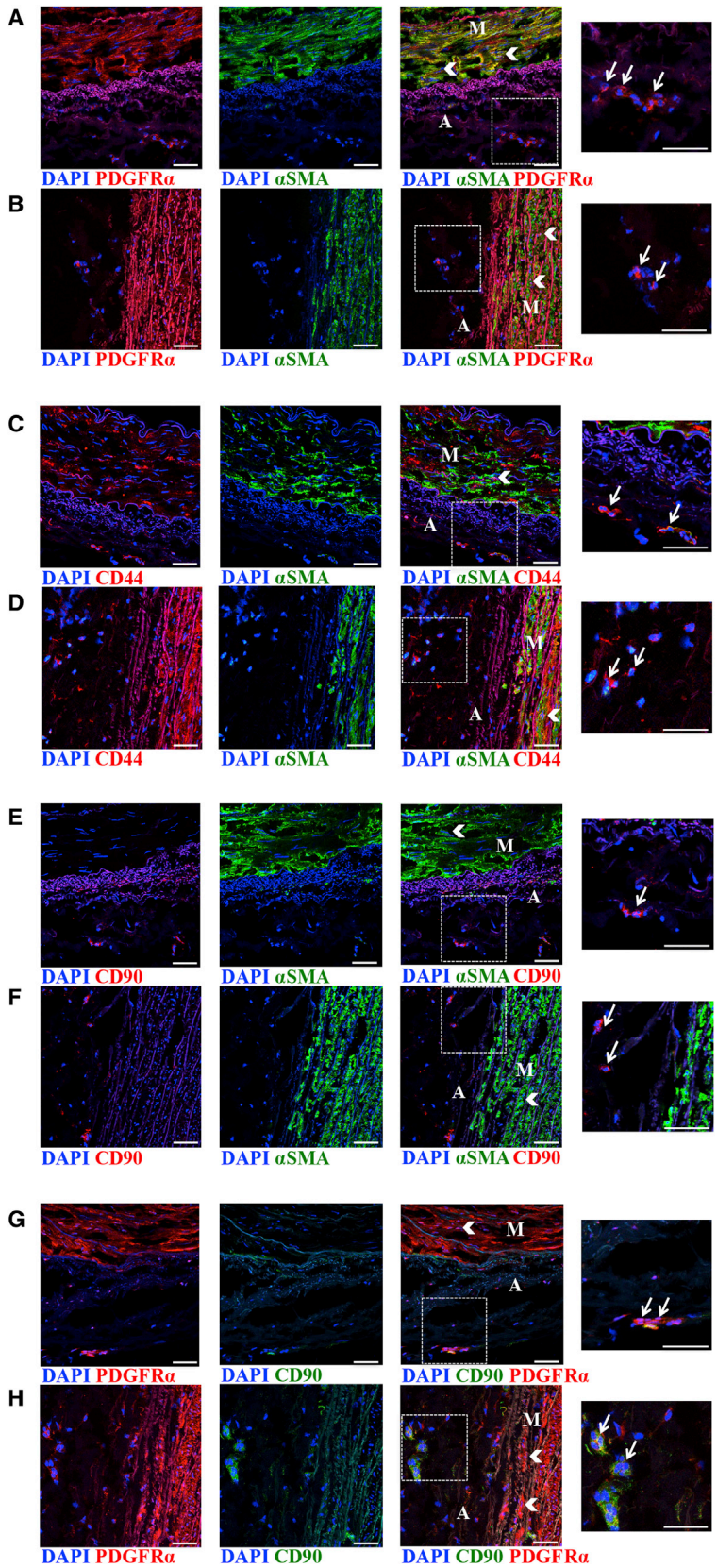


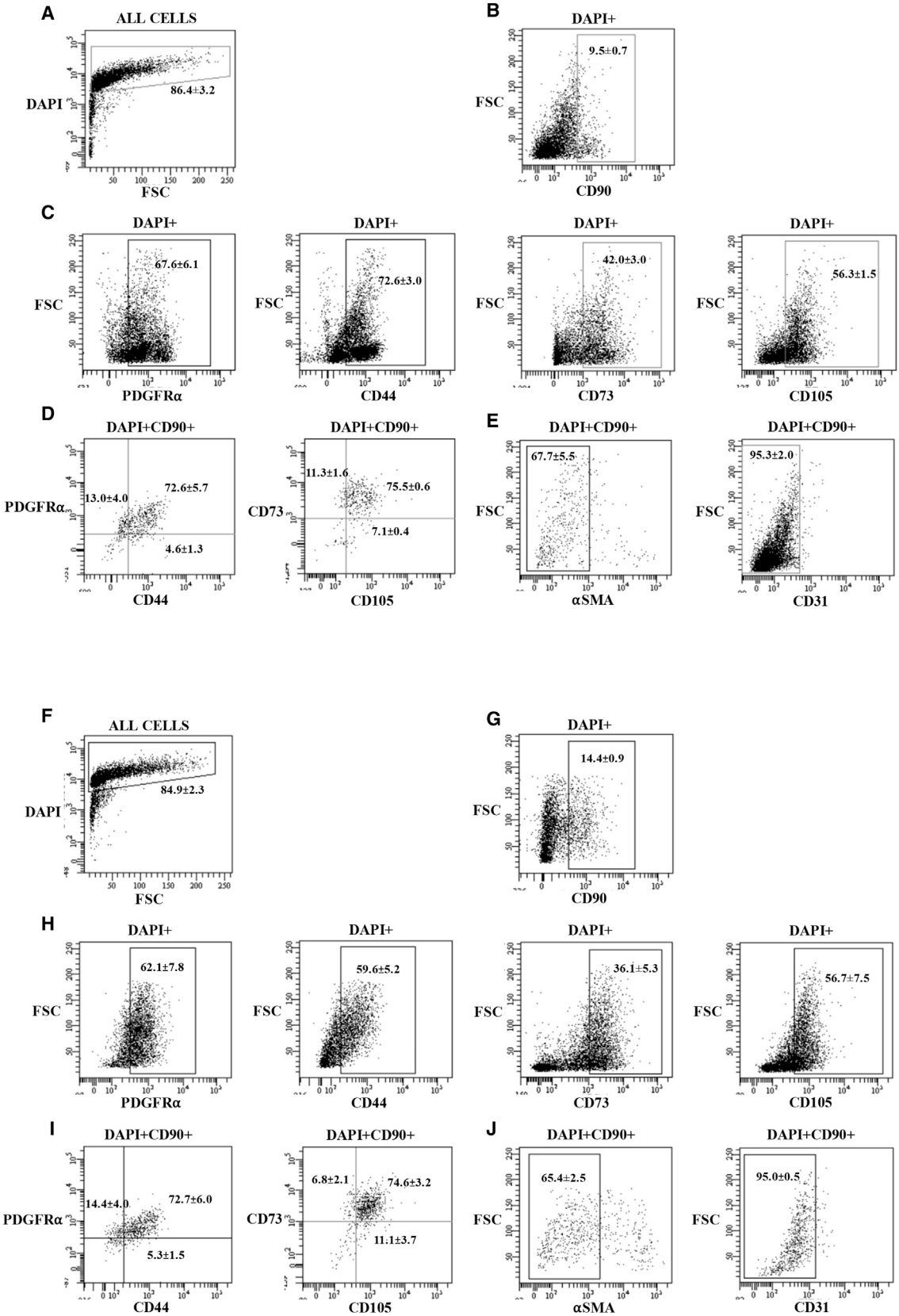
Figure 1. CD90 Marks a Subset of Adventitial Cells in the Human ITA and Healthy Aorta

(A and B) IF staining for PDGFRα in the ITA (A) and in the healthy human aorta (B), demonstrating that PDGFRα is expressed by a subset of cells in the adventitia (A, arrows), but that it is also widely expressed by αSMA+ SMCs in the media (M, arrowheads).

(C and D) IF staining for CD44 in the ITA (C) and also the healthy human aorta (D), demonstrating that CD44 is expressed by a subset of cells in the adventitia (arrows) but also by medial αSMA+ SMCs (arrowheads).

(E) In contrast, CD90 is expressed by only a subset of adventitial cells in the ITA (arrows) and not by αSMA+ medial SMCs (arrowheads).

(F) Similarly, in the aorta, expression of CD90 is limited to a subset of adventitial cells (arrows), and it is not expressed by αSMA+ medial SMCs (arrowheads). In both the ITA (G) and aorta (H), CD90 and PDGFRα are co-expressed by a subset of adventitial cells (arrows), while medial SMCs express PDGFRα only (arrowheads). Scale bars represent 100 μm. Inset panels on the right, representing an enlarged view of the area in the respective dashed squares, show adventitial cells at higher magnification. All images are representative, and consistent results were obtained from the staining of ITA and healthy aorta samples from at least two different subjects of each sample type.



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obtained and then fixed and permeabilized cells, 62.1% ± 7.8% were PDGFRα+, 59.6% ± 5.2% were CD44+, 36.1% ± 5.3% were CD73+, and 56.7% ± 7.5% were CD105+. By comparison, only 14.4% ± 0.9% of all nucleated cells expressed CD90 (Figures 2F–2H). A majority of CD90+ cells from the diseased aorta expressed PDGFRα, CD44, CD73, and CD105 (Figure 2I), while DAPI+ CD90+ cells were generally negative for αSMA (65.4% ± 2.5%) and CD31 (95.0% ± 0.5%) (Figure 2J).

In summary, CD90 is a discriminating surface marker for an adventitial mesenchymal population in the adult human. Conversely, several traditional markers that have been widely assumed to be specific for MSCs, appear to be non-specific for these cells in medium- and large-sized adult human arteries.

Adventitial CD90+ Cells Exhibit Characteristics of MSCs

Fluorescence-activated cell sorting (FACS) was used to isolate live CD90+ cells for further experimentation and characterization. Of the live cell (DAPI−) population that was freshly isolated from the healthy aorta, diseased aorta, and ITA, the percentage expressing CD90 was 4.0% ± 1.2%, 4.8% ± 0.5%, and 5.1% ± 0.9%, respectively (Figures S3A–S3C). Therefore, likely due to differing exposure of the CD90 antigen, the proportion of vascular cells detected as expressing CD90 was 50%–70% lower when direct live cell staining and flow cytometry was performed, compared with staining and flow cytometry with the same CD90 antibody on vascular cells after fixation and permeabilization.

We sought to confirm the MSC characteristics of these CD90+ cells. FACS-isolated live CD90+ cells were spindle-shaped and plastic adherent when grown in culture, exhibited colony formation (Figure S4A), and could undergo extended passaging (>10 passages). *Ex vivo*, purified adventitial CD90+ cells exhibited typical MSC differentiation capacity into adipocytes, osteoblasts, and chondrocytes, which was confirmed by morphologic, immune-histochemical, and gene expression criteria (Figure 3). In addition, at least from passages 2 to 5 (the longest duration of culture prior to further experimental use in this study), our limited *ex vivo* culture of freshly isolated adventitial CD90+ cells was not associated with any appreciable change in the gene expression level of *CD90* (Figure S4B).

Therefore, in addition to surface marker characteristics, freshly isolated CD90+ adventitial cells from adult human vessels fulfill all standard criteria that have previously been established for MSCs (Dominici et al., 2006). Furthermore, *CD90* expression levels were not altered by limited *ex vivo* passaging (passage ≤ 5).

Aorta CD90+ Cells Modulate Angiogenesis and Recovery of Tissue Perfusion after Ischemia

We next aimed to determine the potential role of adult human CD90+ adventitial MSCs in vascular disease. Again, due the smaller amounts of tissue and low cell numbers after sample processing, it was not possible to include cells from the ITA in these experiments.

According to prior reports (Michelis et al., 2014), we first investigated if these cells exhibit paracrine functioning by evaluating conditioned media (CM) and cell lysates from *ex-vivo*-cultured CD90+ cells for protein and cytokine production. Compared with CD90+ cells from the diseased aorta, healthy aorta CD90+ cells secreted 1.7× more vascular endothelial growth factor (VEGF) (1,239 ± 68 versus 740 ± 117 pg/mL; $p < 0.05$) and 3.3× more angiogenin (2,535 ± 187 versus 757 ± 71 pg/mL; $p < 0.0001$) (Figure 4A). There was no significant difference in placental growth factor (PIGF) and hepatocyte growth factor (HGF) production between the two groups, while angiopoietin-2, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), heparin binding EGF-like growth factor, leptin, and platelet-derived growth factor-BB (PDGF-BB) were below

Figure 2. Flow Cytometry Analysis of Freshly Isolated Human Aortic Cells Indicating that CD90 Marks a Population of MSC-like Cells

Freshly obtained samples from the healthy or diseased aorta underwent tissue digestion, and cells were directly fixed and permeabilized to permit flow cytometry analysis of both surface (CD90, CD44, PDGFRα, CD105, CD73, and CD31) and internal (αSMA) markers.

(A–E) Representative flow cytometry analysis of healthy aorta cells. (A) Initial gating was performed to select for nucleated (whole) DAPI+ cells. (B) Only a small fraction of DAPI+ cells from the healthy aorta expressed CD90 (9.5% ± 0.7%). (C) In contrast, DAPI+ cells from the healthy aorta showed broad expression of PDGFRα (67.6% ± 6.1%), CD44 (72.6% ± 3.0%), CD73 (42.0% ± 3.0%), and CD105 (56.3% ± 1.5%). (D) DAPI+ CD90+ cells from the healthy aorta broadly co-expressed PDGFRα, CD44, CD73, and CD105. (E) The majority of CD90+ cells from the healthy aorta were negative for αSMA and CD31. Data represent mean ± SEM from at least three independent experiments, with each experiment performed using cells obtained from a different subject.

(F–J) Representative flow cytometry analysis of diseased aorta cells. (F) Initial gating was performed to select for nucleated (whole) DAPI+ cells. (G) Only a small fraction of DAPI+ cells from the diseased aorta expressed CD90 (14.4% ± 0.9%). (H) In contrast, DAPI+ cells from the diseased aorta showed broad expression of PDGFRα (62.1% ± 7.8%), CD44 (59.6% ± 5.2%), CD73 (36.1% ± 5.3%), and CD105 (56.7% ± 7.5%). (I) DAPI+ CD90+ cells from the diseased aorta broadly co-expressed PDGFRα, CD44, CD73, and CD105. (J) The majority of CD90+ cells from the diseased aorta were negative for αSMA and CD31. Data represent mean ± SEM from at least three independent experiments, with each experiment performed using cells obtained from a different subject. Data shown are mean ± SEM.

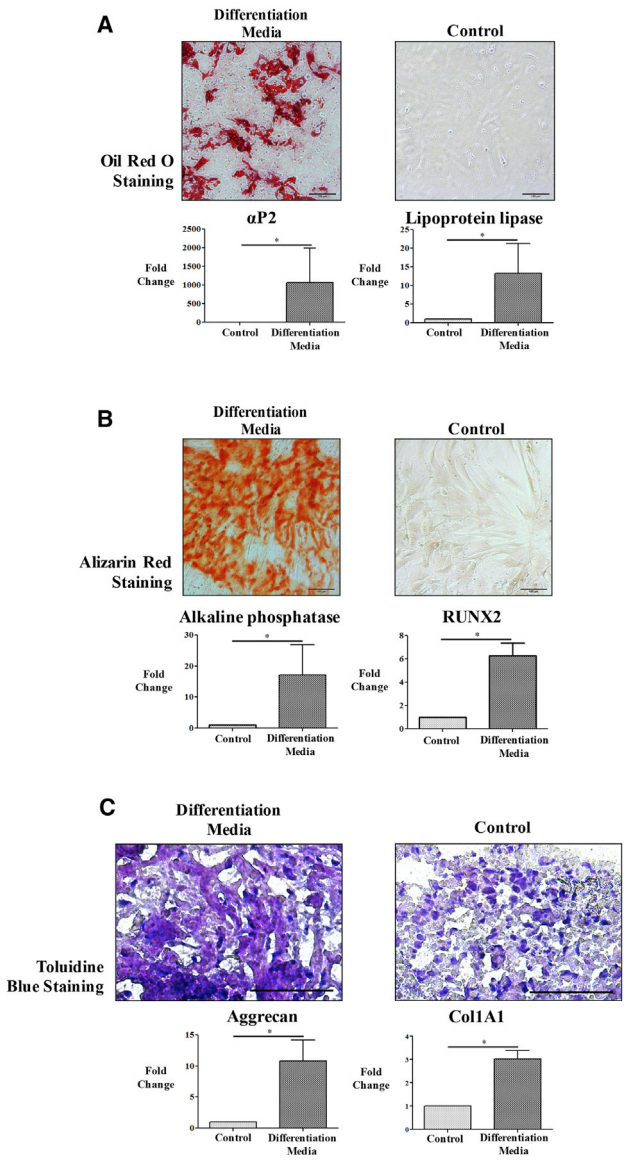

Figure 3. Freshly Isolated CD90+ Cells from the Human Aorta Exhibit MSC-like Differentiation Capacity

Freshly isolated CD90+ cells were cultured *ex vivo* and passaged a maximum of five times prior to the following differentiation experiments.

(A) CD90+ aorta cells cultured in adipogenic differentiation media contained lipid droplets that stained with oil red O. By qRT-PCR, compared with RNA expression of undifferentiated CD90+ aorta cells, under adipogenic differentiation conditions there was a significant increase in the RNA expression of adipogenic genes (αP2, lipoprotein lipase).

(B) CD90+ aorta cells cultured in osteogenic differentiation media contained calcium deposits that stained with alizarin red. By qRT-PCR, compared with RNA expression of undifferentiated CD90+ aorta cells, under osteogenic differentiation conditions there was a significant increase in the RNA expression of osteogenic genes (alkaline phosphatase, RUNX2).

(C) CD90+ aorta cells cultured as a pellet in suspension of chondrogenic differentiation media formed chondrocytes located within lacunae that were visible with toluidine blue staining. By qRT-PCR, compared with RNA expression of undifferentiated CD90+ aorta cells, under chondrogenic differentiation conditions there was a significant increase in the RNA expression of chondrogenic genes (aggrecan and Col1A1). Staining was performed for two different differentiation experiments and representative images are shown. Scale bars represent 100 μm. qRT-PCR data represent mean ± SEM from at least four independent experiments, with each experiment performed using cells obtained from a different subject. *$p < 0.05$.

the detection limit. For intracellular concentrations of angiogenic proteins, there was no difference between the two groups for angiopoietin-2, bFGF, HGF, and PIGF (Figure S5), while angiogenin, EGF, HB-EGF, leptin, PDGF-BB, and VEGF were below the detection limit.

Based on the differential secretion of VEGF and angiogenin, we speculated that aorta CD90+ cells may modulate angiogenesis, and performed a tubulogenesis assay using CM from diseased and healthy aorta CD90+ cells. Human umbilical vein endothelial cells were pre-treated for 24 hr with CM alone or CM plus neutralizing antibodies to both VEGF and angiogenin before seeding onto growth factor-reduced Matrigel. Plates were imaged at 4 hr to assess tubule formation. As measured by tubule branch points, tubule formation was significantly enhanced by pretreatment with CM from healthy aorta CD90+ cells compared with CM from diseased aorta CD90+ cells (Figure 4B). Within these experimental groups (healthy and diseased aorta CD90+ cells), the addition of VEGF and angiogenin neutralizing antibodies effectively reduced tubule formation (Figures 4C and 4D). Collectively, these data indicate that adventitial CD90+ MSCs from the adult human aorta are able to modulate angiogenesis via paracrine mechanisms; specifically, the production and secretion of VEGF and angiogenin. In addition, compared with CD90+ cells from the diseased aorta, CD90+ cells from the healthy aorta exhibit greater angiogenic potential.

To validate these findings *in vivo*, we performed adoptive transfer of human aorta CD90+ MSCs in a well-validated mouse model of limb ischemia (Michelis et al., 2014). Specifically, ischemia was induced in the left hindlimb of severe combined immunodeficient (SCID) mice by ligation and resection of the femoral artery. The ischemic hindlimb was then locally injected with CD90+ cells from the healthy or diseased aorta, or with the same volume of PBS. After femoral artery ligation and resection, blood flow quantified between the ischemic and non-ischemic hindlimbs was immediately reduced by more than 40% by laser speckle imaging to a mean perfusion ratio (PR) of 0.58 ± 0.02 (Figure 5A). By post-surgical day 3, there was a significant difference in recovery between the mice

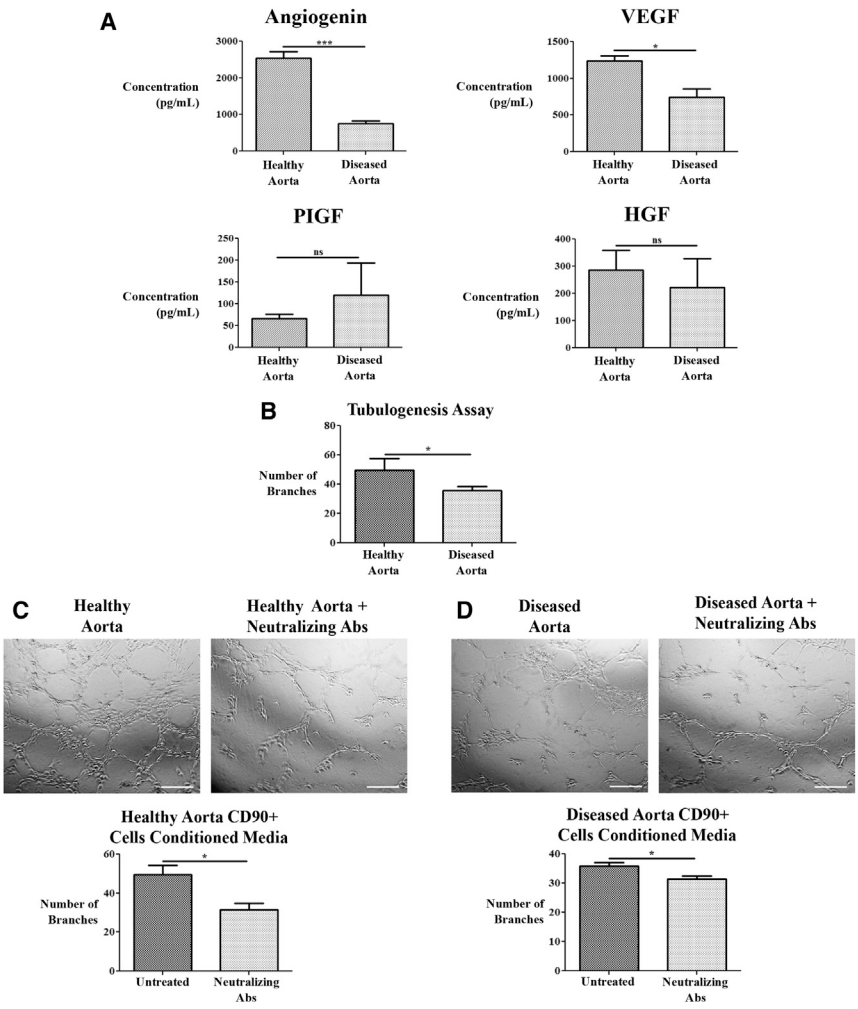

Figure 4. CD90+ Cells Isolated from Healthy Human Aortas Exhibit Angiogenic Capacity *In Vitro*

Freshly isolated CD90+ cells from the healthy and diseased aorta were cultured *ex vivo* and passaged a maximum of three times prior to experiments.

(A) Quantification of angiogenic protein levels in the supernatant of human aorta CD90+ cells in culture was performed. Compared with CD90+ cells isolated from diseased aortas, CD90+ cells from healthy aortas secreted significantly more angiogenin and VEGF (vascular endothelial growth factor). There was no significant difference in HGF (hepatocyte growth factor) or PIGF (placental growth factor) secretion. In addition, angiopoietin-2, EGF (epidermal growth factor), bFGF (basic fibroblast growth factor), HG-EGF (heparin binding EGF-like growth factor), leptin, and PDGF-BB (platelet-derived growth factor-BB) were below the limits of detection. For protein quantification from supernatant, n = 5 different diseased aorta samples and n = 3 different donor aorta samples (each from a different subject) were used for each condition.

(B) Tubulogenesis assay demonstrated that human umbilical vein endothelial cells (HUVECs) pre-treated with conditioned media (CM) from healthy aorta CD90+ cells formed significantly more branch points than those pre-treated with CM from diseased aorta CD90+ cells.

(C and D) Adding neutralizing antibodies specific for angiogenin and VEGF to the CM before pretreatment of the HUVECs resulted in a significant decrease in the number of branch points formed in the healthy aorta CD90+ (C) and diseased aorta CD90+ (D) experimental groups. Representative bright-field images are shown. Scale bars represent 100 μm. For tubulogenesis experiments, n = 5 different diseased aorta samples and n = 3 different donor aorta samples (each from a different subject) were used for each condition, with the experiment for each sample performed in duplicate.

For all panels data shown are mean ± SEM, *$p < 0.05$, ***$p < 0.0001$, and ns, not significant.

injected with healthy aorta CD90+ cells (PR = 0.81 ± 0.03) versus those injected with diseased aorta CD90+ cells (PR = 0.67 ± 0.02) or PBS (PR = 0.70 ± 0.02) ($p < 0.005$). This significant difference persisted at days 7, 11, and 15. At day 21, the curves for each experimental group converged with mean PR of 0.94 ± 0.01 for all groups (Figure 5A). Representative laser speckle images are shown for mice in each group at days 0, 7, and 21 (Figure 5B). To better understand the mechanisms underlying the differences in limb perfusion between these groups, we studied capillary density as assessed by CD31+ staining. Capillary density was quantified in the adductor muscles of the ischemic limbs of mice transplanted with CD90+ cells, and was significantly greater in mice that received healthy aorta versus diseased aorta CD90+ cells at day 21 (Figure 5C). Consistent with our *in vitro* data in Figure 4, this suggested that the favorable effect of healthy aorta CD90+ cells is at least partially due to enhanced angiogenesis. Finally, while it is well described that there is substantial cell death of adoptively transferred cells in such experimental settings (Golpanian et al., 2016), we were nevertheless able to demonstrate the survival of a limited number of transferred human CD90+ cells in the injected hindlimbs at day 21 (Figure S6). Overall, these experiments demonstrate that, compared with diseased aorta CD90+ cells, healthy aorta CD90+ cells are able to favorably modulate angiogenesis and restoration of perfusion after an ischemic injury.

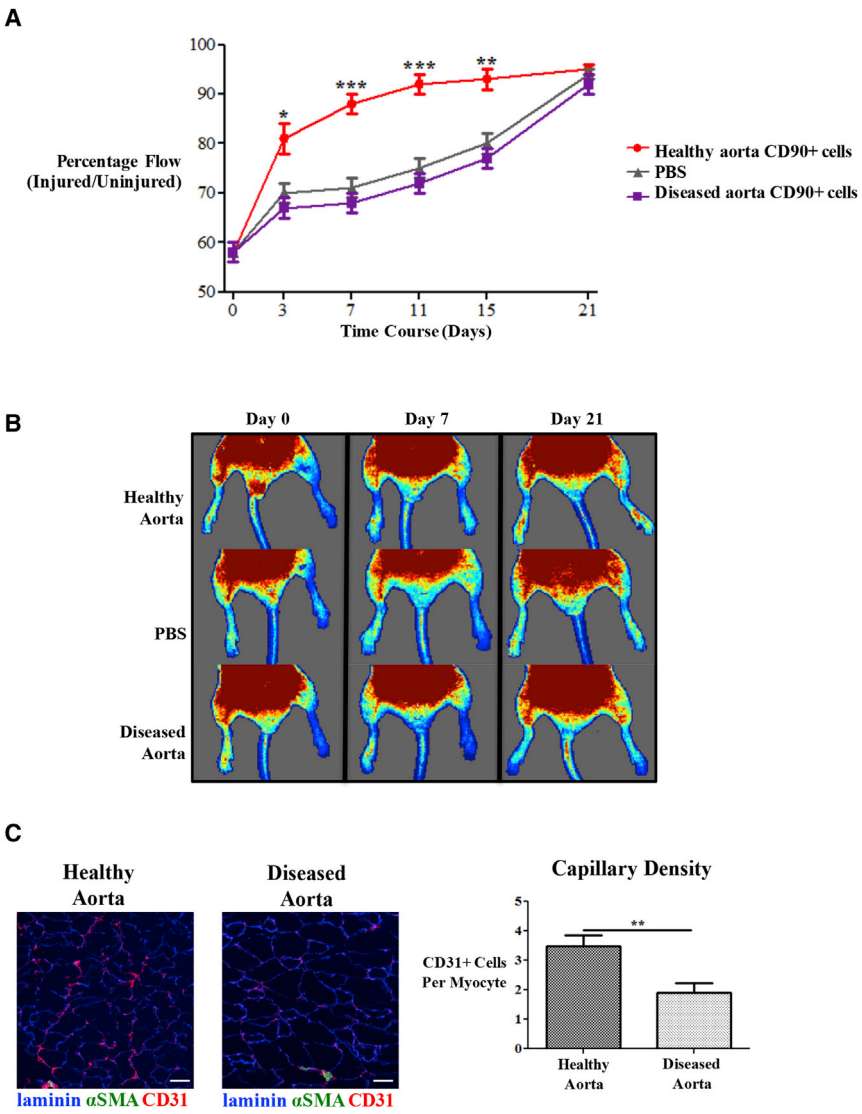

Figure 5. CD90+ Cells Isolated from Healthy Human Aortas Increase Angiogenic Capacity and Tissue Perfusion *In Vivo*

(A) Femoral artery ligation was performed in nude mice, with subsequent local injection of cultured CD90+ cells (passaged $\leq$5 times) or PBS as indicated. Summary of perfusion ratios (PRs) derived from laser speckle imaging of the ischemic limb is shown relative to baseline (prior to surgery), for mice transplanted with 5 × 10^5 CD90+ cells isolated from healthy or diseased aorta or PBS. Mice transplanted with healthy aorta CD90+ cells (n = 7) had augmented perfusion recovery starting at day 3 and persisting through day 15, compared with mice transplanted with diseased aorta CD90+ cells (n = 9) or PBS (n = 6).

(B) Representative laser speckle images for each experimental group at days 0, 7, and 21.

(C) Capillary density was quantitated in the adductor muscles of the ischemic limbs of mice transplanted with CD90+ cells, and was significantly greater in mice that received healthy aorta versus diseased aorta CD90+ cells at day 21. Capillary density is expressed as number of CD31+ cells per skeletal myocyte (identified by laminin staining). At least five micrographs were quantified per mouse, n = 3 mice per experimental group.

For all panels data shown are mean ± SEM, *p < 0.05, **p < 0.005, ***p < 0.0001, and ns, not significant. Scale bars represent 100 μm.

Gene Signature of CD90+ Cells from the Healthy Aorta, Diseased Aorta, and ITA Suggests an Important Role in Vascular Disease Pathogenesis

To gain insights on the role of CD90+ adventitial MSCs in adult human vessels, we performed RNA sequencing (RNA-seq) on freshly isolated (non-cultured) CD90+ cells obtained from the healthy aorta, diseased aorta, and ITA. Using principal component analysis we initially identified a clear separation of these three populations (Figure 6A). Differential gene expression analysis showed that, compared with CD90+ cells from the healthy aorta, those isolated from diseased aorta had significantly altered expression of 993 genes (Table S3). The most statistically significant genes upregulated in the healthy aorta were *MMP19, PTX3, GOS2, LIF, CXCL5, CCL20,* and *THAP2,* while in the diseased aorta it was *RP11-227F19.5* (an uncharacterized gene), *CPAMD8*, *MPPED2*, *KCNJ3*, and *RPL7P15* (Table S3; Figures 6B and 6C). To validate key findings among these data, we stained for MMP19 and CXCL5 proteins in our aortic samples. We selected MMP19 and CXCL5 because they stood out as being two of the most differentially expressed genes between the CD90+ cells of the healthy and the diseased aorta (Table S3; Figures 6B and 6C). Consistent with our RNA-seq data, we found that healthy aorta adventitial CD90+ cells strongly co-expressed both MMP19 and CXCL5, while there was an almost complete absence of expression of these proteins by CD90+ cells in the diseased aorta (Figure 6D). Furthermore, these 993 differentially expressed genes for diseased versus healthy aorta CD90+ MSCs were enriched for multiple gene ontology (GO) terms involved in cellular interactions and trafficking, wound healing, stress response,

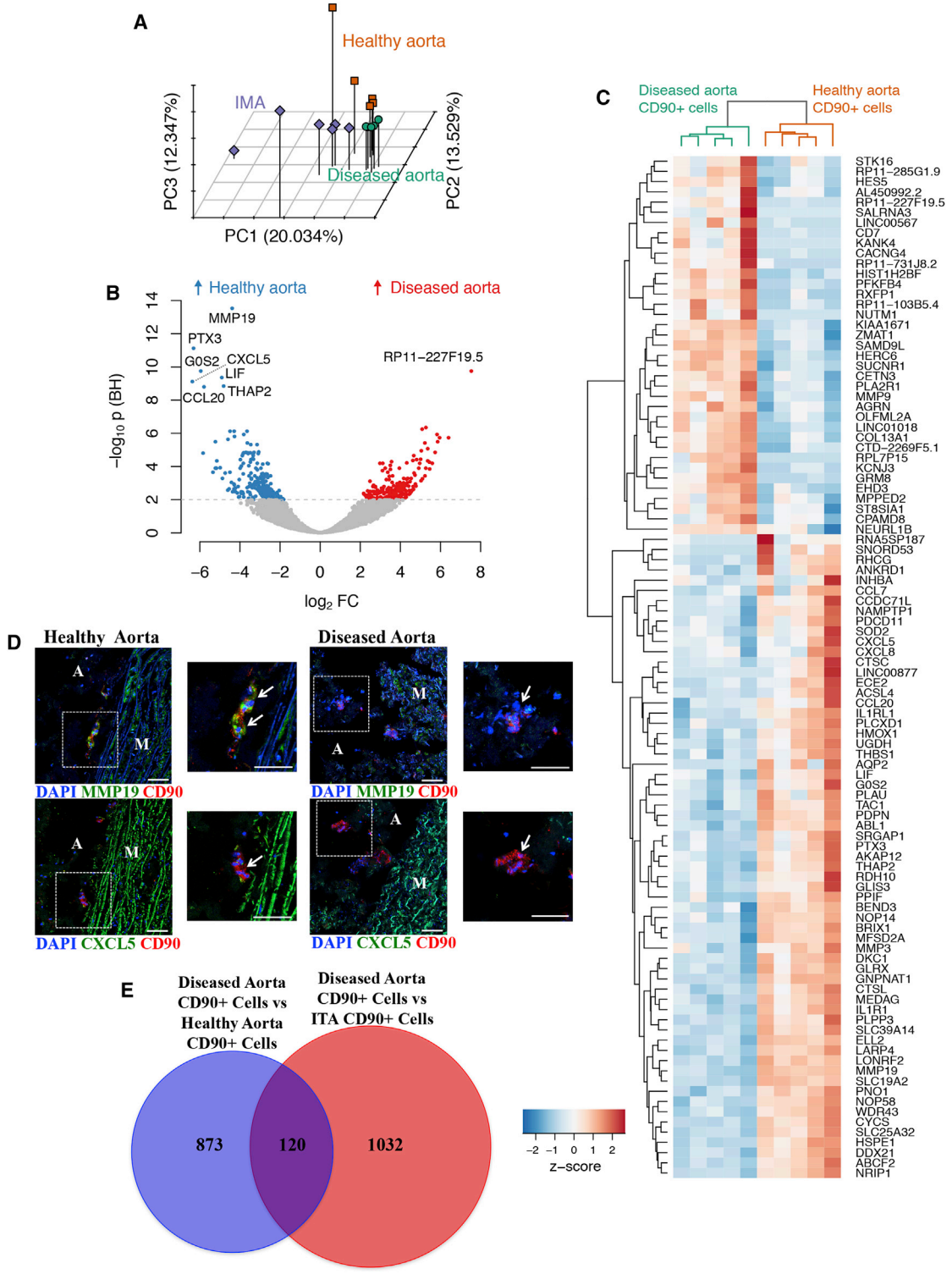

Figure 6. RNA-Seq Analysis of Freshly Obtained, Non-cultured Adventitial CD90+ Cells

(A) Principal-component analysis (PCA) of gene expression profiles of CD90+ cells from the three vascular tissue types (diseased aorta, healthy aorta, and ITA). Percentage of variance explained by each PC is shown in parentheses.

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inflammation, extracellular matrix, coagulation, and vessel formation (Table S4)—processes that are fundamental to the development and progression of atherosclerosis and vascular disease.

We also evaluated differential gene expression between diseased aorta CD90+ cells and ITA CD90+ cells, with 1,152 differentially expressed genes (Table S5), which by GO analysis were broadly related to terms associated with intracellular organization, cell functioning, gene/protein handling, and cell metabolism (Table S6).

As the ITA is known to be protected from developing atherosclerosis (Cameron et al., 1995; Ruengsakulrach et al., 1999; Otsuka et al., 2013), we sought to identify shared transcripts that were differentially expressed in CD90+ cells when obtained from the ITA versus diseased aorta, and the healthy versus diseased aorta (Figure 6E). Our rationale was that if CD90+ cells are involved in atherosclerosis development and progression, then it should be informative to compare CD90+ cells from vessels with no/minimal disease (healthy aorta or ITA) versus CD90+ cells from the diseased aorta. In strong support of the role of CD90+ adventitial MSCs in atherosclerosis, we identified an overlap of 120 common genes that were differentially expressed across both the ITA-diseased aorta and the healthy-diseased aorta comparisons (Figure 6E; Table S7). These 120 common transcripts represent approximately 10% of each set of differentially expressed genes for the two different comparisons (Figure 6E; Table S7). Supporting the biologic importance of this analysis, 115 of these 120 genes (95.8%) were differentially expressed in a concordant fashion in the separate ITA-diseased aorta, and the healthy-diseased aorta comparisons (Figure 6E). In other words, 115/120 transcripts were consistently either upregulated or downregulated across both these healthy-diseased vessel comparisons (Figure 6E), while only 5/120 showed discordant differential expression (Table S7).

Of relevance, among these 120 overlapping genes, some 21.7% (26/120) are already known to play a major role in atherosclerosis and vascular disease, including *ABCG2, ACTA2, CCL5, CX3CR1, MPO, PDGFB, PDGFD, PLAUR,* and *SAA1* (Table S8). Furthermore, the strength of this representation could be considered even stronger, as only approximately 80 of these 120 overlapping genes have been characterized, and therefore among the characterized genes in this overlapping CD90+ cell signature, ~32.5% (26/80) are known to play a role in atherosclerosis and vascular disease. Moreover, 2 of these 120 genes (1.7%), *PLAUR* and *SPHKAP*, are associated with single-nucleotide polymorphisms that have been shown to be related to the development of coronary artery disease (CAD) in genome-wide association studies (Nelson et al., 2017). We also studied the function of these 120 overlapping genes by evaluating their representation among known curated gene sets. Despite the relatively low number of genes, after Bonferroni correction for multiple testing we found significant enrichment of these 120 genes among the following terms: response to wounding (GO:0009611; $p = 0.01$), extracellular region (GO:0005576; $p = 0.01$), and signaling molecule (PC00207; PANTHER protein class; $p = 0.005$).

We further analyzed CD90+ adventitial MSC gene expression within the context of known atherosclerotic gene expression profiles and cross-tissue Bayesian regulatory gene networks (RGNs). For this purpose, we leveraged the recently curated STARNET (Stockholm-Tartu Atherosclerosis Reverse Networks Engineering Task) datasets (Franzén et al., 2016). Patients in the STARNET study had severe CAD requiring coronary artery bypass grafting, and from each patient at the time of surgery whole-blood and various tissue samples were obtained, including; ITA, diseased thoracic aorta, liver, skeletal muscle, whole blood, visceral fat, and subcutaneous fat. STARNET is among the largest genetics-of-gene-expression datasets in existence and currently comprises RNA-seq data from these tissues,

(B) Volcano plot of differentially expressed genes between CD90+ cells of diseased aorta versus healthy aorta. Statistically significant differential expression for each gene (by Benjamini-Hochberg [BH] corrected p value) is indicated by a blue or red dot. Gray dots represent genes not statistically different in expression.

(C) Heatmap and hierarchical clustering of differentially expressed genes between CD90+ cells of the diseased versus healthy aorta. Each column represents an individual sample from a different subject.

(D) To validate top differentially expressed genes from our RNA-seq data (B), IF staining for CD90 with MMP19 and CXCL5 in both donor and diseased aorta was performed. Consistent with our RNA-seq analysis, adventitial CD90+ cells of the healthy aorta exhibited robust expression of both MMP19 and CXCL5 (arrows). Conversely, diseased aorta adventitial CD90+ cells weakly expressed these proteins, if at all (arrows). Scale bars represent 100 μm. Inset panels on the right, representing an enlarged view of the area in the respective dashed squares, show adventitial cells at higher magnification. All images are representative, and consistent results were obtained from the staining of diseased and healthy aorta samples from at least two different subjects of each sample type.

(E) Venn diagram indicating the number of common differentially expressed genes when comparing the diseased versus healthy aorta and the diseased aorta versus ITA CD90+ cell populations. For all RNA-seq analyses there were five samples each from the diseased and healthy aorta, and six samples from the ITA, with each sample being from a different subject. All analyses were performed after adjusting for subject's age and gender.

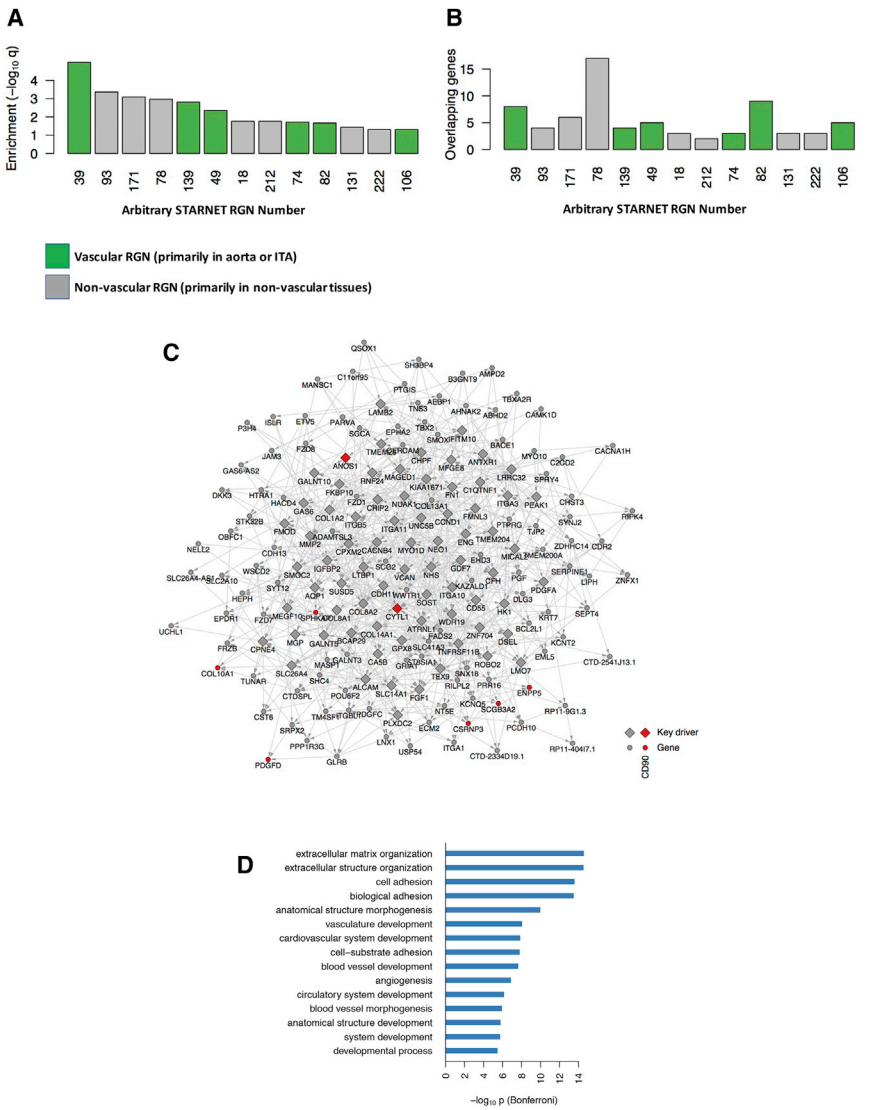

Figure 7. Differentially Expressed Genes from among CD90+ Cells from the Healthy Aorta, Diseased Aorta, and ITA Are Highly Represented in Atherosclerosis and CAD

(A) We looked for enrichment of our 120 CD90+ adventitial MSC genes of interest (Figure 6E) in the STARNET RGNs using a hypergeometric test without, at first, considering the tissue of the RGN. (A) After adjusting the resulting p values using Storey's *Q* values, there were 13 significantly enriched RGNs of which 6 were primarily from the aorta or ITA (green corresponds to STARNET RGNs where the primary tissue is either aorta or ITA). The most significant RGN (RGN 39, $Q = 0.00001$) is an aorta-only module.

(B) The number of genes from our list of 120 differentially expressed CD90+ MSC genes (Figure 6E) represented in each STARNET RGN. For (A) and (B), the x axis represents the arbitrary identification number given to each STARNET RGN.

(C) RGN 39 from the STARNET study. The top hierarchical key driver of this RGN is *CYTL1*. A diamond shape indicates key drivers of this RGN, while a red color indicates any of the 120 common genes that were differentially expressed across both the ITA-diseased aorta and the healthy-diseased aorta CD90+ MSC comparisons (Figure 6E; Table S7).

(D) The top 15 most highly enriched GO biological processes related to STARNET RGN 39.

plus DNA sequence data, for 672 subjects. Recently, these data were used to infer cross-tissue Bayesian RGNs. Such cross-tissue Bayesian RGNs have previously been shown to be causal for atherosclerosis and CAD (Hägg et al., 2009; Ochoa-Callejero et al., 2016; Talukdar et al., 2016).

We queried if our 120 CD90+ MSC genes of interest (Figure 6E; Table S7) are represented in the STARNET cross-tissue Bayesian RGNs, and identified 13 significantly enriched STARNET networks, of which 6 were primarily from the aorta or ITA (Figures 7A and 7B). Across all 13 of these STARNET RGNs, 47 of our 120 (39.2%) CD90+ MSC genes of interest were represented, with aorta and ITA RGNs containing 28 of the 120 genes (23.3%) (Figure 7B). Among these 13 STARNET RGNs, the most significant (RGN 39, $Q = 0.00001$) was an aorta-only network containing the following 8 genes from our 120 CD90+ MSC genes of interest: *SCGB3A2, ENPP5, PDGFD, CSRNP3, CYTL1, SPHKAP, ANOS1,* and *COL10A1* (Figure 7C). Relevant to atherosclerosis and vascular disease, STARNET RGN 39 consists of 182 aorta genes, and by GO analysis this RGN is involved in processes related to angiogenesis, vascular formation, the cardiovascular system, and extracellular matrix/structure organization (Figure 7D). Furthermore, an analysis to determine the key drivers of RGN 39 revealed that its top hierarchical key driver is *CYTL1*—which is among the list of 120 CD90+ adventitial MSC genes of interest (Figure 7C). Moreover, several genes directly downstream of *CYTL1* in RGN 39 have known connections to atherosclerosis or CAD, including *MMP2* (Liu et al., 2006), *FMOD* (*Fibromodulin*) (Shami et al., 2015), *CPNE4* (Lee et al., 2015), and *CD55* (Leung et al., 2009) (Figure 7C). An additional key driver of RGN 39, *ANOS1*, was also

among our 120 CD90+ adventitial MSC genes of interest (Figure 7C). An alternate representation of STARNET RGN 39 showing only its core aspects is provided as Figure S7.

Taken as a whole, these analyses provide evidence of a likely key role of adventitial CD90+ MSCs in atherosclerosis and vascular disease. In particular, analyses of the differential gene expression among CD90+ MSCs from the healthy aorta, diseased aorta, and ITA, are enriched with transcripts that play a critical role in atherosclerosis and CAD.

DISCUSSION

There is an increasing appreciation of the importance of adventitial MSCs in vascular biology and cardiovascular disease (Kovacic and Boehm, 2009; Michelis et al., 2014). While lineage tracking and other tools have made advances in defining these cells and their pathobiologic role in the mouse (Kramann et al., 2015, 2016), our knowledge of these cells *in situ* and *in vivo* in adult human vessels is limited. We undertook this study with the purpose of addressing this knowledge gap. Importantly, the majority of our experiments were performed on fresh cells harvested directly from human vessels. In contrast, prior adult human studies have generally relied on an assortment of heterogeneous *ex vivo* cell outgrowth or expansion techniques prior to cell identification and characterization, which limits the interpretability and biologic relevance of these studies.

The principal finding of this study is that CD90 marks, *in situ* and *in vivo*, a relatively rare population of adventitial cells in medium- and large-sized adult human arteries that fulfill the broadly acknowledged criteria for MSCs (Dominici et al., 2006). The MSC-like features of adult human adventitial CD90+ cells that we verified included typical MSC surface maker expression (CD90, CD73, CD105, CD44, and PDGFRα), plastic adherence, spindle-shaped morphology in culture, colony formation, passage ability, and differentiation into adipocytes, osteoblasts, and chondrocytes. Using IF staining and flow cytometry, we also showed that CD90+ adventitial MSCs are distinct from medial SMCs and endothelial cells. By both functional and genomic profiling, we found evidence to suggest that CD90+ adventitial MSCs may play an important role in atherosclerosis and vascular disease pathogenesis. Our findings are corroborated by prior studies that also found CD90 is expressed by adventitial MSCs in medium- to large-sized adult human arteries, although, in these studies greater reliance was placed on *in vitro* experiments and CD90 was only one of a panel of markers used to identify adventitial MSCs (Pasquinelli et al., 2007; Klein et al., 2011). Furthermore and unlike our study, detailed characterization using RNA-seq or other high-throughput technique was not performed (Pasquinelli et al., 2007; Klein et al., 2011).

As additional important findings to arise from our study, in contrast to other investigators (Klein et al., 2011, 2013), we found that CD44 was not a specific marker for adventitial MSCs, with strong expression of CD44 by medial SMCs. We were also able to corroborate prior work showing widespread PDGFRα expression by SMCs in the media of adult human vessels (Chong et al., 2013b). This finding is of translational importance, because while PDGFRα has emerged as a cardiovascular stem cell marker in the mouse (Chong et al., 2013a; Noseda et al., 2015), our data indicate that it is non-specific as a cellular marker in the human vasculature. Furthermore, our study corroborates recent studies regarding the expression pattern of CD34 in medium- and large-sized arteries and as a marker of MSC-like cells. While in the 2006 position paper that outlined minimal defining criteria it was stated that MSCs do not express CD34 (Dominici et al., 2006), our data and also numerous other studies indicate that this may not hold true in the vasculature, where MSC-like cells may express CD34 (Figure S1) (Campagnolo et al., 2010; Zimmerlin et al., 2010; Corselli et al., 2012). In addition, we were able to extend the findings of Billaud et al. (2017), most notably by refining our understanding of the surface marker expression of adventitial MSCs throughout the entire adventitial layer, and linking these cells to atherosclerosis by transcriptional profiling.

The fact that we identified a specific surface marker for *in situ* adult human adventitial MSCs enabled us to perform transcriptional studies on these cells immediately after live cell isolation. In this way, we avoided genetic and epigenetic changes that occur with cell passaging and varying culture conditions. In contrast, prior studies performed epigenetic and genetic assays on cultured cells that were passaged multiple times (Gubernator et al., 2015). For example, Klein et al. (2013) suggested that the differentiation of CD44+ cells into vascular SMCs was regulated by the methylation status of calponin and TAGLN, which is controlled by HOX expression. However, these conclusions were made using cells that had been cultured over many passages, and the relevance of these findings to *in vivo* vascular biology is unclear.

By profiling freshly obtained CD90+ vascular MSCs, which did not undergo *ex vivo* or *in vitro* culture, we established that there is distinct clustering and differential gene expression between CD90+ adventitial MSCs from the diseased aorta, healthy aorta, and ITA. GO terms and pathways that were identified based on the differential gene expression between the diseased and healthy aorta CD90+ cells, and the diseased aorta and ITA CD90+ cells, were enriched for pathways that are of central relevance in vascular disease; such as cellular interactions and

trafficking, wound healing, stress response, inflammation, extracellular matrix, coagulation, and vessel formation. Furthermore, we identified 120 common genes that showed differential expression when we compared CD90+ cells from vessels with no/minimal disease (healthy aorta or ITA) versus diseased aorta CD90+ cells (Figure 6E). Remarkably, some 21.7% of these genes (26 out of 120; Table S8) are already implicated in atherosclerosis and vascular disease, with several of these already known to play a critical role in disease pathogenesis (Table S8). Important processes governed by these 26 key genes include vascular permeability, inflammation, thrombosis, cell proliferation, cell motility, oxidation, atherogenesis, plaque stability, and angiogenesis (see references in Table S8). As further evidence suggestive of an important role in vascular disease, this genetic CD90+ adventitial MSC profile was highly represented in key RGNs in patients with severe CAD in the STARNET study (Franzén et al., 2016). Notably, we found a particularly strong representation of our 120 diseased-healthy CD90+ adventitial MSC signature genes in STARNET RGN 39 (Figure 7C), with this RGN playing a likely major role in disease-relevant vascular pathobiologic processes (Figure 7D). Furthermore, this representation in RGN 39 of our diseased-healthy CD90+ vascular MSC signature genes included the hierarchical top key driver, *CYTL1*. We interpret these multiple lines of evidence to suggest that CD90+ adventitial MSCs may play a key role in vascular disease pathogenesis. Nevertheless, at the current time this RNA-seq analysis remains hypothesis-generating.

As a point worthy of mention, in our study diseased aorta CD90+ cells were obtained from patients undergoing surgery for TAA. TAA is a disease process that is characterized by degradation of SMCs and elastic fibers that has atherosclerosis-like features (Isselbacher, 2005). Due to the nature of the surgeries typically performed in cardiovascular disease patients, it was not possible to obtain sufficient samples of diseased aorta with "pure" atherosclerosis. While small pieces of aorta may be obtained from the sites of graft anastomoses in patients having coronary artery bypass graft surgery (Franzén et al., 2016), these samples were too small for our purposes. Nevertheless, our use of diseased aorta samples from TAA rather than pure atherosclerotic disease patients had no influence on our major finding of the existence of CD90+ adventitial cells and their characterization as a population of tissue-resident MSCs. While it is conceivable that the gene expression signature of adventitial MSCs from TAA versus pure atherosclerotic aorta may differ, our use of CD90+ adventitial MSCs from TAA patients served to clearly highlight the vascular disease-relevant genetic perturbation of these cells. Indeed, this human adventitial CD90+ MSC disease signature was broadly consistent with a recent study exploring the characteristics of Sca-1+ adventitial progenitor cells in an atherosclerotic mouse model (Kokkinopoulos et al., 2017).

Limitations

We acknowledge several limitations of our study. Although we show that purified adventitial CD90+ cells exhibit differentiation capacity into adipocytes, chondrocytes, and osteoblasts, we did not demonstrate bone nodule formation after osteogenic induction. In addition, our *in vivo* murine data are derived from experiments performed in SCID mice, which lack the immunologic response that is known to be important in responding to ischemia and vascular injury. Furthermore, while our CD90+ adventitial MSCs fulfilled the standard criteria for an MSC population and exhibited differentiation capacity into osteoblasts, chondrocytes, and adipocytes *in vitro*, the ability of adventitial CD90+ cells to differentiate into these cell types *in vivo* was not explored. With respect to transcriptional profiling of CD90+ cells, while we processed freshly obtained and isolated cells without any cell culture steps, it remains possible that minor changes in the gene expression profile may have arisen during the human tissue digestion and cell isolation. Nevertheless, tissue digestion and cell isolation were performed uniformly across all CD90+ cell populations (ITA, healthy, and diseased aorta), and therefore any possible changes in gene expression during cell isolation will be consistent across our analyses. Another limitation is the relatively small sample size, which was a consequence of studying surgical samples from patients. Indeed, it would have been ideal to include a broader range of surgical specimens, including samples from children as performed by Shroff et al. (2008, 2013).

Conclusion

Our study systematically identified and validated, *in situ* and *in vivo*, a population of adventitial CD90+ MSCs in the human aorta and ITA that fulfill the accepted criteria for MSCs. These cells are able to modulate angiogenesis via paracrine mechanisms, and, compared with CD90+ cells from the diseased aorta, CD90+ cells from the healthy aorta have enhanced angiogenic potential. Moreover, across several analyses we identified that CD90+ adventitial MSCs exhibit a gene expression signature that is suggestive of a role in the development and progression of vascular disease. Our identification of this CD90+ resident adventitial adult human MSC population, and the potential role of these cells in vascular disease pathogenesis, opens the door for the future exploration of the specific targeting of these cells to modulate vascular disease and its various clinical sequelae including aneurysm, heart attack, and stroke.

EXPERIMENTAL PROCEDURES

Collection of Human Vascular Tissue

The study was approved by the institutional review board (IRB) of the Icahn School of Medicine at Mount Sinai. Three separate vascular tissues were collected: (1) surplus ITA, which is the vessel typically used for coronary artery bypass grafting of the left anterior descending coronary artery; (2) surplus diseased ascending aorta from patients with atherosclerosis and ascending aortic aneurysm (diseased aorta); and (3) surplus healthy ascending aorta from deceased cardiac transplant donors at the time of cardiac implantation (healthy aorta). Samples of surplus ITA and diseased aorta were obtained with written and signed patient consent prior to surgery. While written and signed consent was obviously not possible for cardiac transplant donors, the collection and use of the surplus healthy aorta samples was covered under our IRB-approved protocol.

Samples were placed in sterile PBS upon harvesting. Processing of all samples was initiated within 60 min of collection, whereupon samples were initially washed in PBS and contaminating fatty tissue was dissected away.

Additional Experimental Procedures

Full details of Experimental Procedures are described in the Supplemental Information, including processing of human tissues, IF staining, antibodies, cell culture conditions and reagents, angiogenesis assays, murine experiments, statistical methods, and RNA-seq analyses.

ACCESSION NUMBERS

RNA-seq data reported in this paper are deposited at GEO and are accessible under the accession number GEO: GSE106116.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and eight tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.06.001.

AUTHOR CONTRIBUTIONS

K.C.M. designed and carried out experiments, analyzed the data, and co-wrote the paper. J.C.K. led the project, designed and oversaw the experiments, analyzed the data, and co-wrote the paper. A.N.-K., L.L., Y.X., M.P.S, V.D., and J.T.L.L. assisted with experimental design and carrying out experiments. O.F. and S.K. performed all bioinformatics analyses with RNA-seq and STARNET data. V.F. and R.H. assisted with conceptual design and revising the manuscript. R.C.R., J.C., P.S., F.F., A.S., and A.A. assisted with the experiments (by providing the surgical specimens). J.L.M.B. oversaw the bioinformatics analyses with RNA-seq and STARNET data. A.N.-K., L.L., O.F., and S.K. contributed equally.

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