Differentiated Smooth Muscle Cells Generate a Subpopulation of Resident Vascular Progenitor Cells in the Adventitia Regulated by Klf4

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Rationale: The vascular adventitia is a complex layer of the vessel wall consisting of vasa vasorum microvessels, nerves, fibroblasts, immune cells, and resident progenitor cells. Adventitial progenitors express the stem cell markers, Sca1 and CD34 (adventitial sca1-positive progenitor cells [AdvSca1]), have the potential to differentiate in vitro into multiple lineages, and potentially contribute to intimal lesions in vivo.

Objective: Although emerging data support the existence of AdvSca1 cells, the goal of this study was to determine their origin, degree of multipotency and heterogeneity, and contribution to vessel remodeling.

Methods and Results: Using 2 in vivo fate-mapping approaches combined with a smooth muscle cell (SMC) epigenetic lineage mark, we report that a subpopulation of AdvSca1 cells is generated in situ from differentiated SMCs. Our data establish that the vascular adventitia contains phenotypically distinct subpopulations of progenitor cells expressing SMC, myeloid, and hematopoietic progenitor-like properties and that differentiated SMCs are a source to varying degrees of each subpopulation. SMC-derived AdvSca1 cells exhibit a multipotent phenotype capable of differentiating in vivo into mature SMCs, resident macrophages, and endothelial-like cells. After vascular injury, SMC-derived AdvSca1 cells expand in number and are major contributors to adventitial remodeling. Induction of the transcription factor Klf4 in differentiated SMCs is essential for SMC reprogramming in vivo, whereas in vitro approaches demonstrate that Klf4 is essential for the maintenance of the AdvSca1 progenitor phenotype.

Conclusions: We propose that generation of resident vascular progenitor cells from differentiated SMCs is a normal physiological process that contributes to the vascular stem cell pool and plays important roles in arterial homeostasis and disease. (Circ Res. 2017;120:296-311. DOI: 10.1161/CIRCRESAHA.116.309322.)

Key Words: adventitia ■ smooth muscle cells ■ vascular remodeling

The static arrangement of concentric layers of intima, media, and adventitia found in most blood vessels fails to capture the dynamics of cell movement between the individual layers. Most studies that address this question have focused on migration of smooth muscle cells (SMCs) into the intimal layer, where cells from the circulation and from the vessel wall interact during progression of atherosclerotic plaques. Little, if any, attention has been paid to the possibility that SMCs may also move in the opposite direction, into the adventitia. In part, this is because of the prevailing notion that the adventitia is an inert bystander tissue whose function is to provide structural support for the artery wall in the form of collagen-rich matrix produced by adventitial fibroblasts. However, this notion is challenged by recent studies showing that the adventitia actually contains a highly dynamic population of leukocytes, microvessels, adipocytes, and resident progenitor cells that collectively both maintain the artery wall and respond robustly to many kinds of vascular injury. In some cases, the adventitia is even the main site of pathological change such as in the formation of tertiary lymphoid organs in diseased
Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>Acta2(α-SMA)</td>
<td>smooth muscle-α-actin</td>
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<tr>
<td>AdvSca1</td>
<td>adventitial sca1-positive progenitor cells</td>
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<td>AdvSca1-MA</td>
<td>non-SMC-derived AdvSca1(+) cells</td>
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<td>AdvSca1-SM</td>
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<td>Cre</td>
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<td>Klf4</td>
<td>kruppel-like factor 4</td>
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<td>Mhy11/SMMHC</td>
<td>smooth muscle myosin heavy chain</td>
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<td>Sca1</td>
<td>stem cell antigen-1</td>
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<td>SMCs</td>
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<td>SRF</td>
<td>serum response factor</td>
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arteries, fibrotic changes that reduce compliance and promote renal damage in hypertension, and as a focus for inflammatory cell accumulation in development of medial dissections and aneurysms. In smaller vessels, the adventitia and associated perivascular cells are important signaling niches for resident progenitor cells in skeletal muscle, kidney, and bone marrow hematopoietic stem cells. The perivascular niche is also a residence site for dormant cancer cells with metastatic potential.

Thus, factors governing the cell composition of the adventitia are important to identify for the health and maintenance of blood vessels and the tissues and stem cell populations they support.

In recent years, reports demonstrating the existence of resident cardiovascular progenitor cells have had profound effects on our understanding of cardiovascular biology and tissue regeneration. Emerging data suggest that several distinct progenitor populations with the capacity to differentiate into endothelial cells, SMCs, fibroblasts, and macrophages reside in a specialized niche in the adventitia at the media–adventitia border. Hu et al described a population of vascular progenitor cells in the aortic root adventitia of ApoE−/− mice that express the progenitor markers Sca1 and CD34 and differentiate to vascular SMCs in vitro (adventitial sca1-positive progenitor cell [AdvSca1] progenitors). Our group showed similar cell clusters in an adventitial domain of sonic hedgehog signaling and, although SMC marker negative, were found to express transcription factors known to activate SMC markers (eg, serum response factor [SRF] and myocardin). They also express high levels of SRF corepressors (eg, Klf4), suggesting that AdvSca1(+) progenitors are specified for the SMC fate, but transcriptional repression maintains their progenitor phenotype. AdvSca1 progenitors were shown to have the potential to self-renew or to differentiate in vitro into SMCs, endothelial cells, osteoblasts, chondrocytes, or adipocytes. These cells are not unique to murine vessels as adventitial-derived CD34(+) progenitor cells have also been isolated from human vessels. In addition, an intriguing finding was the existence of local, resident AdvSca1 myeloid progenitors with hematopoietic potential that reside in a similar adventitial niche. The presence of resident vascular progenitor cells has significant implications for their potential therapeutic use in the treatment of vascular diseases and regenerative medicine. Although accumulating evidence supports their existence, many important questions remain unanswered. Several groups demonstrated that these cells do not originate from bone marrow, and our previous findings demonstrated that adventitial progenitors do not arise from cardiac neural crest. Therefore, the origin of AdvSca1 progenitor cells remains unclear. In addition, the degree of heterogeneity of AdvSca1...
progenitors and the mechanism underlying the maintenance of the AdvSca1 progenitor cell phenotype are also unclear.

Vascular SMCs are specialized cells that express high levels of SMC-specific proteins, such as smooth muscle myosin heavy chain (Myh11/SMHC) and smooth muscle-α-actin (Acta2/α-SMC). Under pathological conditions, such as atherosclerosis and restenosis, however, SMCs are capable of undergoing phenotypic and functional changes resulting in a proliferative, inflammatory phenotype, characterized by decreased expression of SMC contractile proteins and increased production of proinflammatory cytokines. As a result, mature SMCs are major contributors to pathological neointima formation. Our previous report using a highly specific SMC fate-mapping approach in the setting of restenosis demonstrated that the majority of proliferating intimal cells derive from mature SMCs. Remarkably, we also consistently detected reporter-positive, but SMC marker-negative, SMC-derived cells in the arterial adventitia, suggesting that mature SMCs contribute to both intimal and adventitial remodeling. In addition, recent reports demonstrated that a high percentage of SMCs in atherosclerotic lesions lack detectable expression of conventional SMC markers but exhibit a macrophage-like phenotype. These findings suggest that SMCs exhibit an even greater degree of plasticity than previously recognized. In light of our findings that SMCs contribute to adventitial remodeling and because the origin of AdvSca1 progenitor cells remains unknown, we sought to determine whether mature SMCs contribute to the vascular progenitor pool. Using fate-mapping and lineage-tracing approaches, in this report, we demonstrate that a distinct subpopulation of AdvSca1 progenitors derive from differentiated SMCs that undergo a reprogramming-like process in situ to generate multipotent progenitor cells. In addition, we show here that the pluripotency-associated transcription factor, Klf4, regulates the generation of SMC-derived AdvSca1 cells and is essential for the maintenance of the AdvSca1 progenitor cell phenotype.

Methods

Mice

Myh11-CreER² transgenic mice and Rosa26-LaCZ or Rosa26-YFP reporter mice were bred to generate tamoxifen-inducible SMC-specific β-galactosidase– or yellow fluorescent protein (YFP)–expressing mice (Myh11-creERT2-β-gal/YFP). To activate Cre (Cre recombinase), adult male mice received 1 mg IP tamoxifen injections for 5 consecutive days 8 weeks before they were euthanized. Timed pregnant female mice (Rosa26-YFP female mice bred to male Myh11-creERT2-Rosa26-YFP mice) received 1 mg IP tamoxifen injections at e15 and e16 to label developing embryos. SM22α-Cre transgenic mice were bred to Rosa26-YFP to generate SMC-specific YFP-expressing mice (SM22α-Cre-YFP). For in vivo Matrigel plug assays, SMC-derived AdvSca1 cells were isolated as described below, resuspended in 700 μL Matrigel plus 100 ng/mL VEGF (vascular endothelial growth factor) and 100 ng/mL FGF2 (fibroblast growth factor-2), and injected subcutaneously into wild-type (WT) C57BL/6J recipients. Plugs were harvested 14 days postinjection, fixed in 4% buffered paraformaldehyde, and embedded in optimal cutting temperature compound for immunofluorescent staining. To induce vascular injury, mice were subjected to wire-induced femoral artery injury or carotid artery ligation injury, as previously demonstrated. Four weeks after femoral artery injury or 3 days after carotid artery ligation injury, uninjured and injured arteries were harvested, perfused fixed in 4% buffered paraformaldehyde, and embedded in optimal cutting temperature compound for immunofluorescent staining. SM22α-CreKI transgenic mice (TaglnCreKI; JAX stock 006878) were bred to Klf4 floxed mice and Rosa26-YFP mice to generate SMC-specific YFP-expressing Klf4-deficient mice (SM22α-CreKI-YFP knockout). This alternative SMC Cre driver, in which Cre recombinase is activated late in development compared with the traditional SM22α-Cre mouse line (Tagln-Cre; JAX stock 004746), was used to generate SMC-specific Klf4 knockout mice as use of Tagln-Cre embryos. SMMHCα-Cre transgenic embryos. Mice were maintained in the Center for Comparative Medicine, and procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver.

Cell Analysis

Isolated aortic arch plus left and right carotid arteries, descending aorta, and left and right femoral arteries were digested to single cells by digestion at 37 °C for 1 hour in collagenase buffer. For flow sorting, single cell suspensions were incubated with a rat anti–mouse monoclonal allophycocyanin (APC)-Sca1 antibody (eBiosciences); live cells were sorted on a MoFlo high-speed cell sorter based on Sca1-APC and endogenous YFP expression. For fluorescence-activated cell sorting analysis, single cell suspensions were stained with various combinations of rat anti–mouse monoclonal antibodies (PerCP-Cy5.5–Sca1, PE-Cy7-CD45, APC/PE/Cy7-CD115, APC-CD140a, PE-CD140b, and PE-CD31; all antibodies were from eBiosciences). Flow cytometry was performed on a Galios cytometer (Becton Dickenson). Data were analyzed using Kaluza software (Beckman Coulter). To quantify AdvSca1 populations in WT versus SMC-specific Klf4 knockout mice, vessels from 8- to 10-day-old mice were individually digested to single cell suspensions, labeled with anti–APC-Sca1 antibodies, and analyzed for endogenous YFP and Sca1-APC expression. To analyze the effect of Klf4 on maintenance of the AdvSca1 progenitor cell phenotype, AdvSca1 cells were sorted based on Sca1 expression, transfected with nontargeting or Klf4-targeting siGENOME SMARTpool siRNAs (100 nm; Thermof-Scientific), or transduced with an empty vector adenovirus or an adenovirus expressing Klf4 (100 multiplicity of infection; Vector Biolabs, Malvern, PA).

Immunofluorescence, Chromatin Immunoprecipitation, and Quantitative Real-Time Polymerase Chain Reaction

Optimal cutting temperature compound–embedded tissues or fixed cells were permeabilized with MeOH followed by 0.05% Tween-20 in PBS, blocked in 3% horse serum, and sequentially incubated with specific primary and secondary antibodies. Antibodies used included monoclonal rat anti–mouse Sca1 (1:100; BD Pharmingen), monoclonal rat anti–mouse CD45 (1:50; Abcam), Pan-α-SMC (1:200; Abcam), Cy3-conjugated polyclonal monoclonal anti–α-SMA (1:2000; Sigma), monoclonal rat anti–mouse CD45 (1:100; BD Pharmingen), polyclonal rabbit anti-Klf4 (1:100; Abcam), monoclonal rat anti–mouse F4/80 (1:50; Abcam), polyclonal rabbit anti–α-SMA (1:200; Abcam), and polyclonal rabbit anti-SMMHC (1:100; Biomedical Technologies, Inc). Sections/cells were imaged using a laser-scanning confocal microscope (LSM 780 spectral; Carl Zeiss, Thornwood, NY). Tissues were stained for β-galactosidase activity using a kit from GTT, Inc, according to the protocols provided. To quantify cell populations in uninjured compared with injured carotid arteries, total numbers of Sca1 or CD45(−) YFP(−), Sca1 or CD45(+) YFP(−), and Sca1 or CD45(+) YFP(+) adventitial cells were counted in 3 high-powered fields per vessel from n=3 uninjured and injured vessels by 2 independent investigators. Chromatin immunoprecipitation (ChiP) was performed on flow-isolated cell populations as previously described. Antibodies for ChiP include anti-H3K4Me2, anti-H4-Ac (Millipore), and anti-SRF (Santa Cruz Biotechnologies). DNA was purified with QIAquick PCR purification kits (Qiagen). Real-time polymerase chain reaction with Power SYBR Green Mastermix (Life Technologies) and primers designed to flanking CARG elements in the Acta2 and Myh11 promoters were used to analyze ChiP (primer sequences are available in the Online Data Supplement). Data from a minimum of 3 independent experiments were normalized to YFP+.
SMCs. Quantitative real-time polymerase chain reaction was used with total RNA isolated from flow-isolated cell populations as previously described.33 Primer sequences are available in the Online Data Supplement. β-Actin was used for normalization. To compare among individual experiments, data were normalized to YFP+ SMCs for SMC genes (αSMA, SMMHC, SM22α, and myocardin) or AdvSca1-MA cells for progenitor cell genes (Klf4, CD34, VEGF, SDF-1α, CD31, Flk1, and Flt1).

Results
Genetic Fate Mapping Reveals Adventitial SMC-Derived Progenitor Cells

We previously used tamoxifen-inducible Myh11CreERT2 transgenic mice crossed with floxed-stop Rosa reporter mice (Myh11-CreER-βGal/YFP) to fate map mature SMCs in response to vascular injury.33 Tamoxifen given to adult mice before vascular injury genetically, permanently, and efficiently marked only Myh11/SMMHC-expressing SMCs through Cre-mediated reporter knock-in. Because tamoxifen was given before injury and then stopped, SMCs expressing Myh11/SMMHC and their progeny were the only reporter-positive cells throughout the experimental period, allowing tracking of mature SMCs in response to vascular injury even if levels of Myh11/SMMHC (or other SMC markers) were no longer detectable; any non-SMC that potentially gained expression of Myh11/SMMHC after injury would not be labeled as tamoxifen was not in the system to activate Cre. Using this approach, we found that the majority of intimal Acta2/αSMA-expressing cells originate from mature SMCs33 (Online Figure I), establishing that neointimal SMCs derive from mature differentiated SMCs. However, similar to a recent report in the setting of atherosclerosis,33 we also detected populations of YFP(+) SMC-derived intimal cells that expressed no detectable levels of SM markers (asterisks in Online Figure I), suggesting that the contribution of SMCs to intimal hyperplasia is underestimated if solely using expression of conventional SMC marker proteins to identify intimal SMCs. Mice not treated with tamoxifen exhibited no genetic labeling of SMCs or adventitial cells (Online Figure II). In addition to intimal SMCs, we made the entirely unanticipated observation that over time mature SMCs migrate to the adventitia and reside in a niche at the adventitia–media border33 (Figure 1A). Adventitial SMC-derived cells have lost SMC marker expression (Figure...
1B and 1F, Acta2αSMA shown) but gained expression of the stem cell markers, Sca1 and CD34 (Figure 1C through 1E and 1G), suggesting that differentiated SMCs generate AdvSca1 cells to contribute to the resident vascular progenitor cell pool.

Our previous report demonstrated that AdvSca1 progenitors appear in the adventitia of the ascending aorta and pulmonary trunk by e18.5, a developmental time point after the arterial media has acquired its complement of SMCs and has organized structured elastic lamellae. Based on this, we hypothesized that SMC-derived AdvSca1 progenitors are established during the late embryonic-to-earlyphal period. To test this hypothesis, tamoxifen was administered to timed-pregnant female mice at e15 and e16 to obtain restricted labeling of Myh11-expressing SMCs in embryos between e15 and e17, when all vascular beds exhibit strong Myh11/SMMHC expression and before the appearance of AdvSca1 cells. Pups were allowed to develop to 30 days postpartum, then carotid arteries plus aortic arch, descending aortas, and femoral arteries were harvested, digested into single cells, immunolabeled for Sca1, and flow sorted based on Sca1 and YFP expression (gating strategy shown in Online Figure III). Three distinct cell populations were retrieved from each vessel: (1) Sca1(−)YFP(+) mature SMCs; (2) Sca1(+)YFP(−) non–SMC-derived AdvSca1(+) cells (hereafter referred to as AdvSca1-MA); and (3) Sca1(+)YFP(+) SMC-derived AdvSca1(+) cells (hereafter referred to as AdvSca1-SM; Online Figure IVA and IVB).

Using this approach, we found that ≈8% of the total Sca1(+) cell population were YFP-positive SMC-derived cells (Online Figure IVB). In contrast to blood vessels, AdvSca1-SM cells were not detected in peripheral blood mononuclear cells (not shown). This is consistent with our previous finding of a lack of labeled cells in bone marrow33 and a previous report using a comparable SMC-specific labeling approach in which labeled cells were not detected in bone marrow cells or the circulation. Others have used a similar in utero tamoxifen pulse to fate-map yolk sac–derived myeloid progenitors and hemogenic endothelial cells, albeit tamoxifen was administered at much earlier developmental time points (eg, e8.5). In contrast to these studies, on further examination in our system, although 5-day tamoxifen treatment of adult mice resulted in highly efficient labeling of medial SMCs,33 we were unable to achieve similar labeling delivering tamoxifen in utero at these later time points (2 injections failed to efficiently label outer medial SMCs (Online Figure IV) and 2 in utero injections consistently resulted in premature termination of pregnancy), thus precluding the ability to accurately quantify the percentage of AdvSca1 progenitors that originate from SMCs because of lack of genetic labeling. Nonetheless, using a highly

Figure 2. Percentage of total adventitial sca1-positive progenitor cells (AdvSca1) originating from differentiated smooth muscle cell (SMCs). A, Carotid arteries (CA) plus aortic (Ao) arch, descending aorta, and femoral arteries were harvested from 2-mo-old SM22αCre-YFP mice. Arteries from 5 to 7 mice were pooled, digested into single cell suspensions, labeled with an APC (allophycocyanin)-conjugated anti-Sca1 antibody, and flow sorted based on endogenous YFP and Sca1 expression. The percent±SE SMC-derived YFP(+) Sca1(+) (quadrant B; Online Figure V) of total Sca1(+) cells (quadrants A+B; Online Figure V) from 7 independent sorts is shown in the table. B, Representative immunofluorescent stains of carotid artery sections from 2-mo-old SM22αCre-YFP mice for αSMA (red) and YFP (green; top), Sca1 (red) and YFP (green; middle), or CD34 (red) and YFP (green; bottom). Arrows indicate YFP(+) adventitial cells. Dashed lines represent media-adventitia boundary. A minimum of 8 mice were analyzed. Bars=50 µm. C, Higher magnification image of inset box in (B, middle). D, Representative immunofluorescent stain of a carotid artery section from a 5-d-old SM22αCre-YFP mouse for YFP (green), Sca1 (red), and αSMA (white). Arrows indicate SMC-derived AdvSca1+ cells expressing residual levels of αSMA; Arrowheads indicate SMC-derived adventitial cells expressing residual levels of αSMA and low/absent levels of Sca1. Dashed line represent media-adventitia boundary. Bars=50 µm.
selective fate-mapping approach, our data demonstrate that a population of resident AdvSca1 cells derives from mature SMCs.

To accurately quantify the percentage of AdvSca1 cells that originate from SMCs, constitutive, SMC-specific SM22α-Cre transgenic mice were bred to Rosa26-YFP mice (SM22α-Cre-YFP) to label differentiating SMCs at the time in vascular development that SM22α is induced. We previously showed that AdvSca1 cells do not express SM22α. Using this approach, we consistently found that a large percentage of total Sca1(+) cells in the carotid artery plus aortic arch, descending aorta, and femoral artery originate from SMCs (Figure 2A; Online Figure V). By immunofluorescent staining, αSMA(−), but YFP(+)Sca1(+)CD34(+), cells were detected in the adventitia of 30-day-old SM22α-Cre-YFP mice (Figure 2B and 2C). Consistent with in vivo staining, freshly isolated AdvSca1-SM cells from 30-day-old SM22α-Cre-YFP mice express high levels of progenitor cell markers, including Sca1, CD34, and the pluripotency-associated transcription factor, Klf4, but dim to undetectable levels of αSMA (Online Figure VI). To further strengthen that SMCs migrate into the adventitia and to provide evidence of the late developmental timing of SMC reprogramming, we analyzed arteries from 5-day-old SM22α-Cre-YFP mice and detected Sca1(+)YFP(+) adventitial cells still expressing residual SMC markers (Figure 2D). Specific chromatin modifications occur selectively in SMC gene loci of SM markers (eg, Acta2/αSMA and Myh11/SMMHC) and are associated with specification of the SMC lineage during development.40,41

Unlike most that are lost from SMC genes in response to

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**Figure 3. Progenitor cell marker expression in adventitial sca1-positive progenitor cells (AdvSca1).** Total RNA was isolated from cell populations from pooled, digested arteries from SM22α-Cre-YFP mice and analyzed by quantitative real-time polymerase chain reaction for the indicated mRNAs. Shown are fold changes in mRNA copy number±SE from an n=3 independent experiments using arteries from 10 pooled mice per experiment; *P<0.05. β-Actin was used for normalization. A, Smooth muscle cell (SMC) markers. To compare among individual experiments, data were normalized to YFP(+) SMCs. B, Progenitor cell markers expressed by both Sca1+ populations. To compare among individual experiments, data were normalized to AdvSca1-MA cells. C, mRNAs selectively expressed in Sca1(+)/YFP(-) cells. To compare among individual experiments, data were normalized to AdvSca1-MA cells. Shown for all panels are data obtained from cell populations isolated from pooled carotid arteries plus aortic arch. ND indicates not detectable.
SMC phenotypic switching (e.g., H4Ac), 1 chromatin mark in particular, H3K4 dimethylation (H3K4Me2), is retained and serves as a highly specific SMC lineage marker in both mouse and human tissues.\textsuperscript{40,42} As a secondary approach to demonstrate SMC origin of YFP(+)Sca1(+) AdvSca1 cells, ChIP was conducted for expression of the H3K4Me2 mark in the Myh11 and Acta2 loci of isolated YFP(+)Sca1(+) compared with YFP(−)Sca1(+) cells. Similar to SMC-rich intact aortic media (not shown), H3K4Me2 was detected on the Myh11/SMMHC and Acta2/αSMA promoters of isolated YFP(+) mature SMCs and YFP(+)/Sca1(+) AdvSca1-SM cells but not YFP(−)/Sca1(+) AdvSca1-MA cells (Online Figure VIIA).

In contrast, compared with mature YFP(+) SMCs, expression of the SMC differentiation–associated H4Ac chromatin mark\textsuperscript{40} was undetectable in YFP(+)Sca1(+) AdvSca1-SM cells (Online Figure VIIIB left; Myh11 shown). Binding of the transcription factor, SRF, to CArG boxes of SM gene promoters is essential for promoting the SMC differentiation program.\textsuperscript{40} Loss of the SMC differentiation H4Ac chromatin mark was associated with undetectable SRF binding to SM gene promoters in YFP(+)Sca1(+) AdvSca1-SM cells (Online Figure VIIIB right; Myh11 shown). Collectively, using 2 independent genetic fate-mapping systems combined with a SMC lineage histone mark, we established

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\caption{Fluorescence-activated cell sorting (FACS) profiling reveals distinct subpopulations of adventitial Sca1-positive progenitor cells (AdvSca1) cells. Single cell suspensions were obtained from digested arteries from SM22α-Cre-YFP mice. A, Representative flow cytometry plots showing expression of CD45 and Ly6C in YFP(−) and YFP(+) cells. B, Representative flow cytometry plots showing expression of Sca1 in CD45(−) and CD45(+) cells from YFP(−) and YFP(+) cell populations. C, Representative flow cytometry plots showing expression of Ly6C in Sca1(+)CD45(−) and Sca1(+)CD45(+) cells from YFP(−) and YFP(+) cell populations. D, Representative flow cytometry plots showing expression of Ly6C and CD115 in YFP(−) and YFP(+) cells. E, Representative flow cytometry plots for expression of CD140b and CD31 in Sca1(+)CD45(−)-Ly6C(−) and Sca1(+)CD45(−)-Ly6C(+) cells from YFP(−) and YFP(+) cell populations. F, Pie charts illustrating the various subpopulations of Sca1(+) progenitor cells within Sca1(+)YFP(−) and Sca1(+)YFP(+) groups. Shown are data obtained from cell populations isolated from carotid arteries plus aortic arch; n=4 independent analyses using pooled arteries from 5 mice.}
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that differentiated SMCs generate in situ a subpopulation of AdvSca1 progenitor cells.

**Differentiated SMCs Generate Phenotypically Distinct Subpopulations of AdvSca1 Cells**

We used quantitative real-time polymerase chain reaction to further characterize individual cell populations. Mature SMCs, AdvSca1-SM progenitors, and AdvSca1-MA progenitors were isolated by flow sorting as described above. Compared with mature YFP(+)Sca1(−) SMCs, expression of the SMC-specific mRNAs, Myh11/SMMHC, Acta2/αSMA, TagIn/SM22α, and Mycn/myocardin was undetectable in both AdvSca1-SM and AdvSca1-MA progenitors (Figure 3A). No difference in expression of SRF was detected among the cell populations (Figure 3A). However, combined with loss of SRF binding to SM gene promoters by ChIP analysis (Online Figure VII), these results suggest that the SMC differentiation program is repressed in AdvSca1-SM cells. In contrast to SM-specific genes, Klf4, the progenitor cell marker CD34, and progenitor cell-associated cytokines, VEGF and SDF-1α, were highly expressed in both populations of AdvSca1 cells compared with mature SMCs (Figure 3B). However, combined with loss of SRF binding to SM gene promoters by ChIP analysis (Online Figure VII), these results suggest that the SMC differentiation program is repressed in AdvSca1-SM cells. In contrast to SM-specific genes, Klf4, the progenitor cell marker CD34, and progenitor cell-associated cytokines, VEGF and SDF-1α, were highly expressed in both populations of AdvSca1 cells compared with mature SMCs (Figure 3B). Finally, CD31, Flk-1, and Flt-1, commonly associated with endothelial/monocyte progenitor cells, were selectively expressed by AdvSca1-MA, but not by AdvSca1-SM, progenitor cells (Figure 3C), suggesting that 2 distinct populations of AdvSca1 progenitor cells reside in the vessel wall.

We used flow cytometry to further immunophenotypically characterize these subpopulations. Two recent reports identified a population of nonhematopoietic-derived resident adventitial Sca1(+)/CD45(+) macrophage progenitor cells.5,26 Gated YFP(+) and YFP(−) cells (gating strategy shown in Online Figure VIII A) were analyzed for expression of CD45 and the common monocyte progenitor marker Ly6C to determine whether CD45(+) macrophage progenitors reside selectively in the YFP(−) population. Whereas a much higher percentage of CD45(+) cells was identified in the YFP(−) cell fraction, the YFP(+) cell fraction was found to contain a small percentage of CD45(+) cells (Figure 4A and 4F; Online Table I). CD45(−) and CD45(+) cells from YFP(−) and YFP(+) populations were analyzed for Sca1 expression. Distinct populations of Sca1(+)/CD45(−) and Sca1(+)CD45(+) were identified within both SMC-derived YFP(+) and non–SMC-derived YFP(−) cells (Figure 4B; Online Table I). Although the majority of Sca1(+)CD45(−) and Sca1(+)CD45(+) AdvSca1-MA progenitors also expressed Ly6C, only approximately half of Sca1(+)CD45(−) (54.6%) and Sca1(+)CD45(+) (56.1%) AdvSca1-SM progenitors expressed Ly6C (Figure 4C and 4F; Online Table I). Ly6C(+) cells within the YFP(+)Sca1(+) and YFP(−)Sca1(+) populations coexpressed CD115, also a common monocyte progenitor marker (Figure 4D, circled populations). Expression of the platelet-derived growth factor receptors PDGF-Rβ/CD140b and PDGF-Rα/CD140a has been implicated in regulating a variety of progenitor cells and, in particular, specification of a SMC fate. CD31 is a cell surface marker...
expressed by a variety of cells, including mature endothelial cells and endothelial and myeloid progenitor cells. Compared
with AdvSca1-MA cells, the vast majority of both CD45(−)
Ly6C(+) and CD45(−)Ly6C(−) subpopulations of AdvSca1-
SM cells expressed CD140b and CD140a (Figure 4E and 4F;
Online Table I; CD140b shown) but not CD31. In contrast,
CD45(−)Ly6C(+) and CD45(−)Ly6C(−) subpopulations of
AdvSca1-MA cells expressed either CD140b/CD140a or
CD31; expression seemed to be mutually exclusive (Figure 4E
and 4F; Online Table I; CD140b shown). All non-SMC Sca1(+)
CD45(+) cells were negative for CD140b, CD140a, and CD31
(Figure 4F; Online Table I), whereas SMC-derived Sca1(+)
CD45(+) cells either expressed Ly6C and CD115 or CD140b
and CD140a; none expressed CD31 (Figure 4F; Online Table
I). Finally, the remaining YFP(+)Sca1(−)CD45(−) population
(Online Figure VIIIB, circled population in left panel) did not
express the myeloid markers, Ly6C, CD115, or CD31 (Online
Figure VIIIB middle and right panels; CD115 not shown) and
only expressed low levels of CD140b and CD140a (Online
Figure VIIIB right 2 panels; Online Figure VIIIC), verifying
this population as mature SMCs.
To determine whether AdvSca1-SM cells possess multi-
potency potential capable of differentiating into other cell
lineages, in vivo Matrigel plug angiogenesis assays were
performed. AdvSca1-SM cells were recovered from SM22α-
Cre-YFP mice, resuspended in Matrigel plus growth factors,
subcutaneously injected into syngeneic WT mice, and plugs
examined 14 days postimplantation for SMC-, macrophage-, and
endothelial cell–specific markers (Myh11/SMMHC, F4/80, and von Willebrand factor, respectively). Analysis of
YFP(+) cells demonstrated that AdvSca1-SM cells contribute
to perivascular cells of functioning neovessels within Matrigel

Figure 5. Smooth muscle cell (SMC)–derived AdvSca1(+) cells (AdvSca1-SM) cells exhibit multipotency potential. AdvSca1-SM
cells were isolated from SM22α-Cre-YFP mice by flow sorting, embedded in Matrigel, and subcutaneously implanted into syngeneic
wild-type (WT) mice. Matrigel plugs were harvested 14 d post implantation for immunofluorescence analysis. A, Representative stains
of neovessels within plugs for yellow fluorescent protein (YFP; green) and the SMC-specific marker, smooth muscle myosin heavy chain
(SMMHC; red). B, Representative stains for YFP (green) and the macrophage-specific marker, F4/80 (red). C, Representative stain of
an endothelial cord for YFP (green) and the endothelial cell–specific marker, von Willebrand Factor (red). Arrows indicate AdvSca1-SM–
derived endothelial cells. Bars=50 μm. n=4 independent analyses.
plugs that connected to the systemic circulation (Online Figure IX). Many of these perivascular cells coexpressed SMMHC, a marker of differentiated SMCs (Figure 5A), demonstrating that AdvSca1-SM cells serve as SMC progenitor cells. In addition, we observed YFP(+)F4/80(+) cells largely in regions of the plug with recruited host-derived inflammatory cells, demonstrating that AdvSca1-SM cells differentiate to macrophages (Figure 5B). Moreover, although the majority of endothelial cells in neovessels were host derived (eg, YFP-; Online Figure XA), YFP(+)von Willebrand factor(+) endothelial-like cells were observed (Figure 5C; Online Figure XB), suggesting a role for AdvSca1-SM cells as endothelial cell precursors. Finally, undifferentiated YFP(+) cells still expressing Sca1 were observed surrounding newly developing vessels (Online Figure XC). In vitro differentiation assays demonstrated the ability of SMC-derived AdvSca1 cells to also differentiate into adipocytes and chondrocytes (Online Figure XI), thereby supporting the concept that AdvSca1-SM cells exhibit a multipotent progenitor cell phenotype. Collectively, these data suggest that differentiated SMCs can generate distinct subpopulations of multipotent progenitor cells that reside in the vascular adventitia.

**AdvSca1-SM Cells Expand in Number in Response to Vascular Injury**

Although the predominant focus of the vascular response to injury has been on neointima formation and vessel occlusion, previous reports have also demonstrated early activation and expansion of adventitial cells after injury. To determine whether AdvSca1-SM cells contribute to adventitial cell expansion early after vascular injury, carotid artery ligation injuries were performed on 2-month-old SM22α-Cre-YFP mice. Compared with uninjured contralateral controls (Online Figure XII A and XIIB), there was a large increase in the numbers of YFP(+)SMA- adventitial cells 3 days post injury (Figure 6A; Online Figure XIIC). Although this consisted

![Figure 6](http://circres.ahajournals.org/)

**Figure 6. Injury-induced expansion of Smooth muscle cell (SMC)-derived AdvSca1(+) cells (AdvSca1-SM) cells.** Two-mo-old SM22α-Cre-YFP mice were subjected to carotid artery ligation injury and injured left and uninjured right arteries were harvested 3 d post injury for immunofluorescence analysis. A, Representative stains of injured vessels for α-smooth muscle actin (αSMA; red) and yellow fluorescent protein (YFP; green) from 2 independent mice showing expansion of SMC-derived adventitial cells. B, Representative stain of an injured vessel for Sca1 (red) and YFP (green). C, Representative stain of an injured vessel for CD45 (red) and YFP (green). Arrows indicate YFP(+)CD45(+) adventitial cells. A indicates arterial adventitia; and M, arterial media. Arrowheads indicate internal (A) and external elastic laminae. D, Higher magnification images of arrows in panel (C). For A–C, dashed lines represent media–adventitia boundary. E and F, Total numbers of adventitial Sca1(−)YFP(+) , Sca1(+)YFP(+) , or Sca1(+)YFP(−) cells (E) or CD45(−)YFP(+) , CD45(+) YFP(+) , or CD45(+)YFP(−) cells (F) in uninjured compared with injured arteries were quantitated as described in the Methods section. Total numbers±SE are recorded in the graphs. n=3 independent mice; *P<0.05. Bars=50 µm.
of increases in both Sca1+YFP+ and Sca1-YFP+ adventitial cells, the majority of YFP+ adventitial cells coexpressed Sca1 (Figure 6B and 6E). Similarly, there were increased numbers of CD45+YFP+ and CD45-YFP+ adventitial cells in response to injury. Although rare in uninjured vessels, SMC-derived CD45+ adventitial cells increased in response to injury; the majority of CD45+ adventitial cells, however, were YFP–non–SMC-derived cells (Figure 6C, 6D, and 6F). These data are consistent with the concept that resident vascular progenitor cells, and in particular SMC-derived AdvSca1 cells, are activated early and are the dominant source of adventitial remodeling in response to vascular injury.

**SMC Generation of AdvSca1-SM Cells Is Dependent on Induction of Klf4**

Although many somatic cells, including SMCs, have been successfully reprogrammed in vitro to induced pluripotent stem cells using exogenous approaches, endogenous mechanisms directing reprogramming are largely undefined. Klf4, a Kruppel-like transcription factor member, is not expressed in differentiated SMCs (Figure 3), but its induction contributes to SMC phenotypic switching. Klf4 has been shown to be 1 of 4 genes necessary for in vitro induced pluripotent stem cell generation, underscoring its importance in maintenance of a progenitor cell phenotype. Because Klf4 was highly expressed by AdvSca1-SM cells (Figure 3; Online Figure VI), we examined its role in SMC generation of AdvSca1-SM cells. To determine whether induction of Klf4 in differentiated SMCs was required in vivo, aortas from 8- to 10-day-old WT and SMC-specific Klf4-deficient mice (SM22α-CreKI-YFP Klf4 SM-knockout) were examined for the presence of AdvSca1 cells. We found abundant numbers of adventitial Sca1+ cells in WT mice (Figure 7A). In contrast, adventitial Sca1+ cells were barely detectable in aortae from SM22α-CreKI-YFP Klf4 SM-knockout mice (Figure 7B). Fluorescence-activated cell sorting analysis was used to quantify AdvSca1-SM and AdvSca1-MA cells in WT and Klf4 SM-knockout mice. No difference in the numbers of AdvSca1-MA cells was observed, as anticipated because Klf4 was not deleted from non–SMC-derived cells (Figure 7D). Compared with WT mice, however, decreased numbers of AdvSca1-SM cells was confirmed in carotid arteries plus aortic arch and descending aortae of Klf4 SM-knockout mice (Figure 7C), supporting the concept that Klf4 induction is necessary for SMCs to generate progenitor cells. Unfortunately, because of premature death of SM22α-CreKI-YFP Klf4 SM-knockout mice by 4 weeks of age (M.C.M. Weiser-Evans et al, unpublished data, 2014), we were unable to verify this result.

Figure 7. Smooth muscle cell (SMC) reprogramming is dependent on kruppel-like factor 4 (Klf4) induction. Aorta and carotid arteries plus aortic arch were harvested from 8- to 10-d-old SM22α-CreKI-YFP wild-type (WT; A) or SM22α-CreKI-YFP Klf4 knockout (KO; B) mice. Representative immunofluorescent stains of aortic sections for Sca1 (red) and YFP (green); aortae from 2 independent WT and KO mice shown; n=6 per genotype total. Bar=50 µm. Dashed lines represent media–adventitia boundary. C and D, Fluorescence-activated cell sorting analysis was used to quantify SMC-derived AdvSca1-SM (C) or non–SMC-derived AdvSca1-MA (D) cells in WT vs Klf4 KO mice. Each symbol represents data from an individual mouse. n=7 WT and n=5 KO mice.
to demonstrate the biological importance of Klf4 in the expansion of AdvSca1-SM cells after vascular injury. To test whether Klf4 is sufficient to promote a progenitor cell phenotype as defined by expression of progenitor cell markers and repression of SM genes, cultured SMCs were transduced with adenoviruses expressing GFP (control) or Klf4-GFP. By fluorescence-activated cell sorting analysis, we observed ≈50% transduction efficiency (Online Figure XIII A). Compared with GFP-transduced SMCs, Klf4-transduced SMCs expressed increased levels of cell surface Sca1 and CD34 (Online Figure XIIIB and XIIIC). This was associated with downregulation of SMC-specific markers at the level of mRNA and protein (Online Figure XIIIC and XIID).

We previously demonstrated that Klf4 is downregulated as AdvSca1 cells differentiate in culture and acquire SMC markers.4 On the basis of its known function as a co-repressor of SRF-dependent SMC gene transcription and as a regulator of self-renewal in embryonic stem cells, we sought to determine whether Klf4 is critical for maintenance of a progenitor cell phenotype. We used in vitro approaches to silence Klf4 using control nontargeting or Klf4-specific siRNAs or overexpress Klf4 using adenoviruses expressing empty vector (control) or wild-type Klf4. Compared with control siRNA, Klf4 knockdown decreased Sca1 mRNA expression (Online Figure XIV A) and reduced the number of Sca1-positive cells, as evaluated by immunofluorescence staining (Figure 8A and 8B, left). This was associated with a trend toward an increased fraction of cells exhibiting αSMA-positive contractile filaments (Figure 8A and 8B, right), suggesting that loss of Klf4 accelerates differentiation of AdvSca1 cells toward a SMC fate. In contrast to Klf4 silencing, adenoviral-mediated overexpression of Klf4 resulted in increased Sca1 expression and decreased αSMA expression (Online Figure XIVB). This was associated with increased numbers of Sca1-positive cells (Figure 8C and 8D, left) and blocked the differentiation of AdvSca1 cells to SMCs, as measured by decreased fraction of cells exhibiting αSMA-positive contractile filaments (Figure 8C and 8D, right). Collectively, these data are consistent with an essential role for Klf4 in SMC reprogramming, similar to a recent report,11 and in the maintenance of the AdvSca1 progenitor phenotype.

Figure 8. Kruppel-like factor 4 (Klf4) maintains the progenitor phenotype. A and B, Isolated adventitial sca1-positive progenitor cells (AdvSca1) from wild-type (WT) mice were cultured for 24 h, transfected with siRNAs targeting green fluorescent protein (GFP; control) or Klf4, and evaluated 72 h after siRNA transfection. A, Representative images from treated cultures with immunostaining for α-smooth muscle actin (αSMA; green), Sca1 (red), and DAPI (blue). Sca1-positive and αSMA-positive cells from each treatment group were counted and normalized to total cell numbers. n=3 independent experiments; *P<0.05. C and D, Isolated AdvSca1 cells from WT mice were transduced with an empty adenovirus (control) or a Klf4-expressing adenovirus then cultured for 7 d. C, Representative images from treated cultures with immunostaining for αSMA (green), Sca1 (red), and DAPI (blue). D, Sca1-positive and αSMA-positive cells were counted and normalized to total cell numbers. n=3 independent experiments; *P<0.05.
Discussion

In previous experiments to determine the fate of medial SMCs in injured arteries, we made the entirely unexpected observation that some mature SMCs migrate from the media into the adventitia and become SMC marker negative. This effect, using 2 independent SMC genetic fate-mapping systems combined with a SMC lineage-specific chromatin mark, we demonstrate that SMCs moving into the adventitia gain expression of progenitor cell markers (Sca1, CD34, and Ki67) and become residents within the adventitial progenitor niche that we and others have described. We propose that this phenomenon is a type of in situ SMC reprogramming with the evidence to support this as follows: (1) no detectable SMC differentiation marker expression by AdvSca1-SM cells, (2) no detectable binding of SRF to CArG elements in SMC marker gene promoter regions in these cells, (3) loss of the H4Ac differentiation-associated mark on SMC promoter CArG elements by AdvSca1-SM cells, but (4) retention of the H3K4Me2 SMC lineage mark, (5) gain of expression of functional progenitor marker genes by AdvSca1-SM cells, and (6) gain of multipotential fate capabilities as defined by differentiation in vivo into SMCs, macrophages, and endothelial cells. Further study showed that SMC-derived progenitor cells reside in the adventitia of aortic arch and carotid arteries, descending aorta, and femoral arteries, each from distinct embryonic lineages, in numbers representing >60%, 40%, and 30%, respectively, of the total adventitial Sca1+ progenitor cell population of these vessels. Taken together, our data suggest the novel possibility that during vascular development differentiated SMCs migrate from the outer media into the inner adventitia where they become reprogrammed to a progenitor-like state. Because formation of AdvSca1-SM cells was observed in adult mice after treatment with tamoxifen, this would also suggest that SMC reprogramming is a continual process likely serving as a mechanism to replace resident vascular progenitor cells. This activity would be similar, in principle, to the reversion of mature differentiated cells to fate-restricted progenitor cells in regeneration of tracheal cell types after lung injury and stomach crypt cells in response to stem cell ablation.

An important difference and the novel aspect of our findings, however, is that our data support the concept that formation of AdvSca1-SM cells from mature SMCs is a normal physiological process, in contrast to these previous studies in which differentiated cells revert to progenitor cells only in the setting of experimental tissue injury or stem cell ablation. We are aware that Myh11/SMMHC can sometimes be expressed by microvascular pericytes and that some myeloid and myofibroblastic cells express Acta2αSMA and Tagln/SM22α (but not Myh11). At this time, we cannot rule out the possibility that some of the AdvSca1-SM cells we identify here may have an origin from adventitial cells. However, vasa vasorum microvessels are sparse in normal murine adventitia and a substantial origin of AdvSca1-SM cells from these alternate sources seems unlikely.

Although remodeling of the adventitia has been known to occur after many forms of vascular injury, it is widely assumed that medial SMCs do not directly participate in this response. This is likely because of the vast majority of studies in the literature that identify SMCs in injured arteries by their expression of conventional SM marker proteins (eg, Acta2αSMA, Myh11/SM22α). By those criteria, intimal SMCs, many plaque macrophages, and AdvSca1-SM cells, which lose expression of these markers, would not be recognized as SMC derived. Recent advances in SM fate-mapping systems that permanently mark mature SMCs has allowed reliable identification of these cells as SM-derived even if they lose all characteristics of SMCs. Using these systems, our data (Online Figure I) and recent reports by others have conclusively demonstrated that the percentage of SMCs contributing to intimal lesion formation and atherosclerosis is much greater than previously estimated from expression of traditional SMC markers. Although the mechanisms regulating SMC migration into the adventitia remain unclear, it is well known that breaks in the internal elastic lamina facilitate migration of SMCs into the intima. We assert that it would not be completely unprecedented that breaks in the external elastic lamina, as observed during vascular development and after injury to the vessel wall, would result in a similar movement of SMCs into the adventitia. Our ongoing studies are addressing this possibility in more detail.

From their earliest description, it was evident that the AdvSca1-positive cell population in the aortic adventitia was heterogeneous. For example, Hu et al reported that Sca1-positive cells isolated from the aortic root adventitia of adult ApoE−/− mice contained >60% fibroblastic cells, >20% epithelioid cells, and occasional adipocyte-like cells. Passman et al and Gräbner et al found that >50% of freshly isolated aortic AdvSca1 cells from WT C57Bl/6 mice lost Sca1 expression and differentiated into SMC marker–positive cells over 8 days ex vivo, whereas <25% proliferated as Sca1-positive cells without SM differentiation, and the remaining 25% lost expression of Sca1 but did not acquire detectable levels of SMC marker expression. Psaltis et al found that aortic cells isolated from WT C57Bl/6 mice exhibited hematopoietic colony-forming activity predominantly for progenitors expressing a macrophage fate. Further study showed that coexpression of Sca1 and CD45 in the adventitia marked the CFU-GM (colony-forming unit-granulocyte, monocyte) progenitor cells, and these Sca1+/CD45− cells comprised ~36% of the total aortic Sca1-positive cell population. The remaining Sca1+/CD45− cell population was shown in previous studies to possess SMC differentiation potential without hematopoietic colony-forming activity. Our results reported in the present study confirm and extend this previous work, supporting the concept that the aortic adventitia contains at least 2 types of AdvSca1 progenitor cells. On the basis of expression of cell surface markers, our data suggest that SMC-derived AdvSca1 cells express predominantly a myogenic progenitor phenotype (AdvSca1-SM), whereas non–SMC-derived AdvSca1 cells express predominantly a macrophage progenitor cell phenotype (AdvSca1-MA). However, myogenic and macrophage progenitor cell phenotypes were observed within subpopulations of both the AdvSca1-SM and AdvSca1-MA populations, supporting the likelihood of multipotent fate decisions. Indeed, our in vivo Matrigel plug assay and in vitro differentiation assays...
demonstrated the ability of AdvSca1-SM cells to differentiate into SMCs, macrophages, endothelial-like cells, adipocytes, and chondrocytes, further supporting the multipotency of these cells.

Induction of pluripotent stem cells from somatic cells can be accomplished through forced overexpression of the transcription factors, Oct-4, Sox2, Klf4, and c-Myc. Our current and previous results together with our unpublished RNA-Seq data demonstrate high expression levels of Klf4 and c-Myc, but not POU5F1/Oct4 or Sox2, in both populations of AdvSca1 cells, further suggesting that these cells possess a fate-restricted multipotent, but not pluripotent, phenotype. This would be similar to the detection of fate-restricted progenitor cells during regeneration of axolotl limb, zebrafish fin, mouse mouse tail, and neonatal mouse hearts after apex amputation injury. Further, our finding that the AdvSca1-SM subset arises predominantly from pre-existing SMCs offers an explanation for the previously puzzling observation that despite being SMC marker-negative, AdvSca1 cells express a transcription factor profile typically found in mature SMCs. Our findings suggest little contribution of AdvSca1-SM cells to the previously identified CFU-M subset. However, our findings suggest that AdvSca1-SM CD45 cells contribute to the adventitial myeloid cell population, which is consistent with differentiation of AdvSca1-SM cells into macrophages as observed in vivo in Matrigel plug assays, and to expansion of adventitial CD45 cells after vascular injury.

The adventitia is a complex layer of the vessel wall than previously thought that responds rapidly and robustly to many forms of arterial injury. Previous studies point to the adventitial fibroblast as the main responders to vessel injury. For instance, Shi et al and Scott et al reported that adventitial cells, referred to as fibroblasts, proliferated earlier and to a greater extent than medial SMCs in response to balloon arterial injury. Our data demonstrate that AdvSca1-SM, in particular, are robustly activated and expand considerably in numbers early after arterial injury. Mobilization of AdvSca1-SM cells might participate in medial repair by differentiating into medial SMCs, and in neointimal formation. We propose that AdvSca1-SM progenitor cells may also play major roles during injury-mediated adventitial remodeling. In support of this model, and in agreement with our data, a recent report demonstrates that AdvSca1 cells are major sources of angiotensin II-induced adventitial fibrosis, leading to artery wall stiffening and hypertension. Similarly, using the mdx mouse model of Duchenne muscular dystrophy, Ieronimakis et al found that the major collagen-producing cell type associated with late-onset cardiac fibrosis was the coronary AdvSca1 cell. Ongoing studies using fate-mapping approaches to selectively label SMC-derived AdvSca1-SM cells are addressing these possibilities.

The pluripotency-associated transcription factor, Klf4, is also well known to regulate SMC phenotypic changes, and multiple lines of evidence support an important role for Klf4 in vascular disease progression. Using in vivo and in vitro approaches, our studies establish that SMC generation of AdvSca1-SM cells is dependent on induction of Klf4. In vivo targeted deletion of the Klf4 gene in SMCs resulted in selective loss of AdvSca1-SM cells, but not AdvSca1-MA cells, suggesting that failure to induce Klf4 prevents AdvSca1-SM cell generation from SMCs and establishment of this subpopulation of resident AdvSca1 cells. In support of these in vivo findings, overexpression of Klf4 in cultured SMCs promoted a progenitor cell phenotype as defined by loss of SMC differentiation markers and gain of progenitor cell markers. Unfortunately, early postnatal death of SMC-specific Klf4 SM-knockout mice precluded our ability to define the biological importance of Klf4 on expansion of AdvSca1-SM cells in response to vascular injury. We recognize that, compared with WT mice, AdvSca1-SM cell numbers decreased in SMC-specific Klf4 knockout mice by only 50%. This is likely because of the late developmental activation of Cre recombinase and thus SMC deletion of Klf4 in SM22α-CreKI compared with SM22α-Cre mice. If migration into the adventitia and reprogramming occurred before Cre activation and inactivation of Klf4 in a subset of SMCs, the potential for Klf4 to be induced and promote AdvSca1-SM cell generation in this subset would remain. Finally, loss- and gain-of-function studies demonstrated that Klf4 is critical for the maintenance of the AdvSca1 progenitor cell phenotype. Collectively, our data demonstrate that Klf4 is a critical regulator of AdvSca1-SM cell generation and maintenance of the resident vascular progenitor cell pool.

In summary, our findings suggest that the local environment of the inner adventitia directs or stabilizes a Klf4-dependent SMC reprogramming-like process to maintain a vascular progenitor cell pool in the artery wall. Depending on environmental cues, SMC-derived progenitor cells may express cell fates resembling tissue resident macrophages, mural cells, endothelial-like cells, adipocytes, and osteoblasts. Going forward, the identification of factors in addition to Klf4 that regulate SMC-to-progenitor cell transitions in vivo will broaden our understanding of the multiple roles SMCs play in vascular homeostasis and disease.

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Disclosures

None.

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Differentiated Smooth Muscle Cells Generate a Subpopulation of Resident Vascular Progenitor Cells in the Adventitia Regulated by Klf4
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Mice and Vascular Injury. Smooth muscle myosin heavy chain \((\text{Myh11})\)-CreERT\(^{2}\) transgenic mice were obtained from Dr. Stephen Offermanns (University of Heidelberg, Heidelberg, Germany). Klf4 floxed mice were obtained from Dr. Klaus H. Kaestner (University of Pennsylvania). SM22\(\alpha\)-Cre (Tagln-Cre; stock 004746), SM22\(\alpha\)-CreKI (Tagln\(^{m2(cre)}\)Yec; stock 006878), and ROSA26 reporter mice (Rosa26-LacZ [stock 003474] and Rosa26-YFP [stock 006148]) were obtained from Jackson Laboratory. All mice were fully backcrossed to a C57BL/6 genetic background prior to studies. Myh11-CreERT\(^{2}\) transgenic mice and Rosa26-LacZ or Rosa26-YFP reporter mice were bred to generate tamoxifen-inducible SMC-specific \(\beta\)-galactosidase- or YFP-expressing mice (Myh11-CreERT-\(\beta\)-Gal/YFP). Adult male mice received 1-mg IP tamoxifen injections for 5 consecutive days 8 weeks before they were sacrificed. Males were used as the BAC transgene inserted on the Y Chromosome of Myh11-CreERT\(^{2}\) transgenic mice. Timed pregnant females (Rosa26-YFP females bred to male Myh11-CreERT-Rosa26-YFP mice) received 1-mg IP tamoxifen injections at e15 and e16 to label developing embryos. SM22\(\alpha\)-Cre transgenic mice were bred to Rosa26-YFP to generate SMC-specific YFP-expressing mice (SM22\(\alpha\)-Cre-YFP). For \textit{in vivo} Matrigel\(^{\text{TM}}\) plug angiogenesis assays, SMC-derived AdvSca1 cells were isolated from pooled arteries from SM22\(\alpha\)-Cre-YFP mice as described below, resuspended in 700 \(\mu\)l Matrigel\(^{\text{TM}}\) plus 100ng/ml VEGF and 100ng/ml FGF2, and injected subcutaneously into wild type C57BL/6 recipient mice; recipients were anesthetized with isofluorane for injections. Plugs were harvested 14 days post-injection, fixed in 4\% buffered PFA, and embedded in OCT for immunofluorescent staining. To induce vascular injury, mice were subjected to wire-induced femoral artery injury or carotid artery ligation injury\(^{1,2}\). For wire-induced injury, mice (n=6) were anesthetized with 60 mg/kg pentobarbital IP, right femoral arteries were isolated, a small arteriotomy made distal to the epigastric branch, and a 0.015-inch diameter fixed core wire guide (Cook, Inc., Bloomington, IN). The wire was advanced to the level of the aortic bifurcation. After removal, the arteriotomy site and skin incision were sutured. For carotid ligation (n=3), mice were anesthetized with isofluorane and left carotid arteries of two-month old SM22\(\alpha\)-Cre-YFP mice were completely ligated just proximal to the carotid bifurcation. Four weeks after femoral artery injury or three days following carotid artery ligation injury, uninjured and injured arteries were harvested, perfusion fixed in 4\% buffered PFA, and embedded in OCT for immunofluorescent staining. SM22\(\alpha\)-CreKI transgenic mice were bred to Klf4 floxed mice and Rosa26-YFP mice to generate SMC-specific YFP-expressing Klf4-deficient mice (SM22\(\alpha\)-CreKI-YFP KO). This alternative SMC Cre driver was used to generate SMC-specific Klf4 KO mice as use of the traditional SM22\(\alpha\)-Cre mouse line resulted in embryonic lethality. Cre recombinase is activated very late in development in SM22\(\alpha\)-CreKI, in contrast to the traditional SM22\(\alpha\)-Cre mouse line, which activates Cre recombinase earlier during vascular development. Mice were maintained in the Center for Comparative Medicine, and procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver.

Preparation of Single Cell Suspensions, Flow Cytometry, siRNA transfection, and adenovirus transduction. Isolated descending aortas, aortic arch plus left and right carotid arteries, and left and right femoral arteries were digested to single cells by digestion at 37\(^{\circ}\)C for 1hr in collagenase buffer (3.2mg/ml collagenase II, 0.7mg/ml elastase [ Worthington], 0.2mg/ml soybean trypsin inhibitor [Sigma]) in Hank’s buffered saline solution (HBSS), pH7.5). Arteries were harvested under sterile conditions following flushing of the vasculature with sterile heparinized PBS and minced prior to digest. Single cell suspensions were passed through a 70 \(\mu\)m filter and washed twice with sterile PBS + 0.1\% calf serum (CS). For flow sorting, single cell
suspensions were suspended in 100 μl PBS + 0.1% CS and incubated with a rat anti-mouse monoclonal Sca1 antibody (APC-conjugated; eBiosciences); live cells were sorted based on Sca1-APC and endogenous YFP expression. Gating strategy for sorting is shown in Supplemental Figure 2. Sorting was performed on a MoFlo high-speed cell sorter. For FACS analysis, single cell suspensions were stained in various combinations with rat anti-mouse monoclonal antibodies, including anti-Sca1, anti-CD45, anti-Ly6C, anti-CD115, anti-CD140a, anti-CD140b, anti-CD31, and DAPI to stain for dead cells. Isootype matched control antibodies were used. Flow cytometry was performed on a Galios cytometer (Beckman Dickenson). Data were analyzed using Kaluza Software (Beckman Coulter). The following antibodies were used: PerCP-Cy5.5-Sca1, PE-Cy7-CD45, APC/e780-Ly6C, PE-Cy7-CD115, APC-CD140a, APC-CD140b, PE-CD31; all antibodies were from eBiosciences. Gating strategy for flow cytometry is shown in Supplemental Figure 4A. Briefly, live cells were plotted for endogenous YFP expression and DAPI to gate out dead cells and identify YFP(+) and YFP(-) populations. YFP(+) and YFP(-) populations were analyzed separately for various markers as shown in Figure 5. To quantitate SMC-derived and non-SMC-derived AdvSca1 cells in WT versus SMC-specific Klf4 KO mice, carotid arteries and aortae from 8-10-day old mice were individually digested to single cell suspensions, labeled with APC-conjugated anti-Sca1 antibodies, and analyzed for endogenous YFP and Sca1-APC expression. To analyze the effect of Klf4 silencing or overexpression on maintenance of the AdvSca1 progenitor cell phenotype, the total population of AdvSca1 cells was sorted based on Sca1 expression as described above. For Klf4 deficiency, AdvSca1 cells were cultured in DMEM plus 10% FBS for 2 days. Cells were then transfected with Dharmafect4 transfection reagent (0.02 μl per 100 μl transfection medium; Thermo Scientific) and either non-targeting or Klf4-targeting siGENOME SMARTpool siRNAs (100 nm; Thermo Scientific). Cells were incubated for an additional 72 hrs. For Klf4 overexpression, AdvSca1 cells were transduced in suspension with an empty vector adenovirus or an adenovirus expressing Klf4 (100 MOI; Vector BioLabs, Malvern, PA). Cells were then plated in DMEM media with 10% FCS for 7 days. Following culture, cells were fixed in 4% PFA and immunofluorescently stained for αSMA and Sca1 expression, as described below. Total RNA was isolated from separate cultures and analyzed by RT-PCR (siRNA experiments) or qPCR (adenoviral overexpression experiments). Primer sequences for RT-PCR: Klf4: forward (5’-ATTAATGAGGCGGCAACCTG-3’), reverse (5’-GGAGGACGAGGAGGGCTG-3’); Sca1: forward (5’-CTCGAGGATGGACACTTTG-3’), reverse (5’-GGTCTCGAGGAGGACTGGAC-3’); β-Actin: forward (5’-TGTTACCAACTGGGACGACA-3’), reverse (5’-CTCCAGCTGGTTGGTGA-3’). Primer sequences for qPCR are listed below.

**In Vitro Differentiation Assay.**  Sca1(+) YFP(+) cells (AdvSca1-SM cells) were flow sorted from arteries of SM22α-Cre-YFP mice as described above, seeded into 8- (5 x10³ cells/well) or 2-well (8-9 x10³ cells/well) chamber slides in serum-supplemented DMEM (10% FBS, 1% penicillin-streptomycin), and allowed to adhere for 24h at 37°C. To initiate differentiation, cultured cells were exposed to specific induction media. Chondrogenic differentiation was induced using media containing non-essential amino acids (50μg/ml), sodium pyruvate (50μg/ml), dexamethasone (0.1μM; D2915, Sigma, USA), insulin (100μg/ml; 10516, Sigma, USA) and TGF-β (10ng/ml; 7666-MB/CF, R&D Systems, USA). Cells were differentiated into adipocytes using conventional adipogenic cocktail (500μM dexamethasone [D2915, Sigma, USA], 5μM rosiglitazone [71740, Cayman Chemical, USA], 100μg/ml insulin [10516, Sigma, USA], 1nM indomethacin [17378, Sigma, USA], 5μM 3,5′,3′,5′-Triiodo-L-thyronine [T3, T2877, Sigma, USA]). Differentiation media was replenished every 3 days until day 12-14 days. Following differentiation, cells were fixed in 4% PFA and immunofluorescently stained for collagen type II (chondrogenic cells) or FABP4 (adipocytes).
**Immunofluorescence and Confocal Microscopy.** For immunofluorescence staining, arteries or Matrigel™ plugs were fixed in 4% PFA and embedded in OCT for sectioning. Isolated cells were plated on 8-well chamber slides (Lab-Tek) overnight then fixed in 4% PFA. Tissue sections and/or cells were permeabilized with MeOH followed by 0.05% Tween-20 in PBS, blocked in 3% horse serum, and sequentially incubated with specific primary and secondary antibodies. Antibodies used include monoclonal rat anti-mouse Sca1 (1:100; BD Pharmingen), monoclonal rat anti-mouse CD34 (1:50; Abcam), FITC-conjugated polyclonal goat anti-GFP (1:200; Abcam), Cy3-conjugated monoclonal anti-smooth muscle alpha actin (αSMA; 1:2000; Sigma), monoclonal rat anti-mouse CD45 (1:100; BD Pharmingen), polyclonal rabbit anti-Klf4 (1:100; Abcam), monoclonal rat anti-mouse F4/80 (1:50; Abcam), polyclonal rabbit anti-SMMHC (1:100; Biomedical Technologies, Inc.), polyclonal rabbit anti-αSMA (1:200, Abcam), rabbit anti-vWF (1:200; Abcam), rabbit anti-FABP4 (1:100; Cell Signaling), and rabbit anti-collagen type II (1:50; ThermoFisher Scientific). For unconjugated antibodies, antigen:antibody complexes were visualized using Alexa Fluor-488, -568-coupled, or -647-coupled secondary antibodies (Molecular Probes). Coverslips were mounted with VectaShield medium containing DAPI to detect all cell nuclei (Vector Laboratories) and cells imaged using a laser-scanning confocal microscope (LSM 780 spectral, Carl Zeiss, Thornwood, NY) with a x63 or x100 oil immersion objective. Images were analyzed using ZEN LE software. Negative controls included the use of rat or rabbit IgG. To stain for LacZ activity, tissues were fixed in glutaraldehyde and whole mount staining was performed at 37°C overnight using a kit from GTS, Inc according to the protocols provided. Tissues were then paraffin-embedded for histological analysis. To quantitate Sca1(+) CD45(+), and YFP(+) cells in uninjured compared to injured carotid arteries, total numbers of Sca1 or CD45(-) YFP(+), Sca1 or CD45(+) YFP(+), and Sca1 or CD45(+) YFP(-) adventitial cells were counted in three high-powered fields per vessel from n=3 uninjured and injured vessels by two independent investigators.

**Chromatin Immunoprecipitation.** ChIP was performed on flow-isolated cell populations as described above. Cells were pelleted and transferred directly to 37°C 1% formaldehyde in MEM for 10 minutes, washed 3 times with ice-cold PBS, and taken through the rest of the ChIP protocol provided with the ChIP kit (Millipore). Briefly, samples were lysed with SDS, sonicated with a Branson Digital Sonifier 450 (Branson, CT) for 35-45 sec with 5 second increment pulses, centrifuged at 14,000Xg for 10min, 4°C. Samples were then analyzed on 1% acrylamide gels for concentration, and volumes were normalized with SDS buffer. Antibodies for ChIP include anti-H3K4Me2, anti-H4-Ac (Millipore), and anti-SRF (Santa Cruz Biotechnologies). DNA was purified with QIAquick PCR purification kits (Qiagen). Real-time PCR with Power SYBR Green mastermix (Life Technologies) was used to analyze chromatin immunoprecipitation. For each primer set, the ratio of IP to Ref was calculated using the formula: 2^\left(\frac{Ct(\text{Ref})-Ct(IP)}{Ct(\text{Ref})-Ct(\text{no-antibody control})}\right) based on previously described methods^3. Data from a minimum of 3 independent experiments were normalized to YFP+ SMCs (YFP SMC set to “1” to average among independent experiments), averaged and standard errors of the mean were calculated. Primer sequences were as follows: Acta2 forward, 5'- AGCGAGACGAGGATACGAGTGGAAGAGAC-3'; reverse, 5'- CCTCCCACTCGCCTC CCAAACAGGAGC-3'; Myh11 forward, 5'- CTGCCGCGGACCATATTTAGTC AGGGGGAG-3'; reverse, 3'-CTGGGCGGGAGAC AACCACAAAGGCCAGG. PCR conditions were as follows: 15-second denaturation at 95°C, 60-second annealing at 65°C, and 45-second extension at 72°C (40 cycles) as described previously.

**Quantitative RT-PCR.** Total RNA was isolated from flow-isolated cell populations by first digesting in RLT lysis buffer (Qiagen). Samples were then processed with QIAshredder and RNeasy Plus kits (Qiagen) to isolate RNA. First strand cDNA was made using the iScript cDNA synthesis kit (BioRad). Sequence-specific primers were designed: αSMA: forward (5'-
CTGACAGGCGACCACCTGAA-3'), reverse (5'- CATCTCCAGAGTCCAGCACA-3'); SMMHC: forward (5'- GCAGCTTCTACAGGCAAACC-3'), reverse (5'-CTCTCATCCTACCTAGCCTGTCG-3); SM22α: forward (5'-GATGGAACAGGTGGCTCAAT-3'), reverse (5'-TTCCATCGTTTTTGTCAC-3'); Myocardin: forward (5'- CACCCATGGACTCTGCCTAT-3'), reverse (5'-TTGAGGTTGTTTGGTGCG-3'); SRF: forward (5'-GGCCCAACTACCCTCCTTTC-3'), reverse (5'-TAGTCAAGTGTGGGTGCG-3'); CD34: forward (5'-ATGTCGCCCTTCTCATT-3'), reverse (5'-CCAAAGGTCAGAGATGGA-3'); CD31: forward (5'-AGCCACAGCCATACGGTTA-3'), reverse (5'-AGCCTTCCCTCTCTTGTTG-3'); Flk-1: forward (5'-TACACAATTCAGACAGCTGTGTTG-3'), reverse (5'-CTGTTTCCCTCAATGGGATATCTTC-3'); Flt-1: forward (5'-TATAAGGCAGCGGATTGACC-3'), reverse (5'-TCATACACATGCGGAGGTT-3'); VEGF: forward (5'-GCACATAGAGAGAATGAGCTTCC-3'), reverse (5'-CTGTTACCTTAGTACCAAGG-3'); SDF-1α: forward (5'-AAAGGGTCTCTGAGCACAGCT-3'), reverse (5'-TGAGTCAACAGATCCGCG-3'), reverse (5'-TGAGTCAACACAGATCCGCG-3'), Sca1: forward (5'-AGGAGGCAGCAGTTATTGTGG-3'), reverse (5'-TGAGTCAACAGATCCGCG-3'), and β-Actin: forward (5'-AGGGTGTGATGGTGGGTATGG-3'), reverse (5'-CGTTGACCTTAGTACCAAGG-3').

Quantitative real-time PCR was performed as previously described and β-actin was used for normalization. To compare among individual experiments, data was normalized to YFP+ SMCs for SMC genes (αSMA, SMMHC, SM22α, and myocardin) (YFP SMC set to “1” to average among independent experiments) or AdvSca1-MA cells for progenitor cell genes (Klf4, CD34, VEGF, SDF-1α, CD31, Flk1, Flt1) (AdvSca1 cells set to “1” to average among independent experiments).

**Smooth Muscle Cell Culture, Adenovirus Transduction, and Western Blotting.** Primary rat aortic SMCs were isolated and cultured as previously described. Briefly, the aggregate population of aortic medial SMC from adult Sprague Dawley rats was aseptically dissected and SMCs were obtained by digestion in Eagle’s MEM Medium (EMEM) containing collagenase and elastase. Isolated cells were maintained in EMEM containing 10% calf serum (CS) and were used as primary cultured cells through passage 10. SMCs were transduced with mouse Kruell-like factor 4 (KLF4) adenovirus (Vector Bioslabs, Ad-GFP-mKLF4, #ADV-262870), or eGFP adenovirus (Vector Bioslabs, Ad-CMV-GFP, #1060) as control, at 100 MOI. 96 hours after transduction, the cells were harvested and subjected to qPCR (described above), Western blotting, or flow cytometry analysis (described above). qPCR sequence specific primers for rat aortic SMCs were designed: Klf4: forward (GTGAGGAACTCTCTACATGA), reverse (GTAGCCGAAGTCCGATCAG); αSMA: forward (CCAGCCACAGCACAGCCG); SMMHC: forward (GTATTGAACTGATTGAGCGGC), reverse (TGAGCCGTTTCTCACCGA); Calponin: forward (CACCAATCATCGAATCTCA), reverse (CTTCAAGGCGCCAATCTCC); βActin: forward (AGGGTGTGATGGTGCTGGATG), reverse (AATGCGCATTGTTTCAATG). For Western blotting, SMCs were lysed with ice-cold mPER lysis buffer (Thermo Fisher, MA) and protease inhibitors. Solubilized proteins were centrifuged at 14,000 g (4 °C) for 10 min. Supernatants were separated using 10% SDS–polyacrylamide gel electrophoresis and transferred to Immobilon P membranes (Millipore, MA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 140 mM NaCl) containing 0.1% Tween-20 (TTBS) and 5% BSA (Sigma), and then incubated with 5% BSA in TTBS solution containing primary antibodies for 12–16 h at 4°C. Membranes were washed in TTBS, and bound antibodies were visualized with alkaline phosphatase-coupled secondary antibodies and Lumi-Phos WB (Pierce, IL) according to the manufacturer's directions. Antibodies used: Klf4 (R&D Systems #AF-3158, 1:2000), α-SMA
(Abcam, #ab5694, 1:60000), Calponin (Santa Cruz, #sc-28545, 1:1000), and βActin (Sigma, #A5441, 1:60000). For FACS analysis SMCs were detached with trypsin and washed with FA3 buffer (1×PBS, 1mM EDTA, 25mM HEPEs, 0.1% FBS). SMCs were then incubated with Anti-Sca1 antibody (Abcam, #ab95439, 1:100 dilution) or anti-CD34 antibody (Abcam, #ab185732, 1:100 dilution) for 1 hour. Fixable Aqua (Thermo Fisher Scientific, #L34965) was employed to stain dead cells according to manufacturer’s instructions. After washing with FA3 buffer, the cells were fixed and permeabilized with Transcription Factor Fixation/Permeabilization buffer (eBioscience, # 00-5521-00) at 4°C for 1 hour. The fixed cells were subsequently stained with Anti-KLF4 Antibody (R&D Systems # AF3158, 1:200 dilution), and corresponding secondary antibodies Alexa Fluor Cy3-conjugated Donkey anti-Goat IgG (Jackson ImmunoResearch, #705-165-147, 1:200 dilution), Alexa Fluor 647-conjugated Donkey anti-Rabbit IgG (Jackson ImmunoResearch, #711-605-152, 1:200 dilution). All antibody incubations were performed at 4°C for 1 hour. The stained cells were analyzed with Gallio Flow Cytometer (Beckman Coulter) at University of Colorado Cancer Center Flow Cytometry core facility.

Statistical Analysis. Data were expressed as means ± SE. Paired results were assessed using Student’s T-test. Comparisons between multiple groups were analyzed using one-way ANOVA (with post-test comparisons as appropriate). P-values <0.05 were considered statistically significant.
REFERENCES


Genetically label mature SMCs in adult mice

**Stop Tamoxifen**

**Vascular Injury**

**Tissue Harvest**

**d1** **d5** **d15** **4 wks**

**Supplemental Figure I. SMC origin of intimal SMCs.** Two-month old Myh11-CreERT-YFP mice received 1 mg IP tamoxifen injections once a day for five consecutive days followed by a ten-day wash out period and then subjected to femoral artery wire-induced injury. (A) Timeline for experiment. (B) Confocal immunofluorescence imaging for YFP and αSMA on 4-wk injured right femoral artery. Arrowheads = internal elastic laminae; L = arterial lumen; * = YFP(+)/αSMA(-) SMC-derived intimal cells. Scale bar = 20 µm. (C) Confocal immunofluorescence imaging for YFP and αSMA on contralateral uninjured femoral artery. L = arterial lumen. Scale bar = 100 µm. N=3 mice.
Supplemental Figure II. Lack of SMC labeling in untreated, no tamoxifen control Myh11-CreERT-YFP mice. Representative confocal immunofluorescence imaging for YFP and αSMA on aorta from two-month old untreated Myh11-CreERT-YFP mouse. L = arterial lumen. Scale bar = 50 μm. Dashed line represents media-adventitia boundary.
Supplemental Figure III. Gating Strategy for Sca1(+) and YFP(+) cells. Gating was performed on single cell suspensions based on forward and side scatter (left) followed by identification of singlets (middle). Single cells were plotted for DAPI to gate out dead cells (right).
Supplemental Figure IV. In utero SMC labeling. Pregnant female YFP mice bred to male Myh11-CreERT-YFP mice received 1 mg IP tamoxifen injections at e15 and e16 to label SMCs of developing male embryos. (A). Timeline for in utero tamoxifen labeling. (B). Carotid arteries were harvested from 30-day old male Myh11-CreERT-YFP pups. Arteries from 5-7 mice were pooled, digested into single cell suspensions, labeled with an APC-conjugated anti-Sca1 antibody, and flow sorted based on endogenous YFP and Sca1 expression as described in Material and Methods. Representative density plot showing three distinct cell populations, YFP(+), YFP(+)Sca1(+), and YFP(+)Sca1(+). N=6 independent sorts. (C). Arteries were immunofluorescently stained for YFP and αSMA. Representative confocal immunofluorescence imaging showing inefficient YFP knock-in in outer medial SMCs (asterisks). Arrowheads = external elastic lamina. Scale bar = 20 μm.
Supplemental Figure V. Percentage of total AdvSca1 cells originating from differentiated SMCs. Carotid arteries plus aortic arch, descending aorta, and femoral arteries were harvested from two-month old SM22α-Cre-YFP mice. Arteries from 5-7 mice were pooled, digested into single cell suspensions, labeled with an APC-conjugated anti-Sca1 antibody, and flow sorted based on endogenous YFP and Sca1 expression as described in Material and Methods. Representative density plots showing three distinct cell populations, (A): YFP(-)Sca1(+), (B):YFP(+)Sca1(+), and C: YFP(+)Sca1(-).
**Supplemental Figure VI. AdvSca1-SM cells express progenitor cell markers.**

Cell populations were sorted as described in Figure 2, plated onto chamber slides, fixed and immunofluorescently stained for αSMA, YFP, and Sca1 or CD34 (A-C) or YFP, Sca1, and Klf4 (D).
**Supplemental Figure VII. Retention of SMC lineage mark in AdvSca1-SM cells.** Cell populations were sorted from pooled, digested arteries from 10 SM22α-Cre-YFP mice as described in Figure 2. DNA from isolated cells was cross-linked with formaldehyde and recovered from immunoprecipitated samples using anti-H3K4Me2 (A), anti-H4Ac, or anti-SRF (B) antibodies. Immunoprecipitated DNA was subjected to qPCR amplification using primers flanking essential CArG boxes in the *Myh11* and *Acta2* promoters (*Myh11* only shown in panel B). 10% genomic input DNA was used as a positive control. ChIP data are shown as fold enrichment over input DNA; N=3 independent experiments using arteries from 10 pooled mice per experiment; *P<0.05. No antibody controls consistently exhibited no PCR amplification (not shown). Data were normalized to YFP(+) SMCs. Shown are data obtained from cell populations isolated from pooled carotid arteries plus aortic arch. ND = not detectable.
Supplemental Figure VIII. Immunophenotypic profiling for mature SMCs. Single cell suspensions were obtained from digested arteries from SM22α-Cre-YFP mice. (A). Flow cytometry gating strategy. Single cells were plotted for DAPI to gate out dead cells and for endogenous YFP expression to identify YFP(+) and YFP(-) populations. For the following panels and those shown in Figure 4, YFP(-) and YFP(+) populations were analyzed separately. (B). Representative flow cytometry plots demonstrating that YFP(+)Sca1(-) cells (mature SMCs) do not express Ly6C or CD31, but express low levels of CD140b and CD140a. Left plot is from the same experiment as in Figure 4, panel (B). (C). Flow cytometry histograms showing higher expression of CD140b (left) and CD140a (right) on SMC-derived AdvSca1-SM cells compared to mature SMCs. Shown for all are data obtained from cell populations isolated from pooled carotid arteries plus aortic arch; n=4 independent analyses.
Supplemental Table I. Quantitation of subpopulations as assessed by FACS analysis.

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<td></td>
<td>% of total YFP(-)</td>
<td>% of total YFP(+)</td>
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<td>Sca1(+)CD45(+)</td>
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<td>63.4**</td>
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<tr>
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*all CD31(-) CD140a(-) CD140b(-)

**all CD31(-)
Supplemental Figure IX. AdvSca1-SM cells contribute to functional neovessel formation. AdvSca1-SM cells were isolated from pooled arteries from SM22α-Cre-YFP mice (6-8 mice) by flow sorting, embedded in MatrigelTM, and subcutaneously implanted into syngeneic WT mice. MatrigelTM plugs were harvested 14 days post-implantation for histochemical (a&b) or immunofluorescence (c&d) analysis. (a&b) Representative hemaotoxylin stained images showing cross-sectional (a) and longitudinal (b) vessels. * = host-derived red blood cells in neovessels. (c&d) Representative immunofluorescence images for YFP expression showing longitudinal (c) and cross-sectional (d) vessels; DAPI = nuclei. * = autofluorescing red blood cells. Scale bar = 50 μm. N=4 independent analyses.
Supplemental Figure X. Host-derived endothelial cells form the majority of neovessels, but AdvSca1-SM cells exhibit in vivo endothelial cell differentiation potential. AdvSca1-SM cells were isolated from pooled arteries from SM22α-Cre-YFP mice (6-8 mice) by flow sorting, embedded in Matrigel™, and subcutaneously implanted into syngeneic WT mice. Matrigel™ plugs were harvested 14 days post-implantation for immunofluorescence analysis. (A). Representative immunofluorescence images for von Willebrand factor (vWF; red) and YFP (green) expression. Arrowheads = host-derived vWF(+)YFP(-) endothelial cells; arrows = vWF(-)YFP(+) AdvSca1-SM-derived perivascular cells. * = host-derived autofluorescing red blood cells. (B). Representative immunofluorescence images for vWF (red) and YFP (green) expression. Arrows = vWF(+)YFP(+) AdvSca1-SM-derived endothelial cells. (C). Representative immunofluorescence images for Sca1 (red) and YFP (green) expression. Arrows = Sca1(+)YFP(+) AdvSca1-SM cells. * = host-derived autofluorescing red blood cells. Scale bars = 50 μm (panels A&B); 20 μm (panel C). N=4 independent analyses.
Supplemental Figure XI. In vitro adipocyte and chondrocyte differentiation potential of AdvSca1-SM cells. (A). AdvSca1-SM cells were isolated from pooled arteries from SM22α-Cre-YFP mice by flow sorting, cultured in media containing dexamethasone, rosiglitazone, insulin, indomethacin, and 3,3',5-Triiodo-L-thyronine for 12 days, fixed, and immunofluorescently stained for YFP (green) and FABP4, an adipocyte-specific marker (red). (B). AdvSca1-SM cells were isolated from pooled arteries from SM22α-Cre-YFP mice by flow sorting, cultured in media containing non-essential amino acids, sodium pyruvate, dexamethasone, insulin, and TGF-β, for 12 days, fixed, and immunofluorescently stained for YFP (green) and collagen type II, a chondrocyte-specific marker (red). N=4 independent analyses.
Supplemental Figure XIIA&B. Injury-induced expansion of AdvSca1-SM cells. Two-month old SM22α-Cre-YFP mice were subjected to carotid artery ligation injury. Representative immunofluorescence images of contralateral uninjured vessels for (A) YFP expression (green; left) and αSMA expression (red; right) or (B) YFP expression (green) and Sca1 expression (red). M = arterial media; A = arterial adventitia. Scale bar = 100 μm. Dashed lines represent media-adventitia boundary.
Supplemental Figure XIIIC. Injury-induced expansion of AdvSca1-SM cells. Two-month old SM22α-Cre-YFP mice were subjected to carotid artery ligation injury. Immunofluorescence images of injured vessels for YFP expression (green). M = arterial media; A = arterial adventitia. Dashed lines represent media-adventitia boundary.
Supplemental Figure XIII. Klf4-dependent reprogramming of cultured SMCs. Cultured rat aortic SMCs were transduced with control (GFP) or Klf4-GFP-specific adenoviruses (100 MOI). FACS analysis was used to examine transduction efficiency based on GFP expression (A), cell surface Sca1 expression (B), and cell surface CD34 expression (C) in GFP- vs. Klf4-transduced cells. Total RNA (D) or whole cell lysates (E) were analyzed by qPCR (D) and Western blotting (E) for SMC marker expression. N≥4 independent experiments.
Supplemental Figure XIV. siRNA and Adenoviral-mediated loss- and gain-of-expression of Klf4 and Sca1. (A). Isolated AdvSca1 cells were cultured for 24 h followed by transfection with siRNAs targeting GFP (siGFP) or KLF4 (siKlf4). Total RNA was isolated for RT-PCR analysis of Klf4 and Sca1 using gene-specific primers. β-Actin-specific primers were used as controls. (B). Isolated AdvSca1 cells were transduced with an empty adenovirus (Adeno-Empty) or a Klf4-expressing adenovirus (Adeno-Klf4) then cultured for 7 days. Total RNA was isolated for qPCR analysis of Klf4, Sca1, and αSMA using gene-specific primers. β-actin was used for normalization. N≥3 independent experiments.