# Regulation of Adventitia-Resident Progenitor Cells

Jenna Nicole Regan

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Approved by:

Mark Majesky

Victoria Bautch

James Faber

Terry Magnuson

Larysa Pevny

**Abstract**

Jenna Nicole Regan: Regulation of adventitia-resident progenitor cells
(Under the direction of Mark W. Majesky)

Far from the inert structural component that was the prevailing view for many years, the arterial adventitia is a region of active signaling that harbors $Sca1^+$ vascular progenitor cells as well as many other cell types. We have shown that a sonic hedgehog (Shh) signaling domain is restricted to the adventitial layer of artery wall beginning at embryonic day 15.5. Hedgehog (Hh)-responsive cells, colocalize with a circumferential ring of Shh protein concentrated between the media and adventitia. Furthermore, $Sca1^+$ progenitor cells (AdvSca1 cells) reside within the Hh signaling domain and are reduced in number in Shh-/- mice. As a population, AdvSca1 cells express transcription factors thought to be required for smooth muscle cell (SMC) differentiation, including serum response factor (SRF) and myocardin family members, yet the cells do not express SMC marker proteins *in vivo*. However, upon removal of AdvSca1 cells from the adventitial environment, they readily differentiate to SMC-like cells *in vitro*. Repression of SRF-dependent transcription may be mediated by SRF co-repressors such as Klf4, Msx1, and FoxO4, all of which are expressed by AdvSca1 cells *in vivo*. Knockdown of Klf4 in AdvSca1 cells *in vitro* results in extensive down-regulation of Sca1 and defects in $Sca1^+$ cell proliferation. *In vitro* and *in vivo* experiments have shown that AdvSca1 cells possess the potential for SMC, pericyte/mural cell, adipogenic, ostogenic, endothelial, and macrophage differentiation. Thus, AdvSca1 cells are regulated, in part, by Hh signaling and the transcription factor Klf4 and function as progenitors for multiple cell types with physiological and pathological relevance to the artery wall.

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## Table of Contents

## List of Tables

## List of Figures

## List of Abbreviations

| | |
|---|---|
| Sca1 | stem cell antigen 1 |
| Shh | sonic hedgehog |
| Hh | hedgehog |
| AdvSca1 | adventitial Sca1$^{+}$ progenitor |
| SMC | smooth muscle cell |
| SRF | serum response factor |
| Klf | Krüppel like factor |
| R26R | Rosa26 reporter |
| PDGF | platelet-derived growth factor |
| Ihh | Indian hedgehog |
| Dhh | desert hedgehog |
| Ptc | patched |
| Smo | smoothened |
| Sufu | suppressor of fused |
| VEGF | vascular endothelial growth factor |
| FGF | fibroblast growth factor |
| EPC | endothelial progenitor cell |
| SMPC | smooth muscle progenitor cell |
| TGF | transforming growth factor |
| MSC | mesenchymal stromal cell |
| SM-MHC | smooth muscle myosin heavy chain |
| WT | wild type |
| BSA | bovine serum albumin |

| | |
|---|---|
| PBS | phosphate buffered saline |
| BrdU | bromo-deoxyuridine |
| ECM | extracellular matrix |
| HSPG | heparin sulfate proteoglycan |
| EC | endothelial cell |
| WT1 | Wilms tumor 1 |
| DMEM | Dulbecco’s modified Eagle’s medium |
| BMP | bone morphogenetic protein |
| LCA | left common carotid artery |
| RCA | right common carotid artery |
| AHF | anterior heart field |
| LIF | leukemia inhibitory factor |

# CHAPTER 1

## Introduction and Background

## Background

### *The mammalian hedgehog signaling pathway*

The hedgehog signaling network plays diverse roles in development. Beginning with the identification of *hedgehog* and *patch* as Drosophila segment polarity mutants by Nüsslein-Volhard and Wieschaus in 1980 (1), investigation into the components and mechanisms of the hedgehog signaling network has drawn an increasingly complex and ever-evolving picture. The components germane to this work will be briefly described, with mention of additional network components.

Three mammalian homologues of the Drosophila *hedgehog* (Hh) gene have been identified: *Desert hedgehog, Indian hedgehog, and Sonic hedgehog* (*Dhh, Ihh,* and *Shh*, respectively) (2). Although all three proteins bind with similar affinity to Hh receptors, they vary in potency (3) and expression pattern (4), consistent with specialized functions during development. Hh proteins undergo autocatalytic processing, resulting in an amino-terminal product with signaling activity. The cleavage reaction also adds a cholesterol moiety to the amino-terminal domain (5, 6). The amino-terminal signaling peptide is then further modified by the addition of a palmitoyl moiety, catalyzed by the acyltransferase Hhat in mammals. Together, these modifications contribute to the formation of soluble Hh protein multimers which, in turn, permit long-range Hh signaling (7, 8).

*Shh* mutant mice exhibit a variety of defects, including brain, spinal cord, eye, skeletal, and limb development (9). They also have abnormal neural crest cell development, leading to defects in arch artery and outflow tract patterning (10). Only about half of the expected number of $Shh^{-/-}$ embryos survive to term, and none live past birth (11). Similar survival rates are seen in $Ihh^{-/-}$ embryos, with about half dying around midgestation and the remainder perishing at birth due to respiratory failure. $Ihh^{-/-}$ embryos exhibit pronounced skeletal defects, including forelimb and rib shortening, misshapen endochondral bones, and misformed joints (12). In addition, Ihh signaling is critical for proper yolk sac angiogenesis, a

phenotype which will be further discussed below. In contrast to the lethal phenotypes of *Shh* and *Ihh* knockouts, *Dhh*$^{-/-}$ mice are fully viable, although males are infertile due to defects in spermatogenesis (13).

There are two mammalian homologues of the Hh receptor Patched, *Ptc1* and *Ptc2*. Ptc proteins are 12-pass transmembrane proteins (14) with two large hydrophilic extracellular loops that mediate binding to Hh proteins (15). In addition to functioning as receptors for Hh ligands, *Ptc* genes are also transcriptional targets of the Hh pathway and are thus upregulated in response to Hh signaling (14). Therefore, *Ptc* expression is found in many tissues adjacent to the source of Hh signal. Mouse Ptc2 protein shares 56% identity with Ptc1, with divergent regions including the intercellular amino- and carboxy-terminal sequences (16). Although both Ptc proteins can bind Hh ligands, they do not function redundantly. Their expression patterns only partially overlap (17, 18) and the knockout phenotype of *Ptc1*$^{-/-}$ mice is much more severe. *Ptc1*$^{-/-}$ embryos die between embryonic day 9 (E9.0) and E10.5, exhibiting an open neural tube, overgrown head folds, hindbrain, and spinal cord, and an abnormal heart (19). In contrast, *Ptc2*$^{-/-}$ mice are viable, although they develop skin defects including alopecia and epidermal hyperplasia (20, 21).

Although Ptc proteins serve as the receptors for Hh ligands, the downstream signaling is actually transduced by another transmembrane protein, Smoothened (Smo). Smo has seven transmembrane domains and bears some structural similarity to G protein-coupled receptors (22, 23). It is well-established that in the absence of Hh signaling, Ptc proteins exert a negative effect on Smo activity. When Hh ligands bind to the Ptc receptors, it alleviates the repression and allows Smo to activate downstream signaling. Exactly how Ptc exerts its repressive effects on Smo has been unclear for many years. Most recent models favor a mechanism by which Ptc functions as a pump (the Ptc protein sequence shows some homology to the prokaryotic resistance-nodulation-division (RND) family of small-molecule pumps (24)). The pumping action of Ptc may change the concentration or

localization of small molecules, such as oxysterols, which affect the activity of Smo (25-28). *Smo* knockout mice die by E9.5 with severe abnormalities, including failure to undergo embryonic turning, defects in closure of the ventral midgut and looping of the linear heart tube, ventral cyclopia, and holoprosencephaly (29).

The ultimate effectors of Hh signaling in mice are the three Gli transcription factors (*Gli1-3*). All three contain a series of five highly conserved C2H2 zinc fingers (30). Like the *Ptc* genes, the *Gli1* locus is also a transcriptional target of Hh signaling (31-33). Gli1 functions as a transcriptional activator, while Gli2 and Gli3 possess both activating and repressive domains. However, the expression of *Gli1* from the endogenous *Gli2* locus rescues defects related to Shh signaling, suggesting that Gli2 functions primarily as an activator *in vivo* (34). Although Gli3 seems to exert its strongest effects as a negative regulator of Hh-dependent gene expression (35), some evidence suggests that it has important activating functions as well (35-37). In the absence of active Hh signaling, the Gli3 protein is proteolytically cleaved to an 83 kDa repressor form, and this process is inhibited in the presence of Hh ligand (38, 39).

The combination of unique and redundant activities of the Gli factors makes it a challenge to interpret the phenotypes of knockout mice. Surprisingly, mutant $Gli1^{zfd/zfd}$ mice (homozygous for a mutation that removes the Gli1 zinc finger domains, and thus the DNA binding activity) are viable and appear normal (40). $Gli2^{zfd/zfd}$ mutants, however, exhibit perinatal lethality and have multiple developmental defects, including skeletal, neural, and lung malformations (36, 37, 40-42). Spontaneous *Gli3* mutants were originally designated Xt (or XtJ), for extra-toes, referring to the gross polydactyly and syndactyly phenotype observed in homozygotes (43). In addition, $Gli3^{-/-}$ embryos display brain, skeletal, and lung defects, and none survive beyond postnatal day 2 (P2) (32, 37, 39, 43).

Various genetic combinations of *Gli* alleles indicate some level of functional redundancy. Despite the lack of a gross phenotype in $Gli1^{zfd/zfd}$ mice, the additional removal

of one copy of *Gli2* (*Gli1*$^{zfd/zfd}$;*Gli2*$^{+/zfd}$) provokes phenotypes similar to *Gli2*$^{zfd/zfd}$, although not as severe. *Gli1*$^{zfd/zfd}$;*Gli3*$^{+/-}$ double mutants phenotypically resemble *Gli3*$^{-/-}$ mice. These results are consistent with Gli2, but not Gli3, compensating for loss of Gli1 function during development (40). Mutations in *Gli2* and *Gli3* affect the development of many of the same systems/organs (skeletal, neural, lung). Analysis of double mutants indicates that each has both shared and specific functions. For example, *Gli2* and *Gli3* mutants both show disruptions in tooth and palate development, but otherwise affect different subsets of the skeletal system. *Gli2*$^{zfd/zfd}$;*Gli3*$^{+/-}$ mice show synergistic malformations in several skeletal elements, consistent with partially redundant functions of Gli2 and Gli3 during skeletal development (37). Analogous synergistic effects have been observed in lung and foregut development (36).

Multiple additional factors in the Hh signaling network which have been identified and described in the literature will not be discussed in detail here, as their specific significance to the work described herein is minimal. However, any background on Hh signaling would be incomplete without at least a brief mention of the critical role of the primary cilium in pathway signal transduction. The primary cilium is a non-motile structure that can be found on most cell types. Cilium structure depends on a microtubule scaffold which is nucleated from the basal body and extends in a “9+0” configuration. Proteins and vesicles are transported along the cilia by kinesin (anterograde) and dynein (retrograde) motors together with intraflagellar transport (IFT) complexes (44). In 2003, a report by Huangfu et al first identified the requirement for IFT proteins in Hh signal transduction (45). Recent work has shown that multiple Hh signaling pathway components are dependent on the primary cilium for proper regulation. In the absence of Hh signaling, Ptc1 is localized to the membrane along the shaft and around the base of the cilium. When Ptc1 binds Shh, it disappears from the cilium, whereupon Smo begins to localize and concentrate at the cilium (27, 46). Other

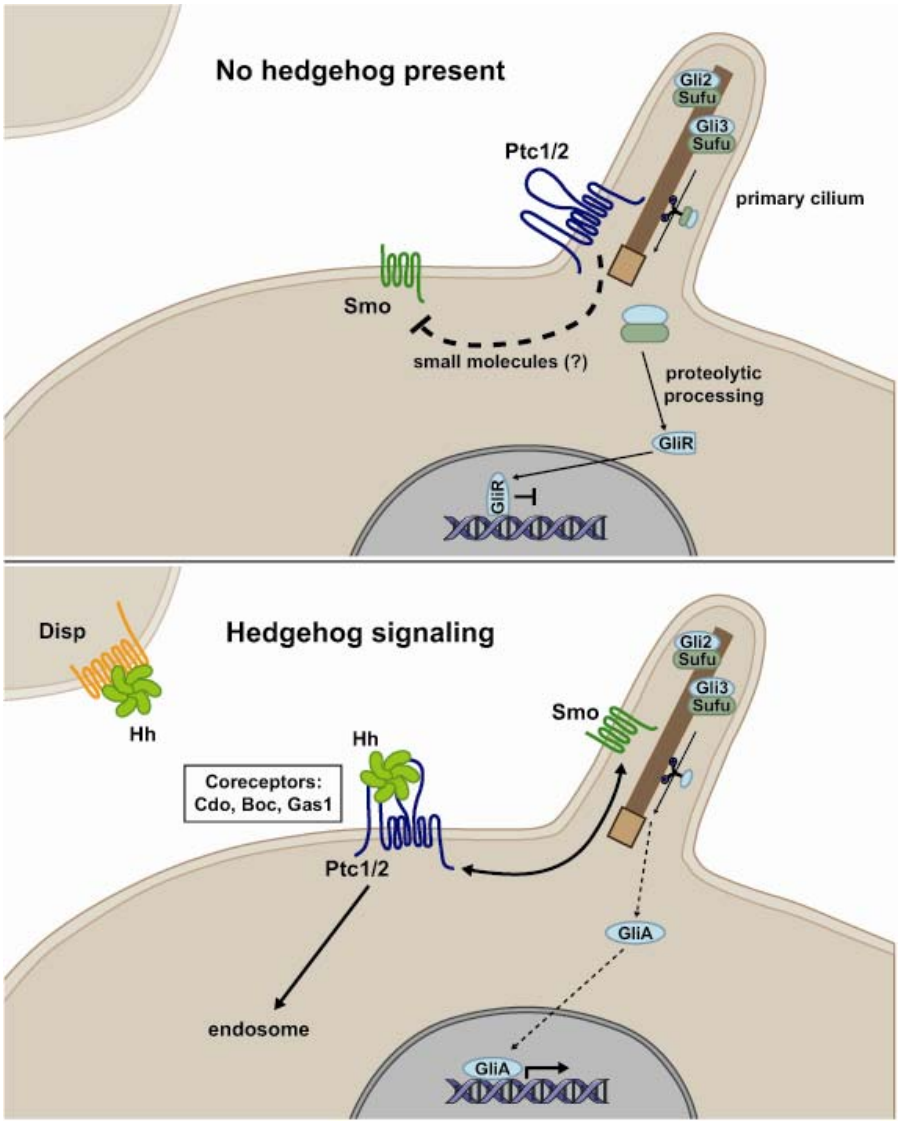


**Figure 1**. *Simplified schematic of mammalian hedgehog signaling*. When no hedgehog ligand is present, Ptc receptors are localized to the shaft and base of the primary cilium. Gli2 and Gli3 transcription factors are bound by suppressor of fused (Sufu), which prevents the proteins from being degraded (47). In the absence of ligand stimulation, Gli3 undergoes limited proteolysis to the shorter repressor form (GliR), which blocks the transcription of Hh target genes. When Hh ligands are produced, they undergo lipid modifications and aggregate into a multimeric protein complex. Release of ligand from Hh-producing cells is assisted by Dispatched1 (Disp) (48, 49). Hh binding to Ptc receptors causes them to move out of the primary cilium, whereupon Smo begins to localize and concentrate at the cilium. Downstream of activated Smo, activator forms of Gli transcription factors (GliA) enter the nucleus and regulate the expression of target genes.

Hh pathway components, such as Suppressor of Fused (Sufu) and the Gli transcription factors, are also found at the primary cilium. Levels of Gli2 and Gli3 at the cilium increase following Hh ligand stimulation (47), and ciliary localization is required for proper activity of both the repressor and activator forms of Gli2 and Gli3 (50, 51). Collection of pathway components at the cilium may therefore be a mechanism which a) facilitates Hh signal transduction by bringing pathway components into close proximity and b) provides an additional level of regulation by controlling the localization of specific factors.

*Hedgehog signaling in the vasculature*

As is evident from the variety of defects observed in mutant mice with disrupted Hh pathway function, proper Hh signaling is critical for multiple developmental processes. Among them, Hh signaling has been linked to both vasculogenesis and angiogenesis. Mouse embryos lacking Smo have severe defects in endothelial tube formation, despite normal numbers of angioblasts (52). Ihh signaling is critical for proper yolk sac angiogenesis, involving assembly of endothelial cells and SMCs into functional channels. *Ihh*$^{-/-}$ yolk sacs display small, disorganized vessels at E10.5. *Smo*$^{-/-}$ yolk sacs arrest at an even earlier stage, having failed to undergo any remodeling of the primitive vascular plexus (53).

A relatively recent body of work indicates that Shh signaling also plays essential roles in the development and maintenance of the coronary vasculature. Cells that comprise the coronary vessels (including endothelial cells, smooth muscle cells, and fibroblasts) are derived from the proepicardium during embryonic development. When proepicardial cells contact the myocardium, they migrate as an epithelial sheet to form the epicardium, which covers the surface of the heart. Myocardial-derived signals initiate epicardial epithelial-to-mesenchymal transition (EMT) and the mesenchymal cells migrate through the matrix-rich subepicardial space, infiltrate the myocardial wall, and begin to assemble the coronary

plexus (54, 55). Lavine et al. reported that *Shh* is expressed in the epicardium at E12.5 and E13.5, during a critical period of coronary plexus growth. The Shh signal is received by perivascular and myocardial cells, which respond by upregulating Hh target genes, including *Ptc1* and *Vegf-A*. The production of VEGF ligands by perivascular and myocardial cells is necessary for proper development of the coronary arteries and veins, respectively (56, 57).

Although vessels in adult mice do express components of the Hh signaling pathway, *Ptc1-lacZ* activity decreases by post-natal day 14 and is maintained at low levels through adulthood ((58) and Chapter II). Remarkably, even this low level of signaling is critical for cardiac function in adult mice. Concurrent genetic ablation of Hh signaling in cardiomyocytes and vascular smooth muscle cells caused severe cardiac dysfunction and loss of coronary microvasculature by five days (59). The Shh signaling pathway can also be robustly upregulated in adult vasculature by the administration of exogenous Shh. Interestingly, the Shh-responsive cells in adult vessels have been identified as interstitial fibroblasts and adventitial cells. In a mechanism similar to that observed in coronary development, the interstitial mesenchymal cells respond to Shh by upregulating angiogenic growth factors such as VEGF-1, angiopoietin-1, and angiopoietin-2 (58). In mouse models of hind-limb ischemia, diabetic wound healing, and myocardial ischemia, delivery of Shh ligand promoted angiogenesis (58, 60, 61).

*Hedgehog signaling functions in progenitor cell maintenance and proliferation*

The involvement of Hh signaling in the establishment, survival, and proliferation of progenitor cells has been observed in multiple tissues both during development and in adulthood. For example, progenitor cells in the adult liver can be identified by *Ptc1* expression. When these cells are isolated and cultured, treatment with cyclopamine, which blocks Hh signaling, induces apoptosis (62). There have also been reports linking Hh signaling to progenitor cell maintenance in the hippocampus and subventricular zone of the

brain (63), progenitor cell self-renewal in mammary epithelium (64), and progenitor cell proliferation in the gut (65), inner ear (66), and skin (67). An elegant model was proposed by Yu et al. for regulation of smooth muscle cell (SMC) progenitors by Shh in the developing kidney and ureter. They found that ureteral mesenchymal cells located immediately adjacent to a source of Shh protein were maintained in an undifferentiated state and that these cells were absent in mice that lacked Shh signaling in the ureter. Furthermore, Shh also had a proliferative effect on SMC progenitors in the ureter, such that mice lacking ureteral Shh showed defective SMC differentiation (68).

*Vascular precursors during development*

The development of all blood vessels begins with a primitive vascular network that is assembled from endothelial cells (vasculogenesis), extended (angiogenesis), and extensively remodeled (69, 70). Endothelial cells themselves arise from mesodermal progenitors known as angioblasts and hemangioblasts. Multiple signaling pathways influence angioblast differentiation, including VEGF and basic fibroblast growth factor (bFGF) (69). Endothelial cells undergo further differentiation, including the adoption of either an arterial or venous identity (70).

A critical step in the maturation of nascent vessels is the addition of supporting mural cells. Diffusible factors secreted by endothelial cells recruit nearby mesenchymal cells, which migrate toward and cluster around the endothelial tube. Knockout mouse studies have shown that PDGF-B is critical for this process, as mice deficient in PDGF-B or PDGFRβ develop fatal hemorrhages due to mural cell deficits (71-73). Mural cells have diverse embryonic origins, reflective of the local mesenchymal tissue from which they are recruited during development. In vessels that develop into major arteries and veins, the mesenchymal cells differentiate into smooth muscle cells, which assemble into multiple layers around the vessel. Mural cells known as pericytes associate with small vessels

(including arterioles, capillaries, and venules). Pericytes usually form no more than a single, often discontinuous, layer around the endothelial tube (69, 74).

*Progenitors for cells of the postnatal vasculature*

In postnatal tissue, multiple triggers can elicit an angiogenic response. Increasing evidence supports the idea that endothelial and mural cells incorporated during the growth of new vessels can derive not only from preexisting differentiated cells, but also from vascular progenitor cells found throughout the body. Precursors for both endothelial cells and smooth muscle cells have been identified in the blood, bone marrow, and resident within tissues such as adipose, heart, skeletal muscle, and vessel wall (75).

Endothelial progenitor cells (EPCs) were first isolated from the mononuclear cell fraction of adult peripheral blood on the basis of CD34 and Flk-1 expression (76). Circulating endothelial progenitor cells were subsequently found to be derived from mobilized bone marrow-resident progenitors (77). Physiological stimuli such as exercise, as well as pathophysiological events like vascular injury and acute myocardial infarction, can provoke the mobilization of EPCs from the bone marrow (78). In addition, there is evidence suggesting that non-bone marrow sources contribute substantial numbers of replacement ECs in transplant arteriosclerosis (79) and circulating EPCs in response to tissue ischemia (80). In the latter study, liver and small intestine transplantation experiments indicated that these organs also harbor tissue-resident EPCs that can be mobilized into circulation, home to sites of ischemia, and contribute to tissue repair (80).

Like EPCs, smooth muscle progenitor cells (SMPCs) have been identified both in the circulation and resident within tissue. Unsurprisingly, the bulk of interest in SMPCs has centered on their contribution to disease states. SMCs are a key component of vascular lesions in atherosclerosis, transplant arteriopathy, vein graft arterisclerosis, and restenosis

after angioplasty (81). However, the relative contribution of various sources of SMPCs remains controversial and may depend on the model in question (82).

There have been several reports of putative SMPCs isolated from postnatal tissue according to various criteria. Majka et al. isolated non-side population (SP) cells from skeletal muscle. These non-SP cells had a spindle-shaped morphology and expressed smooth muscle α-actin, CD45, platelet derived growth factor (PDGF) receptor β, and variable levels of Sca1 and CD34, but did not express desmin. The isolated cells differentiated to smooth muscle *in vivo* when transplanted into skeletal muscle undergoing injury-induced neovascularization. A bone marrow transplantation assay indicated that the skeletal muscle non-SP cells were largely derived from bone marrow (83). Progenitor cells with both endothelial and smooth muscle potential were isolated from the aortas of adult mice by Sainz et al. In this study, the authors focused on SP cells isolated from the aortic media that expressed Sca1 and low levels of c-kit and CD34. The arterial SP cells differentiated to SMCs *in vitro* in response to transforming growth factor β (TGF-β) or PDGF-BB treatment and formed vascular-like structures when cultured on Matrigel (84). There are reports of SMPCs resident within other tissues as well. However, there is a general lack of consistency in the methods and criteria used to isolate tissue-resident SMPCs, consistent with some degree of heterogeneity among the different populations.

*The perivascular progenitor cell niche*

Recently, the model of a perivascular niche for various populations of progenitor cells has generated much interest and excitement. Conceptually, it is an elegant design. Vessels penetrate all types of tissue throughout the body, thus progenitor cells positioned in a perivascular location would be perfectly positioned to move into the surrounding tissue, migrate along the “track” provided by the vessel, or enter the circulation for homing to a distant site. Progenitor cells considered to reside in a perivascular niche may be found

closely apposed to the endothelial cells of the microvasculature or embedded within the multilayered wall of larger vessels.

Pericytes, while traditionally considered a type of mural cell that closely associates with microvascular endothelial cells, are increasingly regarded as functional progenitors themselves. Their differentiation potentials are strikingly similar to those reported for non-bone marrow multipotent mesenchymal stromal cells (MSCs, also known as mesenchymal stem cells) (85). Indeed, recent reports strongly support the idea that MSCs in multiple tissues localize to a perivascular region and may in fact be indistinguishable from pericytes (86-88). Other types of tissue-resident progenitor cells have also been reported to reside within close proximity to blood vessels, including undifferentiated spermatogonia (89), muscle satellite cells (90), and self-renewing brain tumor stem cells (91). In the latter two cases, depletion of capillary vessels reduced the number of progenitor cells, indicating that the perivascular region provides a critical niche environment for maintenance of normal progenitor cell function.

Two examples of progenitor cells residing within the vascular wall of large arteries were provided by Hu et al. and Zengin et al. In work published in 2004, Hu et al. reported that the aortic root adventitia of ApoE-/- mice harbored a significant population of $Sca1^+$ smooth muscle progenitor cells (92). These cells, which we have termed adventitial $Sca1^+$ progenitor cells (AdvSca1 cells) are further discussed in Chapters 2 and 3. A complementary study by Zengin et al. reported that $CD34^+$ EPCs can be isolated from human internal thoracic arteries. Interestingly, these cells were localized in a specific region just outside the external elastic lamina, between the media and adventitia, that the authors termed a “vasculogenic zone”. Progenitors from the vasculogenic zone formed capillary sprouts *ex vivo* and also displayed macrophage differentiation potential (93). The degree of relationship between AdvSca1 cells and human $CD34^+$ cells of the vasculogenic

zone is unclear. However, these studies highlight the adventitial layer of large arteries as an important progenitor cell residence.

*Structure and cellular composition of arterial adventitia*

Surrounding the outside of major vessels is the adventitial layer, which is found immediately adjacent to the external elastic lamina. The adventitia is commonly portrayed as a loosely organized, extracellular matrix-rich layer of the vessel wall composed primarily of fibroblasts. However, multiple cell types in addition to fibroblasts reside within the adventitia of normal arteries. In large muscular arteries, endothelial cells and pericytes

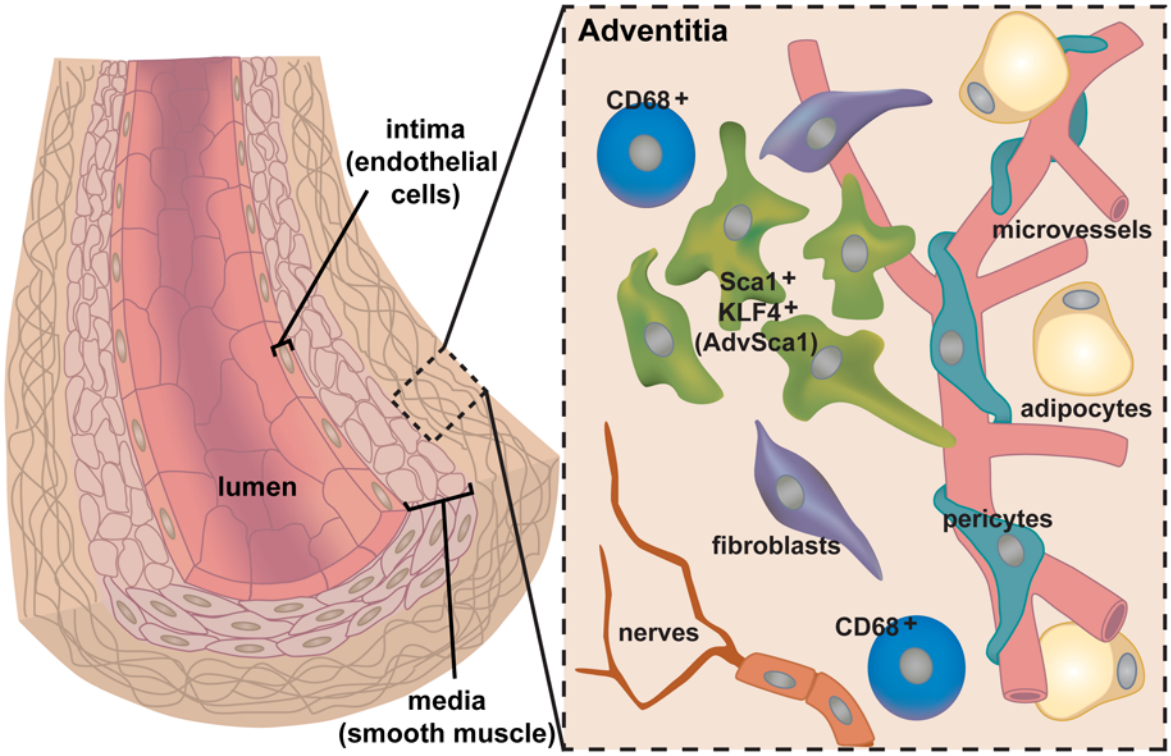


**Figure 2**. *Structure of the artery wall and cell types within the adventitia*.

within the adventitia form a microvasculature network, the vasa vasorum, that penetrates and nourishes the medial and intimal layers of the artery wall (94-96). The adventitia is also

home to perivascular nerves, lymphatic vessels, resident monocytes, macrophages, B and T lymphocytes, and dendritic cells (97, 98). Peridadventitial adipose tissue is found closely surrounding and interspersed with the adventitia.

In comparison to the intima and media, the adventitia has historically received relatively little attention, thus our knowledge about the developmental timing and origins of adventitial cells is limited. A 1997 study by Lee et al reported that in developing chicken aortas, the ratio of the number of medial cells to adventitial cells gradually increases between days 6 and 16 of embryonic development. The proliferation rate in the two compartments exhibits a similar trend, with a media:adventitia ratio of 1:2 from days 6-12 and 3:2 after day 16 (99). The authors used various antibodies against smooth muscle marker proteins to delineate the media, and any surrounding cells were considered adventitia. Thus, much is yet unknown about the behavior of any particular cell type in the adventitia, or how this vessel layer assembles in mammalian systems.

*Adventitial response to vascular injury and contribution to neointima*

Adventitial cells play a key role in vascular wall growth, remodeling, and defense against infection (95, 97). Experimental data shows that the adventitia responds rapidly and dramatically to vascular injury and stress. One of the earliest vascular responses to multiple forms of vascular insult is an increase in adventitial cell proliferation leading to increased adventitial density and development of neoadventitia. This type of adventitial response has been observed in hypertension (100, 101), pulmonary hypoxia (102), and various injury models, including arterial flow reduction (103) and overstretch (104, 105).

Another common adventitial response, particularly to vascular injury, is the adoption of a “myofibroblast” phenotype. In these instances, phenotypic changes may include the expression of SMC marker genes, increased migration, and increased production of extracellular matrix proteins such as collagen. In a balloon injury model of porcine coronary

arteries, adventitial cells in uninjured vessels are negative for smooth muscle α-actin (SMαA) expression. However, at 7 and 14 days after injury cells within the adventitia exhibit strong SMαA staining (104). A similar pattern is observed in rabbit carotid arteries after a balloon injury that does not disrupt medial elastic laminae. At 4 days after injury, adventitial cells expressing SM22α were localized primarily in the inner region of the adventitia (105).

Changes in vascular structure following injury or during atherosclerotic plaque development include both geometric changes in actual vessel diameter and the development of a neointima. It is logical that the adventitia, being the outermost layer of the vessel wall, would play an important role in regulating changes in vessel diameter. Indeed, this is supported by experimental evidence from Labinaz et al, who found that neoadventitia development precedes arterial contracture in balloon-injured porcine coronary arteries (106).

More controversial is the question of whether adventitial cells can participate in neointima formation. In injury models that provoke neointimal development, the results have been mixed. Following mild balloon injury (no disruption of elastic laminae) in rabbit carotid arteries, the authors observed some adventitia-derived cells capable of penetrating the adventitia-media boundary, but did not find evidence that they contributed to neointima formation (105). However, adventitia to neointima migration was observed in a model of severe injury with a complete disruption of the medial layer and exposure of adventitial cells to the lumen (107). Perhaps the most direct evidence of endogenous adventitial cell participation in neointima formation comes from a study by Siow et al in which they directly labeled adventitial cells with adenoviral lacZ. Following balloon injury (without medial dissection) in rat carotid arteries, β-galactosidase-positive cells moved through the medial layer and were found in the neointima by 7 days after injury (108). The variation in results between individual studies could be attributable to differences in the model organism, arterial bed, severity of vascular injury, and method of adventitial cell labeling/detection. A common assumption is that the adventitial cells contributing to the neointima began as

fibroblasts that have undergone a transition to a “myofibroblast” phenotype in response to injury. Yet the labeling methods used are not specific to any particular adventitial cell type, thus we can not say conclusively that “myofibroblasts” are the only adventitial cells participating in neointima formation.

*Transcriptional regulation of smooth muscle cell gene expression*

As cells differentiate to SMCs during development, they begin to express markers characteristic of a SMC phenotype. Common SMC marker genes include SMαA, calponin, SM22α, smooth muscle γ-actin, and smooth muscle myosin heavy chain (SM-MHC) (109). SMαA is one of the first genes to be expressed during SMC differentiation. It is highly abundant in SMCs (up to 40% of total protein) (110) but is also expressed by other SMC-like cell types and is not considered a marker specific to fully differentiated SMCs. At the other end of the spectrum is SM-MHC, which is highly selective for actual SMCs (111).

The transcription of most smooth muscle marker genes is controlled by one or more CArG elements (transcription factor binding sites with a “CC(A/T-rich)GG” motif) found within promoter and/or intronic sequences. CArG elements are bound by homodimers of the transcription factor serum response factor (SRF). Structurally, SRF shares homology with members of the MEF2 family of transcription factors. All include an N-terminal MADS box comprised of a DNA-binding domain, a dimerization domain, and an interface for protein-protein interactions (112). SRF is widely expressed and can regulate cardiac and skeletal muscle genes as well as growth-responsive genes such as *c-fos* (113). SRF-null mice display severe gastrulation defects and do not develop mesoderm, illustrating the widespread importance of this transcription factor (114).

Because SRF is not intrinsically specific for SMC genes, additional mechanisms of regulation must be present in order to achieve specificity and precise control of SMC gene transcription. There is a growing list of proteins that interact with SRF to modulate its

transcriptional activity. These can act as co-activators or co-repressors with regards to SMC gene transcription. Some, such as mHox (115) and Nkx3.1 (116), influence SRF binding to CArG elements. Others associate with the SRF dimer through formation of a ternary complex to either augment (ex. myocardin family members (117) and Csrp1 and -2 (118)) or inhibit (ex. Elk1 (119), Msx1 and -2 (120)) the transcriptional activity of SRF. In the case of myocardin family members MRTF-A (MAL) and MRTB-B, the actin cytoskeleton serves as an additional point of control. MRTF-A and –B bind to actin and are released for nuclear translocation (and thus interaction with SRF) by actin treadmilling (121-123).

Other factors have been shown to act indirectly to inhibit SRF-dependent SMC gene expression. For example, Foxo4 strongly interacts with myocardin, repressing its ability to serve as an SRF co-activator (124). The Krüppel-like factor Klf4 also potently inhibits myocardin-induced activation of SMC genes, both by inhibiting myocardin expression (125) and potentially by recruiting histone deacetylases to regulatory regions of SMC genes (126).

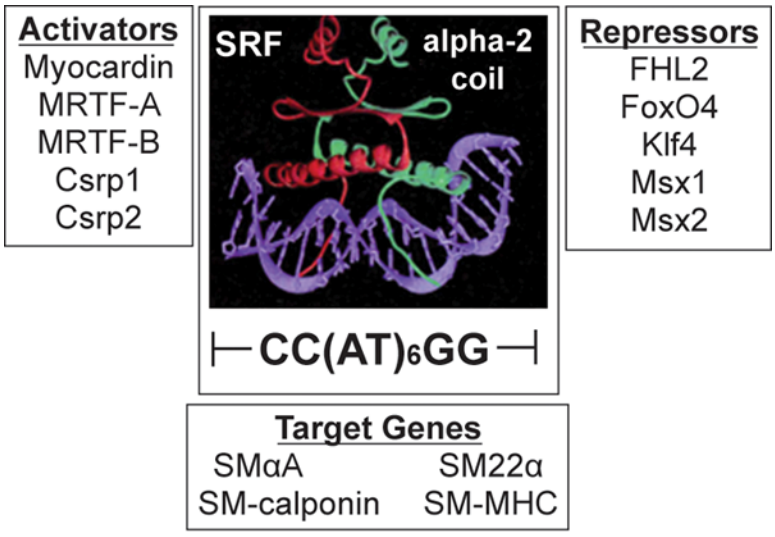


**Figure 3**. *Serum response factor and cofactors*. SRF binds as a homodimer to the consensus CArG element in target gene promoters. The transcriptional activity is modulated by the interaction of co-activators and co-repressors, many of which associate with SRF itself through protein-protein interactions with the MADS domain. The crystal structure of the SRF MADS domain was adapted from Pellegrini et al. (127).

## Introduction and Specific Aims

This research was conducted towards the overall goal of identifying molecular mechanisms that contribute to formation and maintenance of SMC progenitors in the adventitia of artery walls. These precursor cells, which we term AdvSca1 cells, have only recently been recognized as a novel source of SMCs in adult vessels. We hypothesized that paracrine mechanisms which maintain these cells as SMC progenitors are active within a novel signaling domain in the adventitial layer of blood vessels *in vivo*. We found that Sonic hedgehog (Shh) signaling is localized to this domain. Moreover, AdvSca1 cells are significantly reduced in number in Shh knockout mice. Wild-type AdvSca1 cells do not express SMC markers *in vivo*, yet they differentiate to SMC-like cells when removed from their native signaling environment in the artery wall. Despite the absence of SMC marker expression, a subset of AdvSca1 cells express serum response factor (SRF) and myocardin, factors thought to be sufficient for expression of many SMC marker genes. Unlike SMCs, AdvSca1 cells also express potent transcriptional repressors of SRF/myocardin-dependent transcription, including the Krüppel-like zinc finger factor Klf4. These data are consistent with a model in which AdvSca1 cells *in vivo* are poised to become SMCs, but are kept in an undifferentiated state by transcriptional silencers that prevent the expression of SMC marker genes. In addition, AdvSca1 cells are capable of propagating while maintaining a progenitor phenotype. Thus, mechanisms regulating the self-renewal of AdvSca1 cells must also be present. In addition to examining the role of Hh signaling in regulation of AdvSca1 cells, we have characterized their differentiation potential *in vitro* and *in vivo* and investigated the role of the transcriptional co-repressor Klf4 in regulating AdvSca1 self-renewal and SMC differentiation.

**<u>Specific Aim 1</u>: Examine Shh signaling in the adventitia and test for a relationship between Shh signaling and the presence of adventitial Sca1$^{+}$ progenitor cells.**

**<u>Specific Aim 2</u>: Evaluate the progenitor potential and transcriptional regulation of AdvSca1 cells *in vitro* and *in vivo*.**

**<u>Specific Aim 3</u>: Determine if maintenance of undifferentiated AdvSca1 cells depends on the transcriptional co-repressor Klf4.**

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# CHAPTER 2

## A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1$^{+}$ progenitor cells

## Introduction

An adventitia surrounds most blood vessels where it functions as a dynamic compartment for cell trafficking into and out of the artery wall. The adventitia is a site for formation and regression of microvessels that penetrate and nourish the medial and intimal layers of vessel wall (1-3). The adventitia also contains perivascular nerves and lymphatic vessels and is an important domain for NAD(P)H oxidase activity in the vessel wall (4). Adventitial cells participate in vascular growth and repair and are important determinants of lumen size via control of inward or outward wall remodeling processes (2, 5). Recent studies report unexpected roles for the adventitia insofar as it supports resident progenitor cells that can adopt both vascular and nonvascular fates (6-8). Despite these important functional properties, the signals and environmental cues that confer such unique and dynamic properties to the adventitial layer remain largely unknown.

Sonic hedgehog (Shh) is an essential morphogen and growth factor for many developing tissues (9). In the vascular system, Shh signaling is important for specification of arterial-venous identity of endothelial cells (10), remodeling of yolk sac blood vessels (11), and recruitment of mural cells (12). In adult blood vessels, Shh is angiogenic in ischemic hindlimb and corneal micropocket assays (13, 14), promotes perineural neovascularization in diabetic animals (15), and protects the myocardium against chronic ischemia (16). Shh stimulates production of angiogenic factors, including VEGF-A and angiopoietin-1 by interstitial fibroblasts (13, 14), and promotes endothelial cell chemotaxis and tube formation (17, 18). Therefore, Shh plays important roles in cell-cell communication during vascular development and adult wound repair.

Hedgehog (Hh) proteins signal by binding to Patched-1 (Ptc1) or Patched-2 (Ptc2) receptors and Cdo/Boc accessory proteins at the cell surface, resulting in derepression of Smoothened (Smo) (19). Ptc proteins are 12-transmembrane domain receptors that are structurally related to the resistance-nodulation-division (RND) family of bacterial membrane

permeases (20) and the Niemann-Pick C1-like1 cholesterol transporter (21). Ptc proteins contain a conserved sterol-sensing domain, and recent studies suggest that Ptc1 represses Smo by transporting activating sterols out of the cell (22). Hh binding to Ptc1 inhibits transporter activity, thereby allowing activating sterols to accumulate (19, 21). Activated Smo traffics into the primary cilium where Hh signal mediators are concentrated (23, 24) and triggers phosphorylation of Gli factors that then move to the nucleus and stimulate gene transcription (25). Among Hh target genes are *Ptc1*, *Ptc2*, and *Gli1*. Therefore, *Ptc1-lacZ*, *Ptc2-lacZ,* and *Gli1-lacZ* activities are used as sensitive reporters of Hh signaling in transgenic mice (26-28).

The origins and functions of the adventitia are poorly understood. Hu et al. (6) reported that stem cell antigen-1 (Sca1)-positive cells with a potential to differentiate into smooth muscle cells (SMCs) were found in the aortic adventitia of adult ApoE-/- mice (AdvSca1 cells). When transferred to the adventitial side of experimental vein grafts, AdvSca1-derived SMCs made up 30% of cells in the graft neointima after 4 weeks (6).

The adventitia responds to balloon catheter injury with rapid increases in SMα-actin (SMαA) and SM22α expression (29, 30). Endothelial progenitor cells are reported to cluster between the media and adventitia in human arteries (7, 8). These cells formed capillary sprouts in *ex vivo* ring culture assays and were recruited by tumor cells for capillary vessel formation *in vivo*. Given the role of Shh signaling in the maintenance of resident stem and progenitor cells in skin (31), nervous system (32), lymphoid tissue (33), and hematopoietic cells (34), we sought to test for similar roles for Hh signaling in the adventitial tissue surrounding blood vessels.

## Hypothesis

Active Shh signaling in the adventitia contributes to the proper development of the adventitia and to maintenance of adventitial $Sca1^+$ SMC progenitors.

## Materials and Methods

Animals Used: All protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina. Mice used include Ptc1-lacZ (Ptch1tm1Mps, Jackson Laboratories; 003081, described in (26)), Ptc2-lacZ (Ptc2tm1Dgen; Jackson Laboratories; 005827), Gli1-lacZ (Gli1tm2Alj; Jackson Laboratories; 008211), $Shh^{tm1Amc}$ (Jackson Laboratories; 003318), 129/SvEvJ and C57BL/6J (Jackson Laboratories). Noon on the day of vaginal plug was designated E0.5.

β-Galactosidase Assay: Embryos or tissues were fixed in fresh 0.2% glutaraldehyde and stained with a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal substrate at 37°C overnight. Postfixation was performed in 4% paraformaldehyde (PFA) at 4°C overnight. For histological analysis, tissue was dehydrated, cleared (Histo-Clear; National Diagnostics), embedded in paraffin, sectioned, and counterstained with nuclear fast red (Vector Laboratories).

Immunofluorescence Staining: Tissues were fixed for 1 hr in freshly prepared 4% PFA, rinsed in PBS, saturated with 20% sucrose for cryoprotection, embedded in agar, and frozen in OCT. Twelve-micrometer cryosections were fixed in methanol at room temperature for 5 min, permeabilized with 0.05% Tween-20 (in PBS) for 2 min, rinsed in PBS, then blocked in 2% normal goat serum (NGS) or normal donkey serum for 2–8 h. Sections were incubated with primary antibodies at 4°C overnight, rinsed twice in PBS + 0.1% BSA, then incubated with secondary antibodies for 2–8 h at room temperature, protected from light. Nuclei were counterstained with 10 μg/ml Hoechst 33258 in $H_2O$ and slides were mounted in Mowiol with 2.5% DABCO.

Primary antibodies used for these studies included rabbit anti-β-galactosidase (1:500; MP Biomedical 55976), goat anti-Shh (1:100; R&D Systems AF445), rabbit anti-Dhh (1:100; Santa Cruz Biotechnology sc13089), rabbit anti-SM-MHC (1:100; Biomedical

Technologies BT-562), mouse anti-SMαA (1:100; Sigma A2547), rat anti-Sca1 (1:100; BD Pharmingen 553333), rabbit anti-Ihh (1:100; Santa Cruz Biotechnology sc13088), and rat anti-PECAM-1 (1:100; BD Pharmingen 550274). All secondary antibodies were AlexaFluor-conjugated (Invitrogen) and used at a dilution of 1:400 in PBS. Immunofluorescence staining was visualized with a Leica BM IRB inverted epifluorescence microscope, with images captured by a QImaging Retiga 1300 digital camera. Confocal images were obtained with a Zeiss LSM5 Pascal laser scanning confocal microscope and processed with Image J and Adobe Photoshop.

RT-PCR Analysis: Total cellular RNA was isolated by guanidinium isothiocyanate denaturation and phenol/chloroform extraction as described (35). Two-step RT-PCR was carried out with the GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer's instructions. The sequences for primers used for RT-PCR analysis of gene expression are provided in Appendix B. Unless otherwise indicated, primer sequences were designed for this study based on mouse genomic sequence available through Ensembl (release 45, June 2007; (36)). Primers were designed with the aid of Primer3 software (v. 0.4.0) (37).

AdvSca1 Cell Count from Shh-/- Embryos: Embryos were isolated at E18.5. The aorta was isolated and divided by anatomical region into ascending and descending portions (see schematic in Figure 11E). Shh-/- embryos were pooled and matched to a control group comprised of an equal number of WT littermates. Tissue was digested with 14 mg/ml collagenase type 2 (Worthington) and 0.75 mg/ml elastase (Roche) in HBSS for 2 h at 37°C with gentle rocking. The cell suspension was filtered (70 μm), and cells in the filtrate were pelleted at 300xg, rinsed in PBS+0.5% BSA, and counted. $Sca1^+$ cells were isolated by using anti-Sca1 immunomagnetic MicroBeads and a MACS cell separation system (Miltenyi). The number of isolated $Sca1^+$ cells was determined and compared with initial cell

counts to calculate the fraction of $Sca1^+$ cells. This experiment was repeated twice with similar results. Error bars show standard error of the mean.

Cyclopamine treatment of AdvSca1 cells in culture: $Sca1^+$ cells were isolated from the adventitia according to standard protocol. Cells were cultured for 3 days to allow attachment and then cyclopamine (TRC, C988400) was added to a final concentration of 20 μM. Medium containing 0.4% DMSO was used as a vehicle control. Cells were cultured for an additional 7 days, and 10 μM BrdU (BD Pharmingen, 550891) was added to culture medium 24 hrs before fixation.

## Results

### *Hedgehog signaling in postnatal blood vessels*

We examined β-gal activity in blood vessels of Ptc1-lacZ, Ptc2-lacZ, and Gli1-lacZ knock-in mice at various developmental timepoints. LacZ activity in the aortic root and coronary arteries was first detectable at embryonic day 15.5 (E15.5), with the strongest signal present at early postnatal timepoints. In whole-mount hearts at postnatal day 2 (P2), lacZ activity was clearly present in coronary arteries, but not in myocardium or epicardium (Figure 4A-C). The ascending aorta and pulmonary trunk were strongly positive at this time (Figure 4A-C). Both Ptc1-lacZ and Ptc2-lacZ activities declined with age and were detected at low levels in coronary arteries and transverse aorta from adult mice (Figure 4D-E). In contrast, Gli1-lacZ signal in coronary arteries, aorta, and pulmonary trunk remained robust into adulthood (Figure 4F).

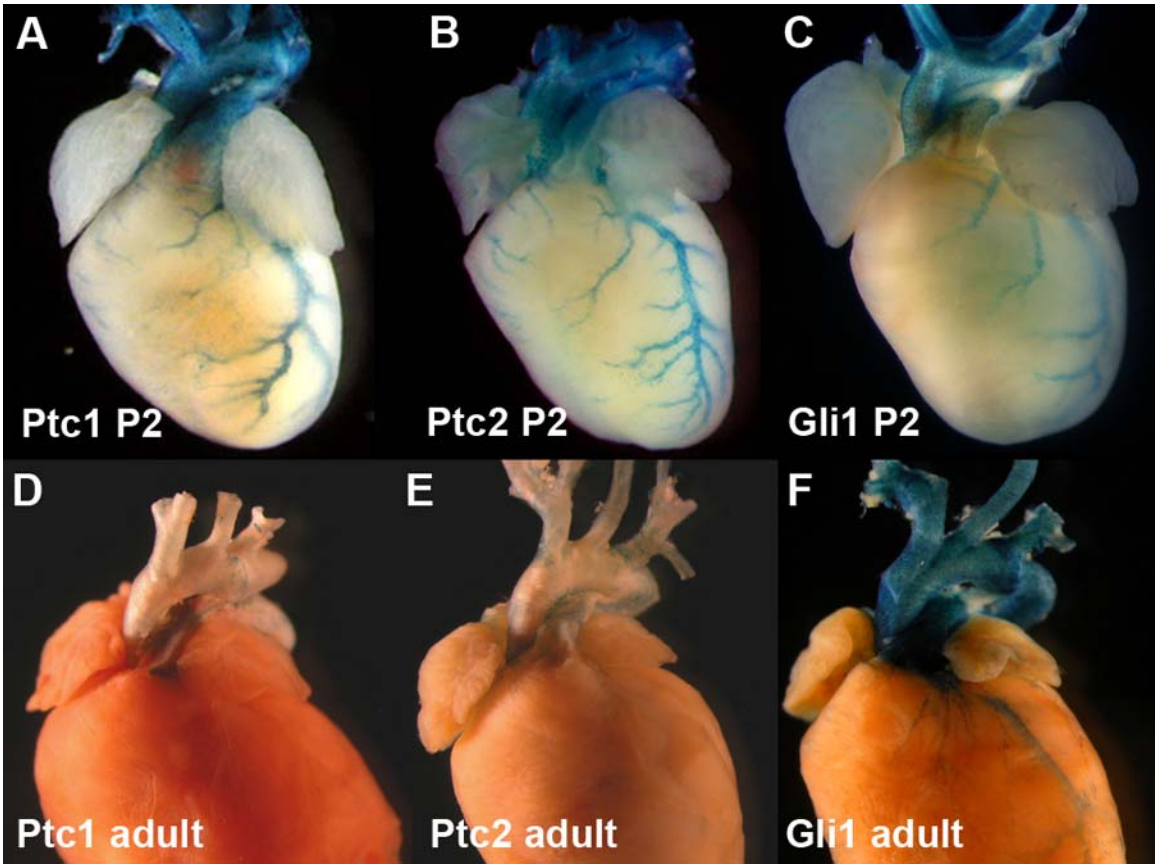


**Figure 4**. *Hedgehog signaling in coronary and outflow tract arteries*. Whole hearts were isolated from Ptc1-lacZ (A, D), Ptc2-lacZ (B, E), or Gli1-lacZ (C, F) mice at either postnatal day 2 (P2; A-C) or adult (D-E) timepoints. At P2, the aorta, pulmonary trunk, and coronary arteries exhibit strong β-galactosidase activity driven by all three promoters. By adult stages, the number of cells with active *Ptc1* and *Ptc2* gene expression has decreased dramatically, while *Gli1* continues to be expressed.

Ptc1-, Ptc2-, and Gli1-lacZ activity was present throughout the body in large and medium-sized arteries, including mesenteric (Figure 5A-C), intercostal (Figure 5D-F) and femoral (Figure 5G-I) arteries. In the mesenteric arcade, lacZ staining of arteries was more intense than that of veins (Figure 5A-C), whereas lymphatic vessels were negative. In cross-sections of the aortic root and coronary arteries at P2, it is apparent that the majority of lacZ activity is restricted to the adventitia, with only an occasional positive cell found in the media or intima (Figure 6). Thus, active Hh signaling is found in large and medium-sized arteries and veins in the perinatal period and is localized to the adventitia of these vessels.

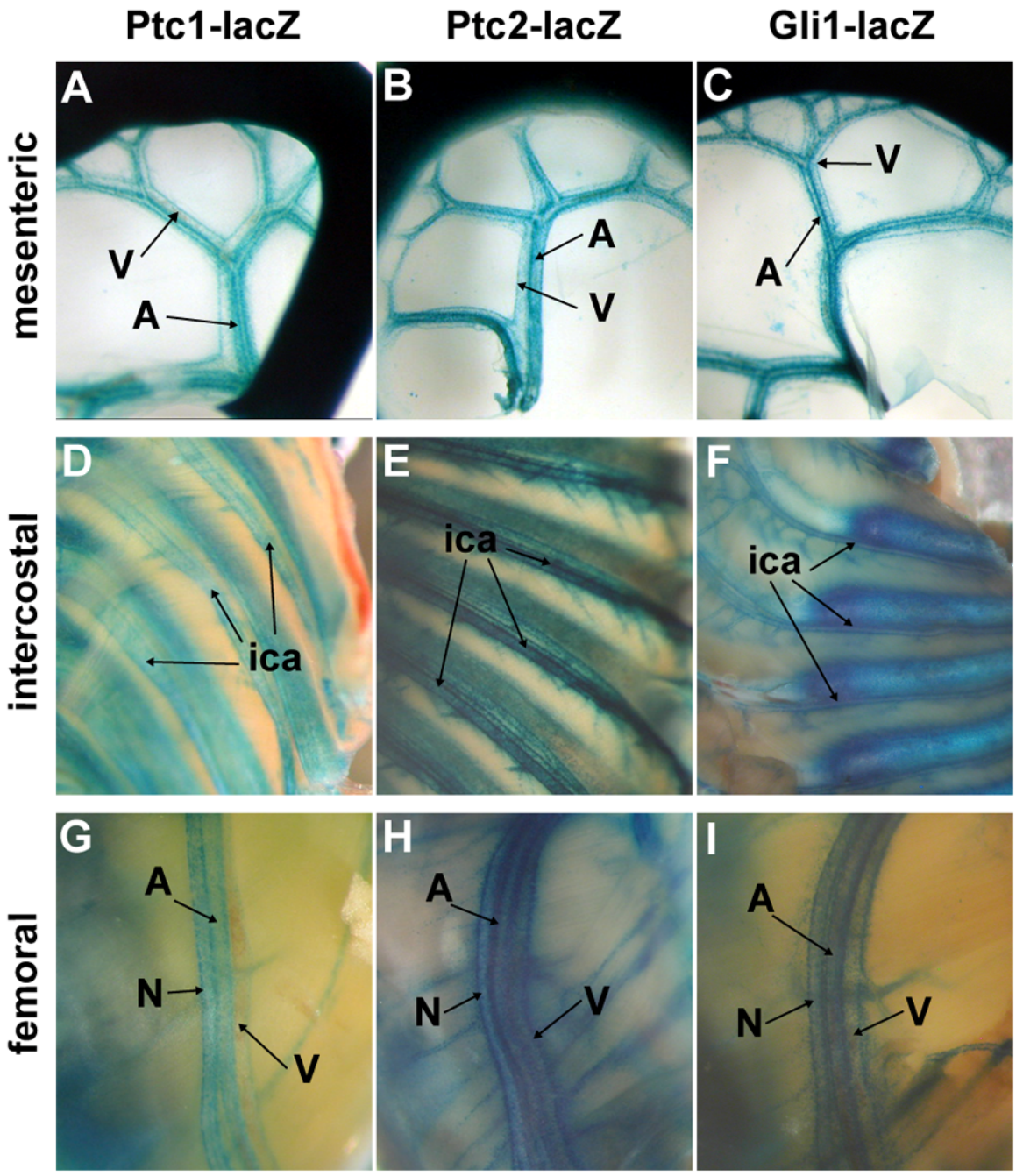


**Figure 5**. *Hedgehog signaling in other arterial beds*. Tissue was isolated from Ptc1-lacZ (A, D, G), Ptc2-lacZ (B, E, H), or Gli1-lacZ (C, F, I) mice at P2 and stained with X-gal. All major arteries examined, including mesenteric (A-C), intercostal (D-F), and femoral (G-I), were positive for lacZ expression. V, vein; A, artery; ica, intercostal artery; N, nerve.

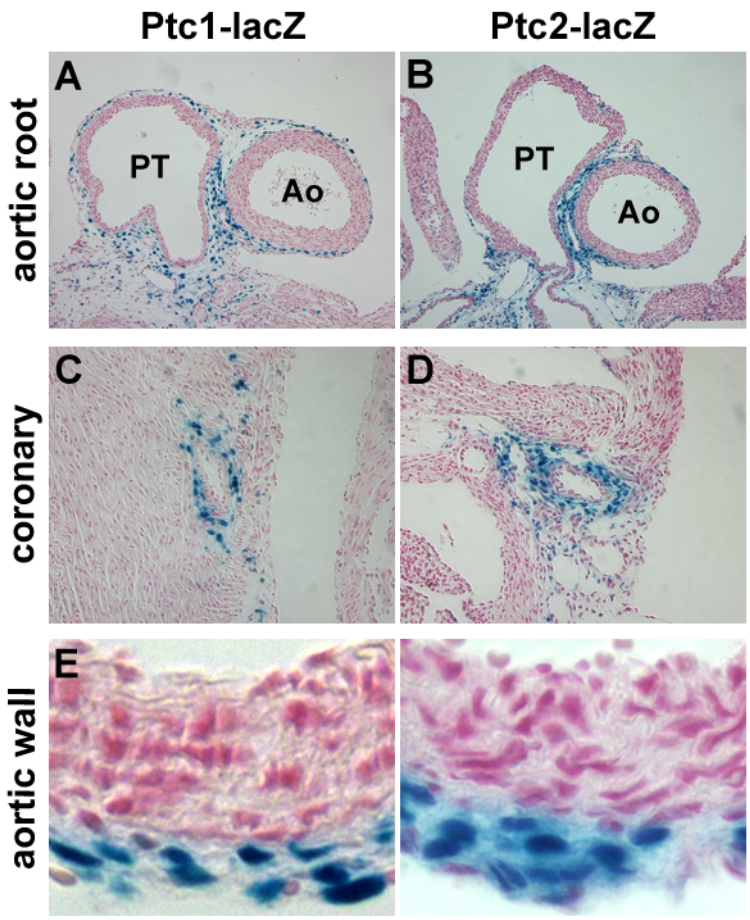


**Figure 6**. *Hedgehog-responsive cells in the adventitia of major arteries*. Tissue from Ptc1-lacZ (A, C, E) and Ptc2-lacZ (B, D, F) mice at P2 was isolated and stained with X-gal. Cross-sections through the aorta (A-B, E-F), pulmonary trunk (A-B), and coronary arteries (C-D) reveal that Hh-responsive $lacZ^+$ cells are restricted to the adventitia during early postnatal development. Staining patterns in Gli1-lacZ mice (not shown) were virtually identical. PT, pulmonary trunk; Ao, aorta.

*Sonic hedgehog in the arterial adventitia*

Shh protein was localized to the interface between the media and adventitia in large and medium-sized arteries at 2 days after birth (Figure 7). This pattern of Shh distribution colocalized with Ptc1-lacZ (Figure 7B, inset) and Ptc2-lacZ activities. We could not detect Indian hedgehog (Ihh) in postnatal arteries and found only low levels of desert hedgehog (Dhh) in the endothelium (data not shown). These results indicate that Ptc1-lacZ and Ptc2-

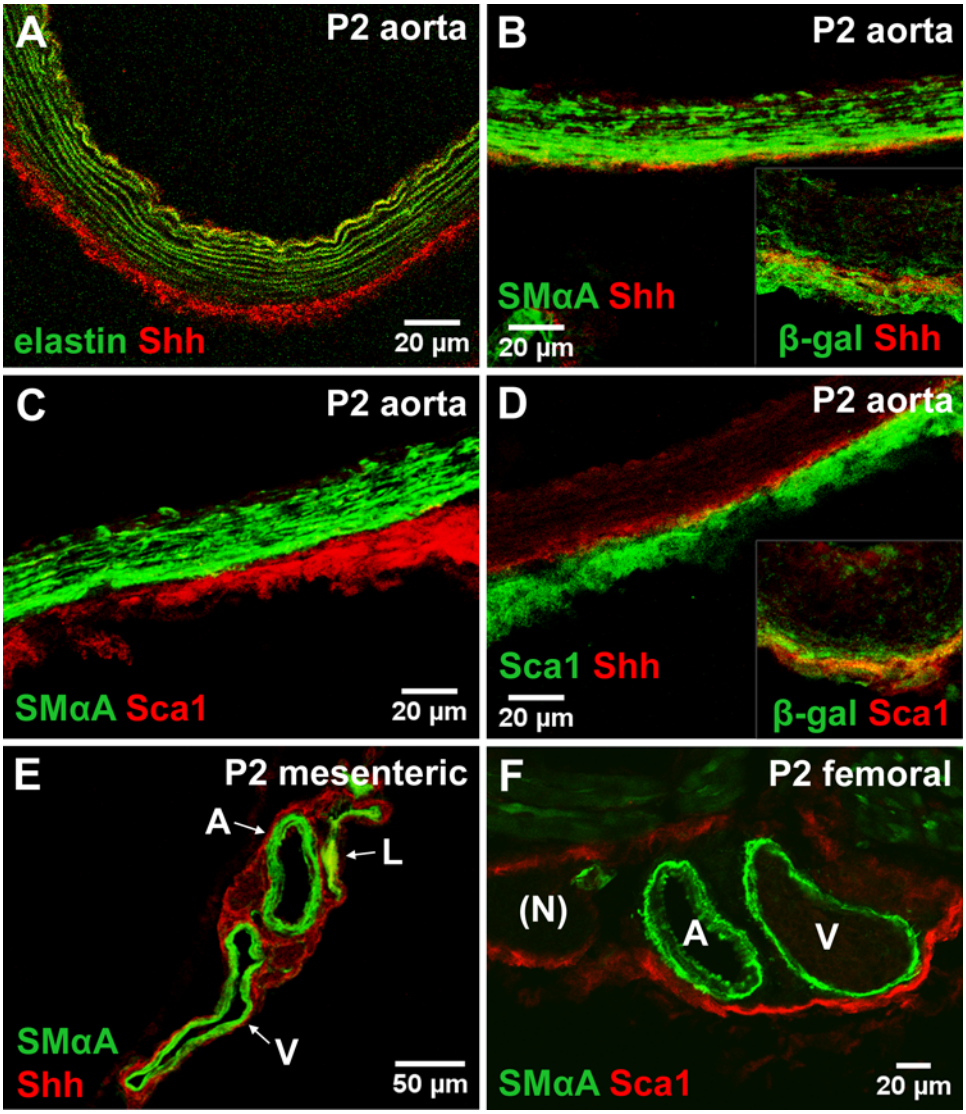


**Figure 7**. *Distribution of Shh and Sca1$^+$ cells in the adventitia.* (A) Shh protein is found outside the aortic external elastic lamina (marked by green autofluorescence) at P2. (B) Shh protein (red) is found between the media (SMα-actin, green) and adventitia of descending aorta. (B, inset) Shh (red) is colocalized with β-gal in aortic sections from Ptc1-lacZ mice. (C) Sca1$^+$ cells (red) reside outside the media (SMα-actin, green) in P2 aorta. (D) Sca1$^+$ cells (green) are found in close proximity to Shh (red) and are embedded within a β-gal-positive domain (inset) in aortic adventitia from Ptc1-lacZ mice. (E) Mesenteric vessels stained for SMα-actin (green) and Shh (red). (F) Femoral arteries stained for SMα-actin (green) and Sca1 (red). Images are stacked Z-plane sections from confocal microscopy. Adventitial localization of Sca1$^+$ cells correlates with the distribution of Shh protein and active Shh signaling *in vivo*.

lacZ activities in the artery wall correlate with localized deposition of Shh protein in a circumferential ring in the extracellular matrix (ECM)-rich space between the media and adventitia.

Despite the localization of Shh protein at the media/adventitia interface, Ptc1-, Ptc2-, and Gli1-lacZ activity suggests that only adventitial cells are Hh-responsive. We tested the expression of Hh receptors and co-receptors in isolated adult medial or adventitial cells by RT-PCR (Figure 8). The expression of *Ptc1*, *Ptc2*, *Cdo*, and *Boc* is significantly higher in adventitial cells than in media.

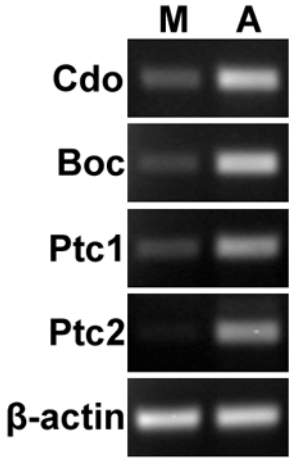


**Figure 8**. *Expression of hedgehog pathway components in media and adventitia*. RNA was isolated from adult aortic media (M) or adventitia (A) and used for RT-PCR. Key hedgehog signaling factors are more highly expressed in the adventitia.

*Sca1$^+$ cells in the adventitia*

A previous report (6) described Sca1$^+$ cells in the adventitia surrounding the aortic root in adult ApoE-/- mice. One possible role of Shh may therefore be to maintain Sca1$^+$ progenitor cells within this domain of artery wall. To gain insight into the development of AdvSca1 cells in artery walls *in vivo*, we examined mice from embryonic day 12.5 (E12.5) to

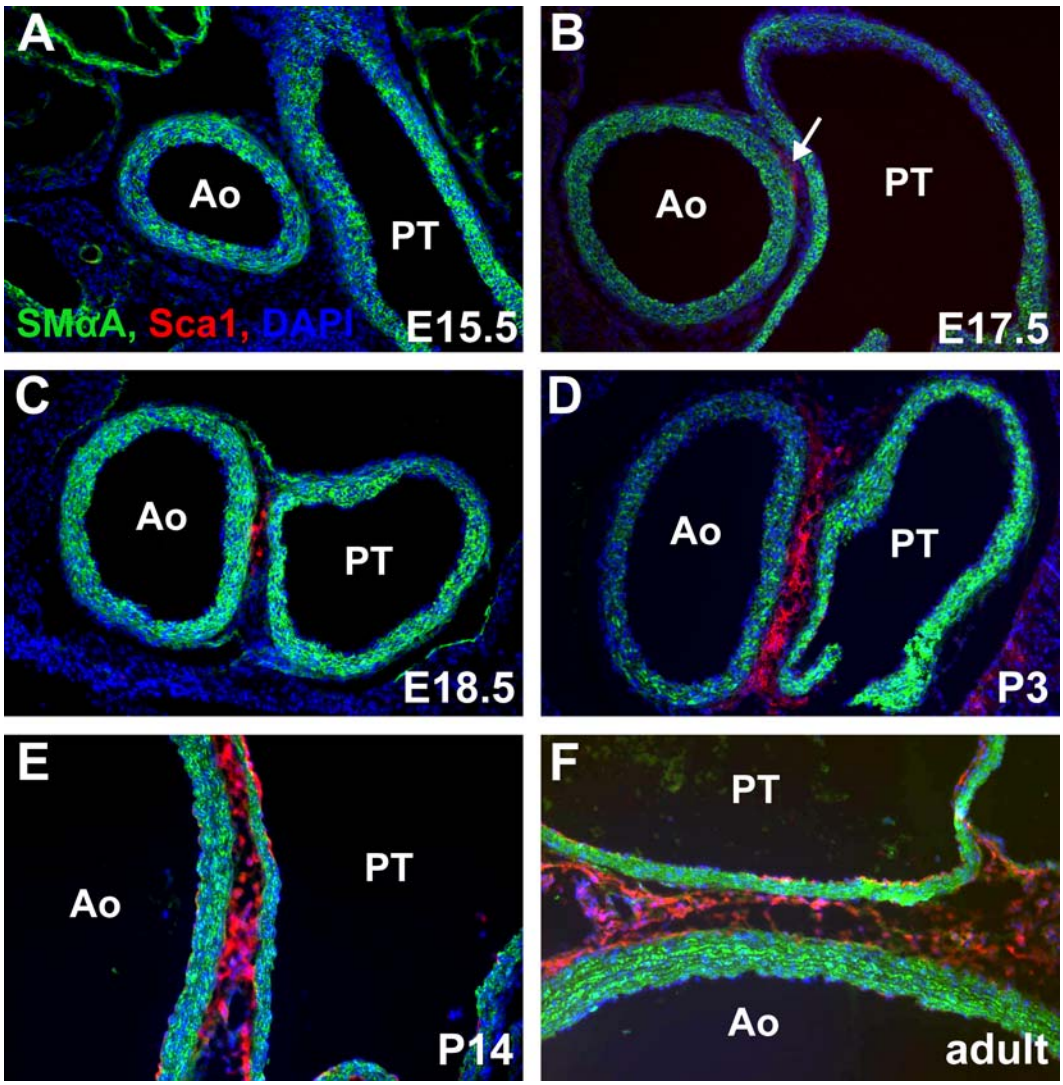


**Figure 9**. *AdvSca1 cells in developing aorta*. (A–F) Tissues were obtained at the time points indicated. Cross-sections through the aortic root were stained for Sca1 (red), SMα-actin (green), and DAPI (blue). In the adventitial space between the aorta and pulmonary trunk, AdvSca1 cells first appear between E16.5 and E17.5 (white arrow in B) and persist in this location into adulthood. Ao, aorta; PT, pulmonary trunk.

12 weeks after birth. In the perivascular space between the ascending aorta and pulmonary trunk, AdvSca1 cells first appeared between E16.5 and E17.5 (Figure 9B, arrow). This is well after the SMCs of the tunica media are established, which is evident by E15.5 (Figure 9A). AdvSca1 cells were present within the adventitia at the aortic root at all subsequent

time points examined, and they increased in number with postnatal growth of the artery wall (Figure 9C–F). As shown in Figures 7 and 9, $Sca1^+$ cells are restricted to the adventitia (AdvSca1 cells) and exhibit little or no overlap with SMα-actin-positive cells in the media. A similar distribution was found in mesenteric and femoral arteries (Figure 7F and data not shown). Close proximity was found between Shh protein and AdvSca1 cells (Figure 7D). Extensive overlap was observed between β-gal-positive cells and AdvSca1 cells in the aortic adventitia of Ptc1-lacZ mice (Figure 7D, inset). These findings suggest that a zone of Shh signaling in the adventitia identifies a unique domain of artery wall within which resident $Sca1^+$ vascular progenitor cells are found *in vivo*.

AdvSca1 cells were isolated by immunoselection and examined for progenitor cell markers. AdvSca1 cells exhibited a $CD34^+$/c-$kit^{low}$/$CD140b^+$ marker profile (Figure 10A) and were negative for CD45 and CD68 (data not shown), similar to the profile reported by Hu et al. (6). Freshly isolated AdvSca1 cells consistently expressed markers of Shh signaling including *Ptc1*, *Ptc2*, *Smo*, *Gli1*, *Gli2*, and *Gli3* (Figure 10B). The distribution of Shh in the extracellular space is governed by multiple binding and transport proteins including hedgehog interacting protein-1 (Hhip1), Cdo, and Boc (reviewed in (19)), all of which are expressed by AdvSca1 cells (Figure 10B). In addition, AdvSca1 cells contain mRNA for *Shh* and *Dhh*, with few, if any, transcripts detectable for *Ihh* (Figure 10B). These results suggest that AdvSca1 cells both produce and respond to Shh, and that the restricted distribution of Shh in the artery wall is, in part, locally controlled by factors produced by AdvSca1 cells themselves.

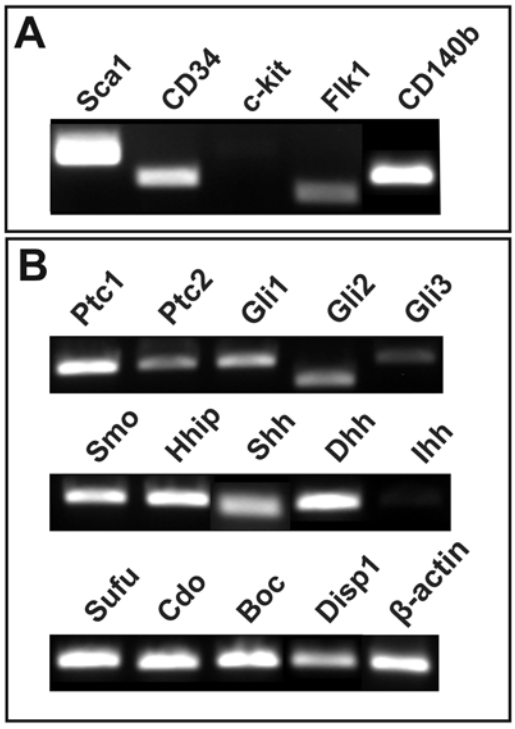


**Figure 10**. *Expression profile of isolated AdvSca1 cells*. RT-PCR analysis of (A) common stem cell markers and (B) hedgehog pathway components in immunomagnetically isolated AdvSca1 cells from adult mice. Components of a functional Hh signaling network are present in AdvSca1 cells.

*Analysis of AdvSca1 cells in Shh$^{-/-}$ mice*

To determine whether Shh was required for the formation or maintenance of AdvSca1 cells, we examined Shh-/- mice. Two anatomically defined sites were studied at E18.5, as Shh mutants do not survive birth. The presence of AdvSca1 cells at the aortic root, where they are abundant both during development and in adult mice (Figure 9 and (6)), was confirmed in wild-type mice (Figure 11A). By contrast, we could not detect AdvSca1 cells at the aortic root in Shh-/- embryos (Figure 11B). A small number of AdvSca1 cells were found in the transverse portion of thoracic aorta (Figure 11F). An absence of septation of the common truncus arteriosus in Shh-deficient embryos has been described (38).

AdvSca1 cells were present in the descending thoracic aorta of Shh-/- embryos, but always in reduced numbers (Figure 11D, F).

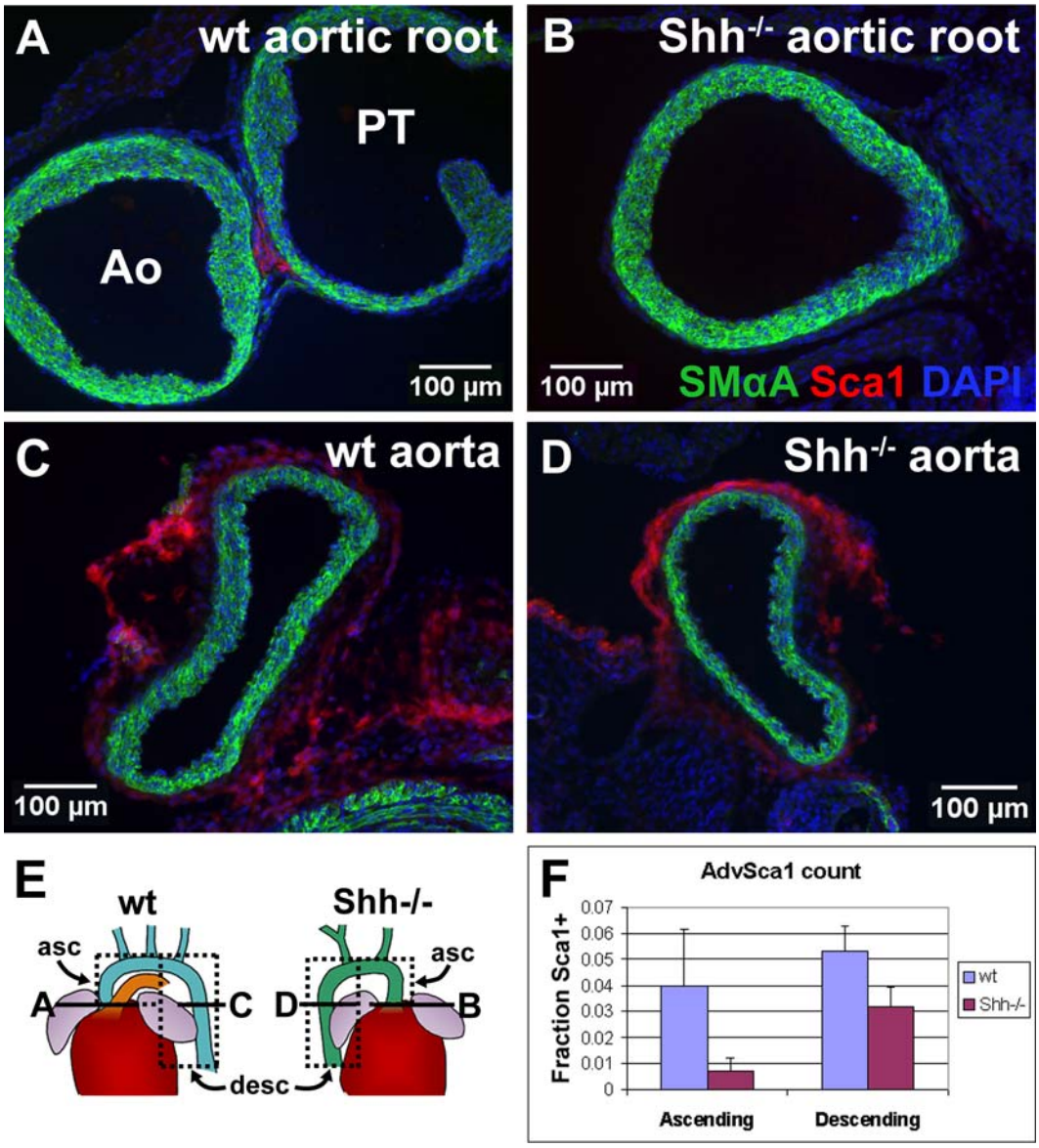


**Figure 11.** *AdvSca1 cells in Shh-/- arteries*. (A–D) Aortic tissues from WT (A and C) and Shh-/- (B and D) embryos at E18.5. Cross-sections through aortic root (A and B) and descending aorta (C and D) were immunostained for Sca1 (red), SMα-actin (green), and DAPI (blue). (E) Solid lines indicate relative positions of sections in A–D. (F) Dotted line boxes in E correspond to aortic segments shown here. AdvSca1 cells are absent in aortic root, greatly reduced in ascending and transverse aorta (termed ascending), and diminished in descending aorta of Shh-/- embryos.

In addition to AdvSca1 cells, multiple other cell types within the adventitia may be dependent on active Hh signaling during early development for proper establishment, maintenance, and/or function. In order to determine whether Shh-/- embryos exhibit a widespread defect in aortic adventitia development, we examined cross-sections through the aortic root. In regions where AdvSca1 cells are absent in Shh-/- vessels, adventitial cells are present in numbers comparable to wild-type vessels (Figure 12, compare C and D).

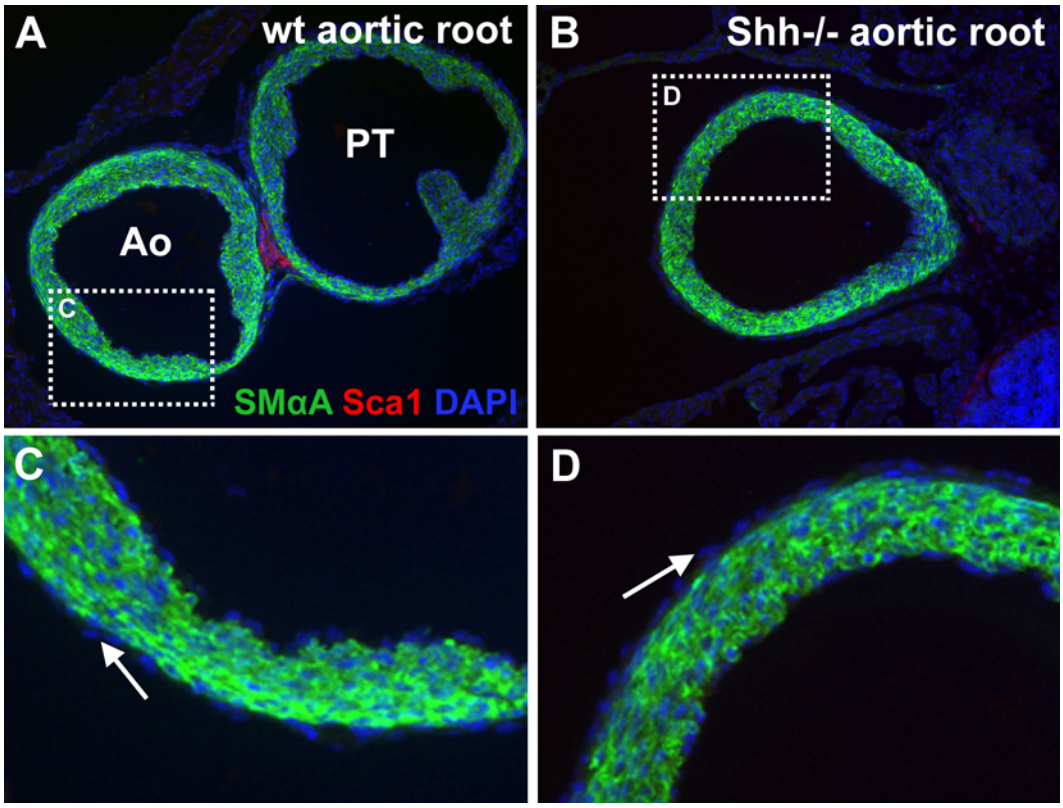


**Figure 12**. *Development of aortic adventitia in Shh-/- mice.* (A and C) Aortic root images from wild type mice at E18.5. The boxed segment in A is shown at higher magnification in C. The adventitia is identified as DAPI-positive nuclei (blue, indicated by white arrows) outside the SMα-actin-positive medial layer (green). Although the adventitial layer is still forming at E18.5, a similar number of DAPI-positive nuclei are found in the adventitia surrounding the aortic root in both WT (A and C) and Shh-/- mice (B and D). wt, wild type; Ao, aorta; PT, pulmonary trunk

The reduced numbers of AdvSca1 cells observed in Shh-/- vessels could be the result of defective recruitment of AdvSca1 cells from extravascular sites, a decrease in AdvSca1 cell proliferation within the adventitia, or a defect in AdvSca1 cell survival. We used cyclopamine, a small-molecule inhibitor of Smo activity, to test the response of AdvSca1 cells to Hh signaling blockade *in vitro*. Addition of cyclopamine to cell culture medium caused a drastic decrease in AdvSca1 cell numbers (Figure 13A-C). The proliferation index of AdvSca1 cells in the presence of cyclopamine is decreased by ~50%, as measured by BrdU incorporation (Figure 13D). There was no increase in apoptosis levels by TUNEL assay (data not shown).

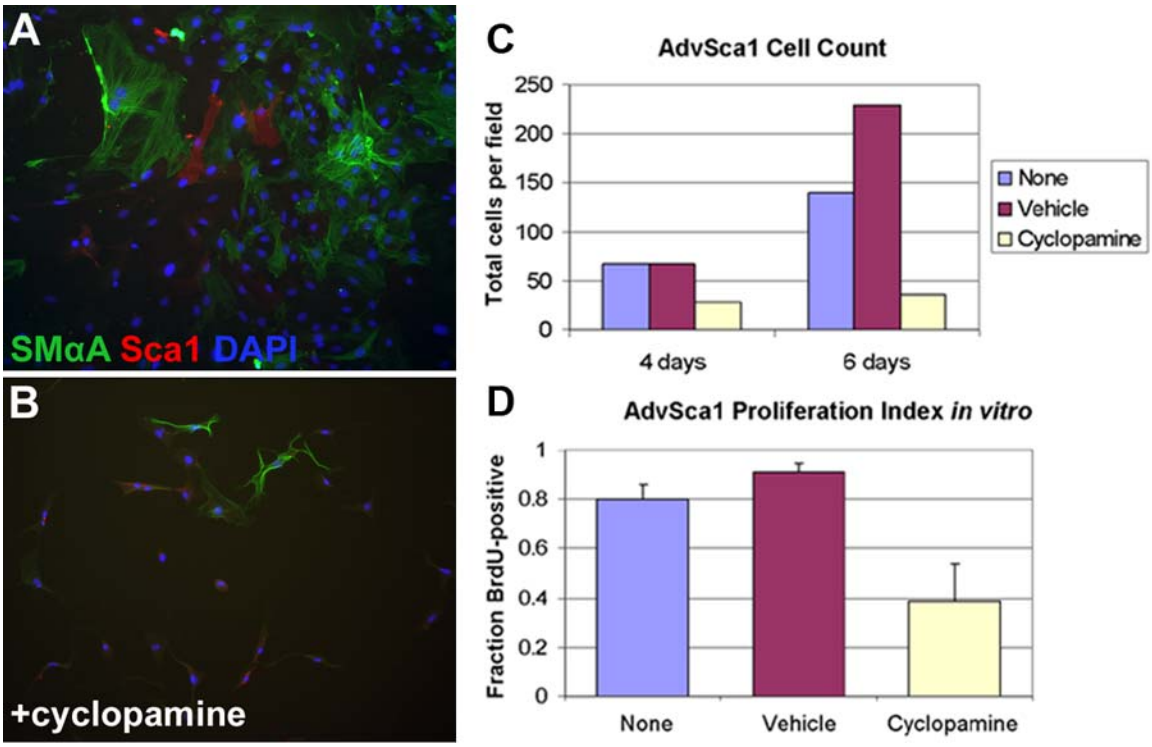


**Figure 13**. *Cyclopamine treatment of AdvSca1 cells in vitro*. (A-B) Isolated Sca1 cells were cultured under standard conditions (A) or with the addition of 20 μm cyclopamine (B). Immunofluorescence staining highlights the difference in overall cell numbers between culture conditions. The change in cell number (C) can be attributed to a reduction in the proliferation of AdvSca1 cells, as measured by BrdU incorporation (D).

## Discussion

### *Hedgehog signaling in the artery wall*

Ptc1-lacZ and Ptc2-lacZ activities in the adventitia of large and medium-sized arteries were greatest between 1 and 10 days after birth. Although the basic structure of major arteries is established at birth, substantial growth and remodeling occur in the perinatal period. For example, the number of SMCs in rat aorta increases 2.5-fold, wall thickness doubles, and collagen and elastin contents increase ~3-fold in the first month after birth (39). These changes in wall structure are adaptations to large perinatal increases in blood flow and blood pressure that are accompanied by corresponding increases in smooth muscle contractile protein mass (40). After 3 months of age, growth of the artery wall is diminished, cell proliferation rates are low, and wall structure has reached an adult state. We found Ptc-lacZ activities and Shh protein levels were maintained at low levels in adult mouse arteries, suggesting that robust adventitial Shh signaling activity correlates with rapid postnatal growth and remodeling of the artery wall.

At this time, we can only speculate about the mechanisms that maintain robust Gli1-lacZ expression in the adventitia of adult arteries, at a time when Ptc1- and Ptc2-lacZ levels are at a low baseline. A similar phenomenon was reported in a genetically-induced model of mouse prostate cancer. During tumor progression from 6-16 weeks, the expression of both *Shh* and *Ptc1* was significantly decreased. In contrast, *Gli1* expression was not significantly altered (41). Further evidence supports the notion that *Gli1* expression may differ from other Hh reporter genes, such as *Ptc1* and *Ptc2*, due to regulation by Hh-independent mechanisms. Nolan-Stevaux et al. generated mice with a genetic ablation of *Smo* in the pancreatic epithelium. Surprisingly, the expression levels of *Gli1* and *Ptc1* in the pancreatic ducts were not significantly affected by the loss of Smo activity. Based on a previous report that TGF-β signaling can induce the expression of *Gli1* and *Gli2* (42), the authors tested the effects of TGF-β treatment of *Smo-/-* cells in vitro. Both *Gli1* (4.5-fold) and *Gli3* (>25-fold)

were upregulated in response to TGF-β. However, *Ptc1* expression was decreased by 40% under these conditions (43). Given the marked upregulation of *Gli3* and its known role as a transcriptional repressor, it is tempting to speculate that the differential response of *Gli1* and *Ptc1* promoters to TGF-β treatment may be attributed to Gli3 repression of *Ptc1* expression. Yet each promoter contains a canonical Gli binding site, so it may be necessary to look beyond Gli-mediated mechanisms of regulation and conduct a more careful examination of the surrounding sequences in the promoter regions in a search for alternate transcription factor binding sites (44, 45) or regions of epigenetic regulation (46).

*Distribution of Shh in the adventitia*

Ptc1-lacZ and Ptc2-lacZ activities were colocalized with Shh protein that accumulates at the border between the media and adventitia. Once secreted, movement of Hh proteins in the extracellular space is limited and regulated by multiple mechanisms which contribute to the formation of a prototypical morphogen gradient (47). Covalent lipid modifications at both amino and carboxyl-terminal domains are required for the aggregation of Hh proteins into multimers, and thus for proper gradient formation and long-range signaling (48-53). Secreted Hh protein multimers interact with heparan sulfate proteoglycans (HSPGs), which further regulates their movement (54, 55). Chan et al. used mice carrying a mutation in the N-terminal Cardin-Weintraub motif of Shh to specifically investigate the requirement for Shh-proteoglycan interaction during development. Interestingly, disruption of Shh-proteoglycan interactions affected growth regulation in multiple organs but not tissue patterning. Neural progenitor proliferation in the developing cerebellum, spinal cord, subventricular zone, and subgranular layer of the hippocampus was significantly impaired in mutant mice. The authors further concluded that Shh-proteoglycan interactions are important both in delineating the progenitor cell niche and in modifying the response of individual cells to Shh ligand (56). Finally, there are multiple mammalian

receptors that can bind Hh ligands at the cell surface, thus limiting ligand diffusion. The expression of several of these receptors is regulated by Hh signaling, thus comprising a feedback mechanism. Ptc1 and Hhip both bind and sequester Hh ligand at the cell surface, which causes a reduction in Hh ligand sensitivity of the individual Ptc1- and Hhip-expressing cell as well as reducing the availability of free ligand to neighboring cells. Mice lacking Ptc1 and Hhip exhibited multiple defects that were consistent with an increased magnitude and range of Shh signaling (57).

Our data show that Shh protein is concentrated between the media and adventitia, outside the external elastic lamina. The sequestration of high protein levels in this region may result from local synthesis, secretion, and retention of Shh multimers by ECM components, such as the HSPG perlecan (58), in the artery wall. Ptc-lacZ-positive cells are located throughout the adventitia, whether directly adjacent to Shh protein at the media-adventitia interface or several cell layers removed. This suggests that Shh ligand is available throughout the adventitia, although it would be reasonable to expect a concentration gradient extending from the high protein levels found at the media-adventitia boundary. The immunofluorescence staining methods that we use to visualize Shh protein localization may limit our ability to detect a subtle gradient in the artery wall. Other data from our lab (not shown) suggests that multiple cell types throughout the adventitia both produce and respond to Shh ligand. Therefore, it will be interesting to examine the specific localization of ECM components within the adventitia to determine if Hh-binding components such as HSPGs are preferentially concentrated near the media-adventitia border.

*Role of Shh signaling in establishing AdvSca1 niche*

The presence of residual AdvSca1 cells suggests that factors other than Shh are involved in homing and/or maintenance of these cells. Because Dhh expression was detected in the aorta, low levels of Dhh may allow some of these cells to escape a

requirement for Shh in the artery wall. As expected, we have found in preliminary experiments (not shown) that multiple other growth factor ligands, receptors, and pathway components are expressed in the adventitia. One or more of these pathways may also partially compensate for the loss of Shh and permit some AdvSca1 cells to reside within the adventitia.

Shh-deficient embryos do not form a septation complex in the truncus arteriosus (38). We cannot at this time distinguish between direct effects caused by the loss of Shh signaling in AdvSca1 cells themselves versus indirect effects of Shh deficiency on the developing outflow arteries as the cause of the loss of AdvSca1 cells from the adventitia surrounding the vessels shown in Figure 11. The lack of truncus arteriosus septation in Shh-/- outflow tracts is a result of defective migration of cardiac neural crest cells during early stages of cardiovascular development (38). Lineage tracing experiments (see Chapter 3) indicate that AdvSca1 cells are not themselves derived from neural crest. However, this does not rule out the possibility that neural crest-derived cells secrete factors or ECM that is critical for the establishment of a functional adventitial niche.

Hedgehog signaling has been shown to function cell-autonomously to regulate multiple aspects of progenitor cell behavior. Progenitor cells in the adult liver can be identified by Ptc1 expression. When these cells are isolated and cultured, treatment with cyclopamine, which blocks Hh signaling, induces apoptosis (59). There have also been reports linking hedgehog signaling to progenitor cell maintenance in the hippocampus and subventricular zone of the brain (60) and to progenitor cell proliferation in the gut (61), inner ear (62), and skin (31). Our data now points to a role for Hh signaling in promoting the proliferation of AdvSca1 cells. In contrast to the robust effects of Hh pathway inhibition, we found that addition of exogenous Shh protein to AdvSca1 cells *in vitro* did not affect their differentiation or proliferation (Figure 19). This suggests that AdvSca1 cells in culture are exposed to adequate concentrations of Hh factors, either through self-production and

secretion or as supplied by serum-containing medium. Whether the effect of Hh pathway activity on AdvSca1 cell proliferation is cell-autonomous remains to be investigated and will likely require the development of genetic tools permitting *in vivo* cell-specific targeting of AdvSca1 cells (see Chapter 5).

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# CHAPTER 3

## Adventitial Sca1$^{+}$ cells are maintained in an undifferentiated phenotype *in vivo*

## Introduction

In many adult tissues, stem or progenitor cells are maintained locally and can be stimulated to participate in physiological and pathological tissue regeneration and repair. Vascular progenitor cells (including endothelial cell (EC) and smooth muscle cell (SMC) progenitors) have been identified in a variety of locations throughout the body, including blood, bone marrow, and adipose tissue (1).

Stem cell antigen 1 (Sca1) is a glycosyl-phosphatidylinositol-anchored cell surface protein that was originally identified as an antigen upregulated on activated lymphocytes. Sca1 expression is commonly used as a method to identify and enrich hematopoietic stem cells, although it is also expressed by a variety of stem, progenitor, and differentiated cell types (2). Defects observed in Sca1-null mice (which are viable) indicate that the protein does have a functional role in some cell types, including hematopoietic cells (3), mesenchymal stem cells (4), and myoblasts (5, 6).

A novel population of $Sca1^+$ progenitor cells was identified within the aortic adventitia of adult ApoE-/- mice by Hu et al. in 2004. The adventitial $Sca1^+$ (AdvSca1) cells also expressed the stem cell markers c-kit and CD-34. Interestingly, bone marrow transplant experiments showed that AdvSca1 cells were not bone marrow-derived. Isolated AdvSca1 cells responded to platelet-derived growth factor BB (PDGF-BB) treatment *in vitro* by differentiating to SMCs. AdvSca1 cells that were transferred to the adventitial surface of an irradiated vein graft *in vivo* underwent SMC differentiation and contributed to neointima formation (7).

The process of smooth muscle differentiation has important implications in both developmental and disease processes. The transcription of most smooth muscle marker genes is controlled by one or more CArG elements found within promoter and/or intronic sequences. CArG elements are bound by dimers of the transcription factor serum response factor (SRF). However, SRF is not intrinsically specific for SMC genes; it is widely

expressed and can also regulate cardiac and skeletal muscle genes, as well as growth responsive genes such as *c-fos* (8). In order to achieve specificity and precise control of SMC gene transcription, additional mechanisms of regulation must be present. Several SRF cofactors have been shown to modulate its transcriptional activity (see Chapter 1). These co-activators and co-repressors can augment or inhibit SRF transcriptional activity through physical association, modulating SRF-CArG binding, or indirectly inhibiting SRF-dependent gene expression.

During development, the smooth muscle cells and pericytes that are recruited by developing vessels arise from diverse embryonic origins (9). Studies in avian and mouse systems have shown that there are at least four distinct populations contributing medial SMCs to the aorta, including cells derived from secondary heart field (10, 11), cardiac neural crest (12, 13), somites (14-16), and a transient population of SMCs originating from splanchnic mesoderm (17). Cells from the proepicardial, serosal, and pleural mesothelia contribute SMCs to coronary, mesenteric, and pulmonary vessels, respectively (18-20). Several studies have suggested that SMCs of different developmental origins may also exhibit functional disparities. For example, avian neural crest-derived SMCs responded very differently to TGF-β1 treatment *in vitro* than did mesoderm-derived SMCs (21).

For years, investigations into the origin of SMCs in vascular disease were based on the idea that new SMCs arise from preexisting SMCs via dedifferentiation (22). There have been multiple recent reports, however, that suggest new SMCs can also arise from adult progenitors which are found within the vessel wall itself (7, 23-27). Resident progenitor cells within the arterial adventitia are perfectly poised to respond to local inductive signals and undergo smooth muscle differentiation to participate in vessel wall repair or disease processes. Therefore, we have more closely examined the regulation of smooth muscle differentiation in AdvSca1 cells.

## Hypothesis

AdvSca1 cells *in vivo* express a "pre-SMC" phenotype that is poised for signal-responsive SMC differentiation. While normally maintained as progenitors within the vessel wall, AdvSca1 cells will respond to differentiation stimuli by upregulation of SMC marker genes and differentiation to a mural cell phenotype.

## Materials and Methods

Animals Used: All protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina. Mice used include Wnt1-cre (Tg(Wnt1-cre)11Rth Tg(Wnt1-GAL4)11Rth/J; Jackson Laboratories; 003829), Nkx3.2-cre (gift of Warren Zimmer, Texas A&M University, described in (28)), WT1-cre (gift of John Burch, Fox Chase Cancer Center), Tie2-cre (Tek-cre1Ywa/J; Jackson Laboratories; 008863), Rosa26-reporter (B6.129S4-Gt(ROSA)26Sortm1Sor/J; Jackson Laboratories; 003474), Ptc1-lacZ (Ptch1tm1Mps, Jackson Laboratories; 003081, described in (29)), Ptc2-lacZ (Ptc2tm1Dgen; Jackson Laboratories; 005827), Rosa26 (B6.129S7-Gt(ROSA)26Sor/J; Jackson Laboratories; 002192), SM22α-lacZ (gift of Li Li, Wayne State University, described in (30)), VE-cadherin-cre (B6.Cg-Tg(Cdh5-cre)7Mlia/J; Jackson Laboratories; 006137), tdTomato (B6.129(Cg)-Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J; Jackson Laboratories; 007676), and C57BL/6J (Jackson Laboratories). Noon on the day of vaginal plug was designated E0.5.

β-Galactosidase Assay: Embryos or tissues were fixed in fresh 0.2% glutaraldehyde and stained with a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal substrate at 37°C overnight. Postfixation was performed in 4% paraformaldehyde (PFA) at 4°C overnight. For histological analysis, tissue was dehydrated, cleared (Histo-Clear; National Diagnostics), embedded in paraffin, sectioned, and counterstained with nuclear fast red (Vector Laboratories).

Immunofluorescence Staining: Tissues were fixed for 1 hr in freshly prepared 4% PFA, rinsed in PBS, saturated with 20% sucrose for cryoprotection, embedded in agar, and frozen in OCT. Twelve-micrometer cryosections were fixed in methanol at room temperature for 5 min, permeabilized with 0.05% Tween-20 (in PBS) for 2 min, rinsed in PBS, then blocked in 2% normal goat serum (NGS) or normal donkey serum for 2–8 h. Sections were incubated with primary antibodies at 4°C overnight, rinsed twice in PBS + 0.1% BSA, then incubated with secondary antibodies for 2-8 h at room temperature, protected from light. Nuclei were counterstained with 10 μg/ml Hoechst 33258 in $H_2O$ and slides were mounted in Mowiol with 2.5% DABCO.

For cells in culture, cells were fixed by incubating in freshly prepared 4% PFA for 15 min at room temperature, then permeabilized with 1:1 methanol/acetone for 1 min. Cells were blocked in 2% NGS for 1-4 h at 4°C, then incubated with primary antibodies at 4°C overnight. After two rinses in PBS + 0.1% BSA, cells were incubated with secondary antibodies 1-4h at room temperature protected from light. Nuclei were counterstained with 10 μg/ml Hoescht 33258 in $H_20$. For BrdU dual-labeling experiments, cells were first incubated with other primary and secondary antibodies, then postfixed in 4% PFA for 15 min at room temperature. To access BrdU antigen, DNA was denatured in 2M HCl for 5 min at room temperature. Cells were rinsed twice with PBS to neutralize residual acid, and then stained according to standard protocol.

Primary antibodies used for these studies included rabbit anti-β-galactosidase (1:500; MP Biomedical 55976), mouse anti-BrdU (1:400; BD Pharmingen 555627), rabbit anti-SM-MHC (1:100; Biomedical Technologies BT-562), mouse anti-SMαA (1:100; Sigma A2547), rat anti-Sca1 (1:100; BD Pharmingen 553333), rabbit anti-SRF (1:100; Santa Cruz Biotechnology sc335), and rat anti-PECAM-1 (1:100; BD Pharmingen 550274). All secondary antibodies were AlexaFluor-conjugated (Invitrogen) and used at a dilution of 1:400 in PBS. Immunofluorescence staining was visualized with a Leica BM IRB inverted

epifluorescence microscope, with images captured by a QImaging Retiga 1300 digital camera. Confocal images were obtained with a Zeiss LSM5 Pascal laser scanning confocal microscope and processed with Image J and Adobe Photoshop.

AdvSca1 Cell Isolation and Culture: Mice were sacrificed by cervical dislocation and perfused through the left ventricle with PBS. The thoracic aorta was cut at the diaphragm and periadventitial fat was cleared from around the vessel. The aorta was gently lifted away from the dorsal body wall, with intercostal arteries trimmed even with aortic wall. In arch region, the superior vena cava and pulmonary artery were cleared and branching arteries and ductus arteriosus were trimmed even with the aortic wall. At the junction with the myocardium, the aorta was cut flush with the ventricular wall and placed in HBSS at 37ºC. Before digesting, any remaining fat and coagulated blood were cleared from the tissue.

Whole aortas were briefly digested in 0.4 mg/mL collagenase in HBSS at 37°C for 6 min. Tissue was immediately removed and placed in fresh HBSS at 37°C. Adventitia was gently separated from the media of each aorta by peeling the layer up and over the vessel. Adventitias were placed in the second digestion solution of 15 mg/mL collagenase II (Worthington, 4176) and cut into small pieces. Tissue was digested for 2-2.5 hrs at 37°C with constant rocking to achieve a single-cell suspension, which was then passed through a 30 µm pre-separation filter (Miltenyi, 130-041-407). Filtrate was collected and cells were pelleted at 300xg then rinsed in PBS + 0.5% BSA. Aortic $Sca1^+$ cells were isolated using anti-Sca1 immunomagnetic MicroBeads (Miltenyi ) and a MACS cell separation system (Miltenyi ) according to manufacturer's instructions. Cells were passed over two consecutive columns to increase purity of the isolation. Isolated $Sca1^+$ cells were cultured in DMEM (Sigma) plus 10% FBS (HyClone) and 1x antibiotic/antimycotic solution (Gibco) at 37°C, 5% $CO_2$. Cells were seeded in 48-well tissue culture plates at a density of $8x10^3$-$1.5x10^4$ cells per well, depending on the assay.

Growth factor treatments and induction of adipocyte differentiation: In some experiments, isolated AdvSca1 cells were treated with growth factors, including recombinant human BMP2 (PeproTech, 120-02); recombinant human PDGF-BB (R&D Systems, 220-BB); recombinant mouse Sonic hedgehog (C25II) N-terminus (R&D Systems, 464-SH); recombinant human TGF-β1 (R&D Systems, 240-B); and recombinant human FGF-basic (Peprotech, 100-18B).

For adipocyte differentiation, AdvSca1 cells were grown to confluency under standard conditions. Cells were then treated with MDI induction medium (11.5 µg/mL isobutylmethylxanthine (Sigma, I7018); 1 µg/mL insulin (Sigma, I5500); 1 µM dexamethasone (Sigma, D4902) in DMEM + 10% FBS) for two days followed by treatment with 1 µg/mL insulin in DMEM + 10% FBS for two days. Cells were then incubated under standard conditions for an additional four days before Oil Red O staining.

RT-PCR Analysis: Total cellular RNA was isolated by guanidinium isothiocyanate denaturation and phenol/chloroform extraction as described (31). Two-step RT-PCR was carried out with the GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer's instructions. The sequences for primers used for RT-PCR analysis of gene expression are provided in Appendix B. Unless otherwise indicated, primer sequences were designed for this study based on mouse genomic sequence available through Ensembl (release 45, June 2007; (32)). Primers were designed with the aid of Primer3 software (v. 0.4.0) (33).

Cell Proliferation and Differentiation Assays: For experiments to determine whether AdvSca1 cells can express SMC differentiation markers in the absence of cell proliferation, AdvSca1 cells were isolated by immunoselection with magnetic microbeads and a MACS cell separation system (Miltenyi Biotech) and cultured for 5 days in DMEM + 10% FBS + 1x antibiotic/antimycotic (Sigma) to allow cells to attach and spread on the culture substrate. At 5 days, cell proliferation was arrested by the addition of 50 µM aphidicolin (in DMSO; Sigma

A0781) or 8 mM hydroxyurea (in $H_2O$; Sigma, H8627) and cells were cultured for an additional 10 days. To verify growth arrest, 25 μM BrdU (BD Pharmingen 550891) was added to each well with or without inhibitors.

Histological Staining: Alizarin red S and von Kossa staining procedures were similar to those described (34). For alizarin red staining, cultures were washed three times in 150mM NaCl, then fixed in ice-cold 70% ethanol for 1 h. Cells were rinsed with $ddH_20$, incubated with alizarin red staining solution (2% alizarin red in $H_2O$, pH 4.3) at room temperature for 10 min, then washed five times in $ddH_2O$ and photographed. For von Kossa staining, cultures were rinsed twice in $ddH_2O$ then incubated in 1% (wt/vol) silver nitrate under UV illumination for 45 min. After two rinses in $ddH_2O$, cells were treated with 3% (wt/vol) sodium thiosulfate to remove unreacted silver. Cells were then rinsed once in $ddH_2O$, counterstained in van Gieson solution (0.6% picric acid, 0.0375% acid fuchsin in $H_20$) for 5 min, then washed in 70% ethanol, dried, and photographed.

For Oil Red O staining of lipids, medium was removed from cultured cells and cells were fixed in 10% formalin in PBS for 5 min at room temperature. The solution was changed to fresh 10% formalin, and cells were incubated 1 hr. Formalin was removed, and wells were rinsed with $ddH_2O$. All liquid was removed from wells, and cells were incubated in 3.5 mg/mL Oil Red O (Sigma, O0625) in isopropanol for 10 min. After removing Oil Red O solution, cells were immediately rinsed 5x in $ddH_2O$ and photographed.

Matrigel plug assay: AdvSca1 cells were isolated according to standard procedure and counted. Aliquots of $1.5x10^5$-$2x10^5$ cells were resuspended in ice-cold Matrigel (growth-factor-reduced, phenol red-free; BD Biosciences, 356231) with or without growth factors, as indicated in text. Matrigel solution was drawn into a pre-chilled 1 mL syringe fitted with a 23-gauge needle and kept on ice. Under isoflurane anesthesia, female C57BL/6J mice, aged 8-16 weeks, were shaved in two patches on the upper dorsal area, just lateral to the midline. Matrigel was injected subcutaneously on either side of the midline, and mouse was revived.

At the indicated timepoints, host mice were sacrificed, and the Matrigel plugs were carefully dissected from the subcutaneous connective tissue. Depending on the experiment, plugs were processed for X-gal staining according to standard protocol or fixed for one hour in fresh 4% PFA and then processed for cryo-embedding according to standard protocol.

Carotid ligation surgery: Blood flow reduction in the left common carotid artery was performed as previously described (35, 36). Briefly, Ptc2-lacZ mice aged 3-4 months were anesthetized with isoflurane and maintained at 37°C on a heating pad. Using sterile technique, connective tissue was gently separated to expose ~0.5-mm lengths of the left external carotid artery distal to the thyroid artery and the left internal carotid/occipital artery pair. The internal and external carotid arteries were ligated with 6-0 silk suture and the area was rinsed with anti-bacterial 0.9% saline solution before closure. Sham operations followed the same procedure, but artery ligation was omitted. Mice received antibiotic (50 mg/kg cephazolin) and analgesic (10 mg/kg im Pentazocine) and were monitored for one week post-surgery.

## Results

### *Developmental origins of AdvSca1 cells*

Following multiple lineage tracing studies in both avian and mouse systems, the developmental origins of smooth muscle cells can be plotted on a lineage map with some confidence (Figure 15A, reviewed in (9)). In contrast, however, very little is known about the embryonic origins of adventitial cells, including adventitial $Sca1^+$ progenitor cells (AdvSca1 cells). Hu et al. performed bone marrow reconstitution experiments in which an irradiated adult mouse received a transplant of isolated AdvSca1 that were genetically marked by constitutive β-galactosidase expression. After six months, no $Sca1^+\beta\text{-gal}^+$ cells were identified in the adventitia, indicating that AdvSca1 cells were not renewed by marrow-derived cells within this time period (7).

Based on the proximity of AdvSca1 cells to underlying medial SMCs, we hypothesized that AdvSca1 cells may derive from the same embryonic origin. Cells of neural crest origin are responsible for establishing the septation between the aorta and pulmonary trunk during midgestation (12) and populate portions of the pulmonary trunk and ascending and transverse aorta. We used Wnt1-cre transgenic mice (13) to label cells of neural crest origin. Wnt1-cre-activated β-gal reporter (R26R) labeled neural crest-derived SMCs in proximal aorta, pulmonary trunk, common carotid arteries, and ductus arteriosus, but did not label the adventitial layer or AdvSca1 cells in these vessels (Figure 14B-C, Figure 15). Distal to the segment of aortic media labeled by Wnt1-cre, SMCs of sclerotome origin are β-gal$^{+}$ in Nkx3.2-cre/R26R mice (37) (Figure 14D). Somite-derived SMCs are also found in the outer layers of the aortic media and within the pulmonary trunk at the level of the aortic root, but adventitial cells are β-gal$^{-}$ (Figure 14E). Wilms tumor 1 (WT1) is a marker of mesothelial cells (38) and Tie2 (TEK) is expressed by endothelial cells and hematopoietic stem cells (39). Both populations represent reasonable sources of AdvSca1 cells, yet neither WT1-cre/R26R nor Tie2-cre/R26R mice display significant adventitial staining in areas where AdvSca1 cells are known to be abundant (Figure 14F-G). Tie2-cre does label scattered SMCs in the pulmonary trunk and occasional adventitial cells (Figure 14G). However, regions known to harbor abundant AdvSca1 cells at this stage, such as the adventitia between the aorta and pulmonary trunk, are negative for Tie2-cre-labeled cells. Thus, the developmental origins of AdvSca1 cells differ from their neighboring SMCs and are as of yet unknown.

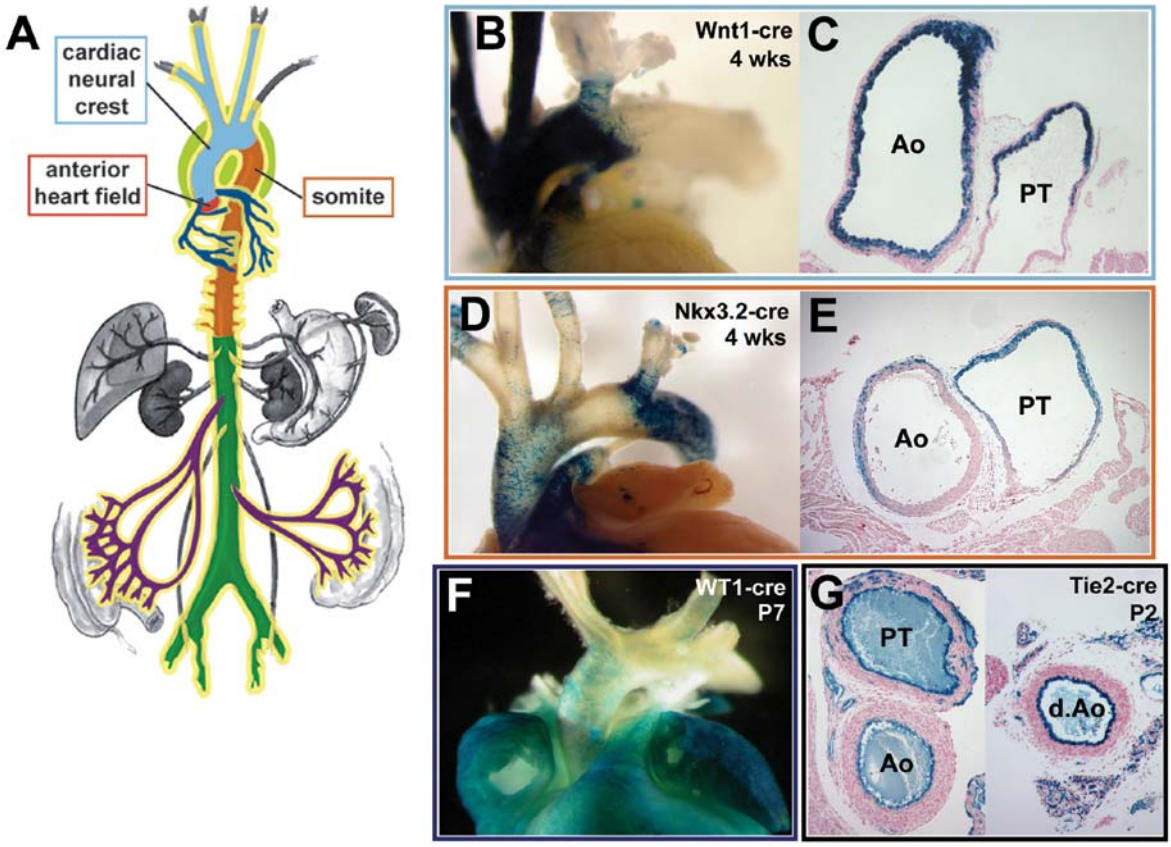


**Figure 14**. *Lineage tracing of arterial cells*. (A) Schematic of known embryonic origins for vascular smooth muscle cells, adapted from Majesky, 2007 (9). In the aortic region where AdvSca1 cells are abundant, adjacent SMCs are derived from cardiac neural crest (CNC), anterior heart field, somites, and proepicardium. (B,D,F) Whole-mount images of mice carrying cre under the (B) Wnt1, (D) Nkx3.2, or (F) Wilms tumor-1 (WT1) promoters. When crossed with Rosa26-reporters (R26R), cells of the (B) cardiac neural crest, (D) sclerotome, or (F) proepicardial lineages are identified by expression of β-galactosidase, as revealed by X-gal staining (blue cells). (C,E,G) Cross-sections of X-gal stained tissue from the aortic root region, counterstained with nuclear fast red. (B-C) In proximal aorta and pulmonary trunk, subsets of SMCs are derived from CNC. SMCs in the aortic root region are β-galactosidase-positive (blue) while adventitial cells are negative (C). (D-E) Labeling of cells derived from the Nkx3.2-expressing splanchnic mesoderm lineage. Note that positive cells are found primarily in the outer layers of medial smooth muscle in the aorta, complementary to the pattern of CNC-derived SMCs (compare C and E). However, the majority of adventitial cells in the aortic root region are still negative. (F) A scattering of SMCs (but not adventitial cells, data not shown) in the proximal aortic root are derived from a WT1-cre-expressing lineage. (G) Tie2-cre labels endothelial and hematopoietic cells. Note: the large β-galactosidase$^{+}$ clusters on the outside of the pulmonary trunk are areas of coagulated blood that persisted through dissection and tissue preparation. While some adventitial cells appear positive, the region between the aorta and pulmonary trunk where AdvSca1 cells are particularly abundant is negative, indicating that most, if not all, AdvSca1 cells originate from a different source during development. Ao, aorta; PT, pulmonary trunk; d.Ao, descending aorta.

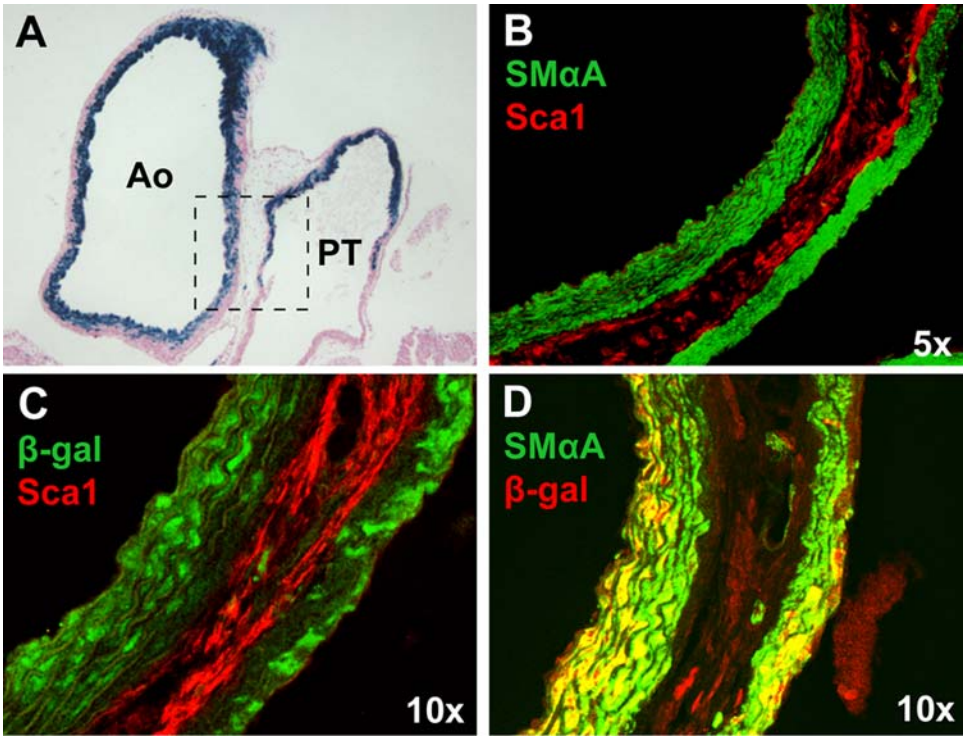


**Figure 15**. *Analysis of β-galactosidase and Sca1 expression in Wnt1-cre/R26R vessels.* (A) Cross-section of X-gal-stained aortic root from a Wnt1-cre/R26R mouse at 4 wks. Dashed box indicated general region shown in B-D. (B-D) Cryosections of Wnt1-cre/R26R vessels, with aorta to the top/left and pulmonary trunk to the bottom/right. Immunofluorescence staining is as indicated. Note the lack of overlap between β-galactosidase and Sca1 (C), confirming that AdvSca1 cells in this region are not derived from cardiac neural crest. Ao, aorta; PT, pulmonary trunk.

*Analysis of isolated AdvSca1 cells*

To characterize the properties of AdvSca1 progenitor cells, we used immunomagnetic beads to isolate $Sca1^+$ cells from aortic adventitial tissue (see Materials and Methods). AdvSca1 cells *in vivo* do not express markers of a differentiated phenotype (Figure 16A). However, when isolated cells were cultured in DMEM containing 10% serum, many AdvSca1 cells (~30–50%) lost expression of Sca1, gained expression of SMα-actin, SM22α, calponin, and SM-MHC, and adopted an elongated, mesenchymal cell shape (Figure 16B).

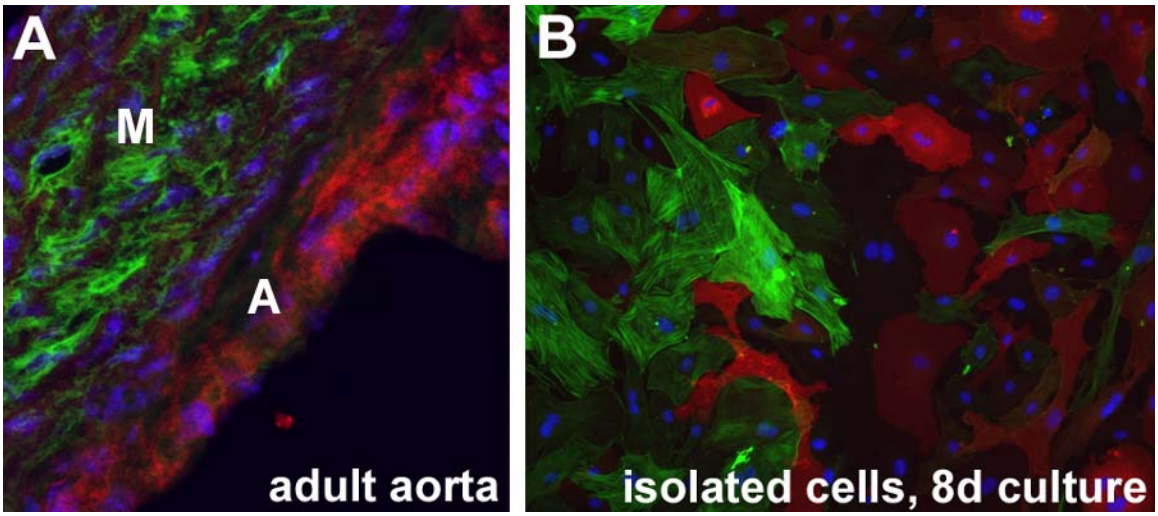


**Figure 16**. *Differentiation of AdvSca1 cells in vitro*. (A) In a cross-section through the adult aorta, $Sca1^+$ cells (red) are evident in the adventitia, distinct from SMα-actin-expressing cells (green) of the media. (B) After 8 days in culture, a subset of AdvSca1 cells has lost Sca1 expression and upregulated SMα-actin. When removed from the adventitia and cultured, AdvSca1 cells upregulate SMC markers without additional exogenous treatment. M, media; A, adventitia.

To rule out the possibility that up-regulation of SMC markers resulted from expansion of a pool of medial SMCs carried over in the AdvSca1 cell isolation, we repeated our experiments in the presence of aphidicolin or hydroxyurea, inhibitors of cell proliferation. After verifying growth arrest, we observed up-regulation of SMC marker proteins over a similar time course and to a similar extent as seen in the absence of cell cycle inhibitors (Figure 17). We conclude that AdvSca1 cells can directly differentiate into SMC-like cells, and that this differentiation does not require cell proliferation.

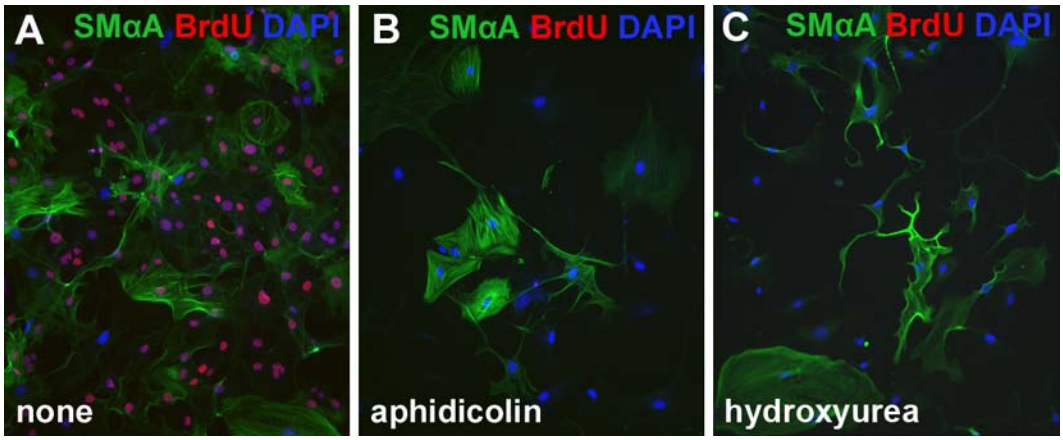


**Figure 17**. *AdvSca1 cell differentiation in the absence of cell division*. (A-C) Isolated AdvSca1 cells were cultured in serum-containing medium in the presence of 10μM BrdU. Proliferating cells are identified by BrdU immunostaining (red nuclei). The addition of 50μM aphidicolin (B) or 8 mM hydroxyurea (C) strongly inhibited cell proliferation, as indicated by the absence of BrdU staining. The up-regulation of SMα-actin (green) in AdvSca1 cells does not require cell proliferation.

We tested the response of isolated AdvSca1 cells to treatment with factors commonly associated with the modulation of smooth muscle differentiation, including transforming growth factor β1 (TGF-β) and platelet-derived growth factor BB (PDGF-BB). In contrast to results from Hu et al, we did not observe a significant response of AdvSca1 cells to PDGF-BB treatment, as assessed by immunostaining for Sca1 and SMα-actin or by semi-quantitative RT-PCR (Figure 18B,E). Following TGF-β treatment, we consistently observed a dramatic downregulation of Sca1 expression, indicating that AdvSca1 cells are TGF-β-responsive. Since AdvSca1 cells reside within a Shh-responsive environment in the adventitia and are at least partially dependent on Shh signaling for proper development (see Chapter 2), we also tested for an *in vitro* response to exogenous Shh ligand. No changes in Sca1 or SMα-actin expression were induced by Shh treatment (Figure 18C and data not shown). These results suggest that Shh signaling may not be critical for regulating AdvSca1 differentiation, but do not rule out other roles, such as regulation of proliferation (see Chapter 2).

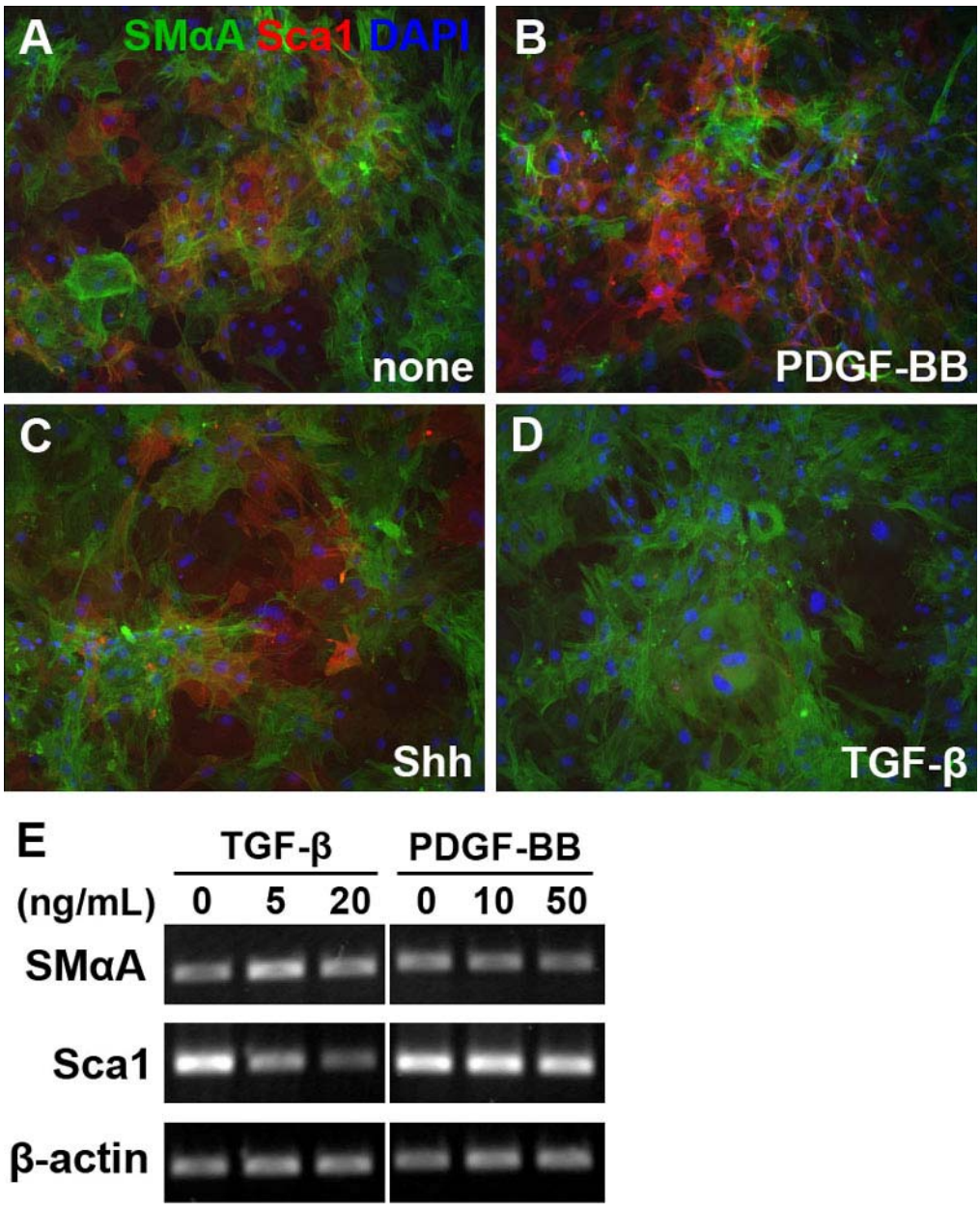


**Figure 18**. *AdvSca1 cell response to growth factor treatments in vitro*. (A-D) Isolated AdvSca1 cells were cultured for 6 days to allow attachment, then treated with (A) no additional factors, (B) 10 ng/mL PDGF-BB, (C) 1 µg/mL Shh(C25II)-N, or (D) 5 ng/mL TGF-β for an additional 8 days. Cells were fixed and stained for SMα-actin and Sca1. (E) In a separate experiment, isolated AdvSca1 cells were cultured for 6 days to allow attachment and then treated with TGF-β (0, 5, or 20 ng/mL) or PDGF-BB (0, 10, or 50 ng/mL) for 48 hrs. RNA was collected, and the expression of SMα-actin and Sca1 was evaluated by RT-PCR. β-actin was used as a loading control. Treatment of established AdvSca1 cultures with TGF-β induces downregulation of Sca1, but PDGF-BB does not provoke a robust response.

Multipotent adult mesenchymal stem cells have been characterized by several criteria, including the capacity to differentiate to bone, cartilage, and adipose tissue (40). Having established the inclination of AdvSca1 cells to differentiate to SMC-like cells when placed in culture, we sought to test whether AdvSca1 cells also harbored the potential for other mesenchymal cell types. When isolated AdvSca1 cells were incubated with BMP2 (50 ng/ml, 20 days), colonies formed that stained with alizarin red or von Kossa's stain (~1

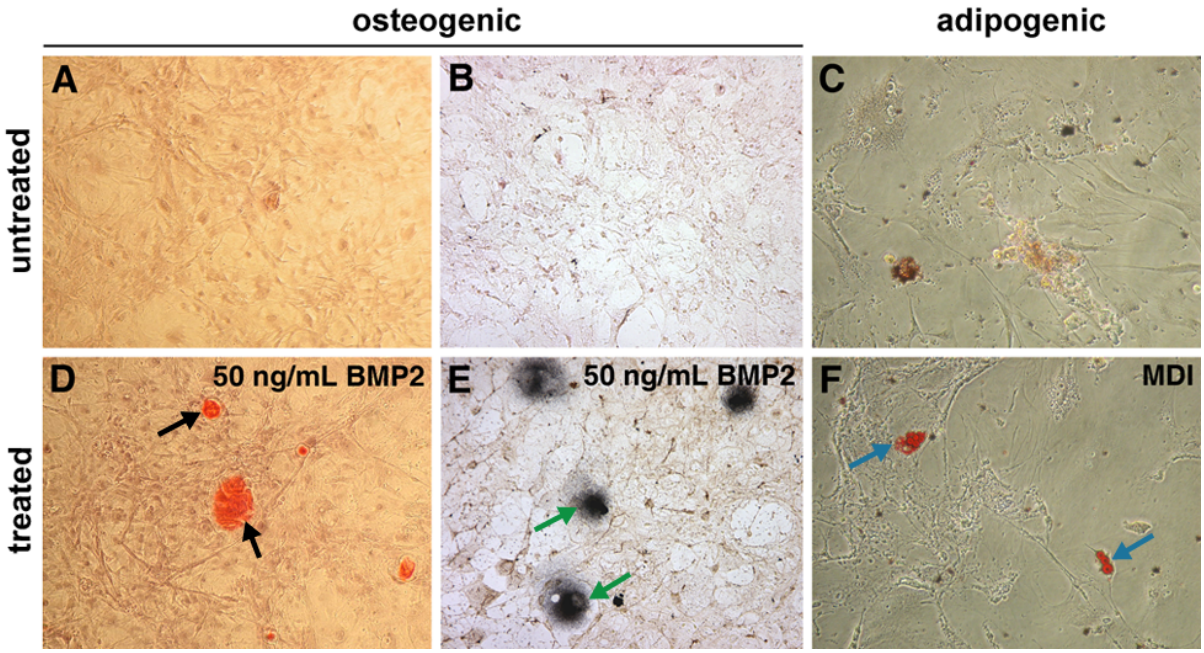


**Figure 19**. *Other possible mesenchymal fates of AdvSca1 cells in vitro*. Isolated AdvSca1 cells were cultured in medium without (A-C) or with the addition of BMP2 (50 ng/ml, 20 days) (D-E) or adipogenic induction factors (8 days total, see Materials and Methods) (F). (A,D) Alizarin red forms a red-orange complex with calcium ions indicating the formation of calcified nodules (black arrows in D). (B,E) Von Kossa staining indicates the presence of mineralized colonies by the formation of silver salts, which are reduced to black metallic silver (green arrows in E). (C,F) Oil Red O stains lipid droplets produced by adipogenic cells (blue arrows in F). The overall frequencies of AdvSca1 differentiation to either osteogenic or adipogenic fates upon appropriate treatment were at least 10-fold lower than that observed for differentiation to SMα-actin-positive cells in standard AdvSca1 cultures.

colony per 400 cells plated), consistent with differentiation of a subset of AdvSca1 cells to osteogenic cells (Figure 19D-E). Under conditions commonly used to promote adipogenic differentiation (see Materials and Methods), oil red O staining revealed lipid-rich clusters in

AdvSca1 cultures (~1 colony per 700 cells plated, Figure 19F). AdvSca1 cells as a population are thus multipotent and have the capacity to differentiate to other mesenchymal cell types, although at a much lower frequency than that observed for SMC differentiation.

*AdvSca1 cells regulate the expression of smooth muscle genes*

AdvSca1 cells do not express SMC marker proteins *in vivo* (Figures 9 and 16). However, they contain mRNAs for transcription factors involved in SMC differentiation,

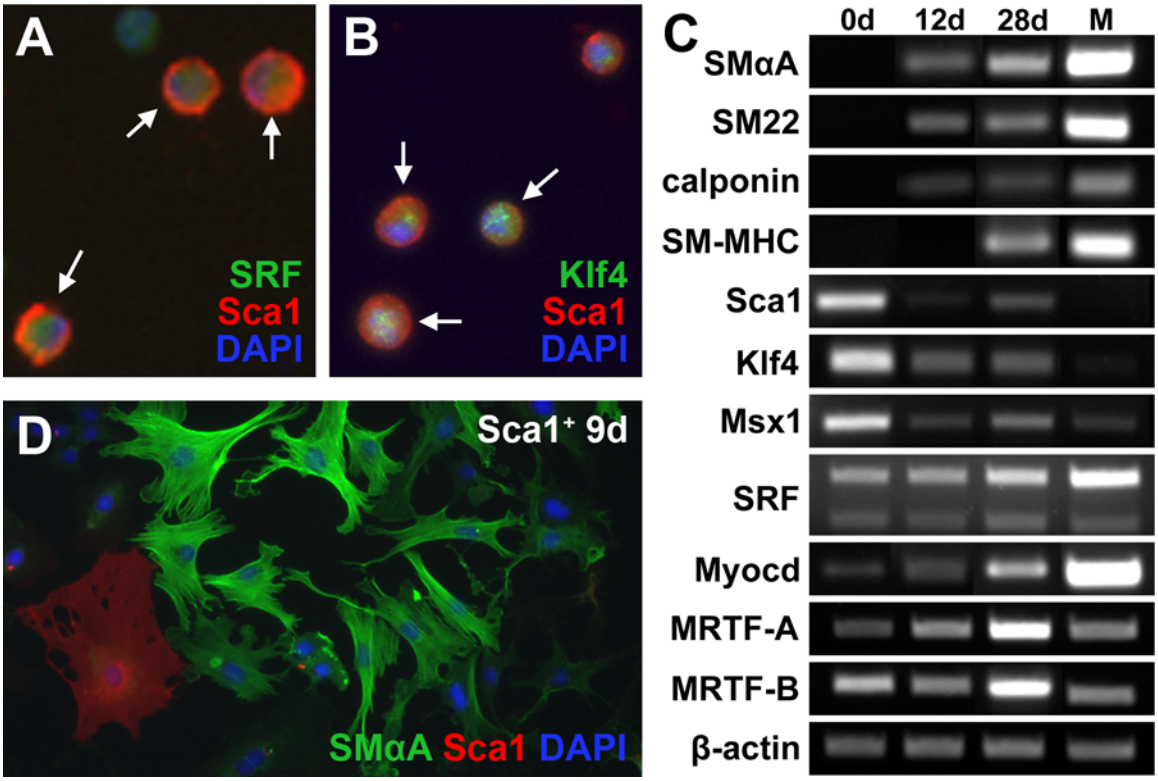


**Figure 20**. *Analysis of AdvSca1 gene regulation during in vitro differentiation*. (A) Freshly isolated AdvSca1 cells (red) contain SRF (green) colocalized with DAPI-stained nuclei (blue) (arrows). (B) Freshly isolated AdvSca1 cells (red) contain Klf4 (green) colocalized with DAPI-stained nuclei (blue) (arrows). (C) RT-PCR analysis of AdvSca1 cells cultured for 0, 12, or 28 days. M is total RNA from aortic media used as a positive control for SMC markers. This analysis was performed three times with similar results. (D) AdvSca1 cells cultured in serum-containing medium for 9 days. Many cells down-regulate ScaI (red) and up-regulate SM α-actin (green). As AdvSca1 cells differentiate *in vitro*, repressors of SMC gene expression are downregulated and activators become more highly expressed.

including serum response factor (SRF), myocardin family members, and cysteine-rich LIM proteins Csrp1 and Csrp2 (Figure 20C). In addition, a subset of freshly isolated AdvSca1 cell nuclei were immunopositive for SRF protein (Figure 20A). However, AdvSca1 cells also express potent silencers of SRF-dependent transcription, including Msx1, Klf4, and FoxO4 (Figure 20C and data not shown). Each of these factors can inhibit SRF and myocardin-dependent SMC gene expression (41-43), and they are down-regulated as AdvSca1 cells acquire SMC markers and increase SRF co-activator expression (Figure 20C). Therefore, a subset of AdvSca1 cells may be maintained as SMC progenitors by expression of silencers of SRF-dependent transcription.

*Cells derived from AdvSca1 progenitors contribute to vascular structures in Matrigel plugs*

Our results indicate that a subset of AdvSca1 cells readily differentiates to SMCs and SMC-like cells *in vitro*. To evaluate the behavior of isolated AdvSca1 cells *in vivo*, we suspended the cells in Matrigel containing basic fibroblast growth factor (FGF2) and implanted them subcutaneously into a host mouse. The combination of Matrigel and FGF2 is known to promote neovascularization within the plug (44). We found that the presence of AdvSca1 cells in the Matrigel plugs promoted a greater degree of organized vascularization compared to plugs without cells added (Figure 21, compare A and B). Using AdvSca1 cells isolated from mice that constitutively express β-galactosidase from the Rosa26 promoter reveals that AdvSca1-derived cells integrate into the microvascular network (Figure 21C,D). Cross-sections did not offer enough resolution to differentiate between AdvSca1-derived endothelial cells and mural cells (Figure 21D), so we took advantage of genetically modified mice to label endothelial cells and SMC-like cells.

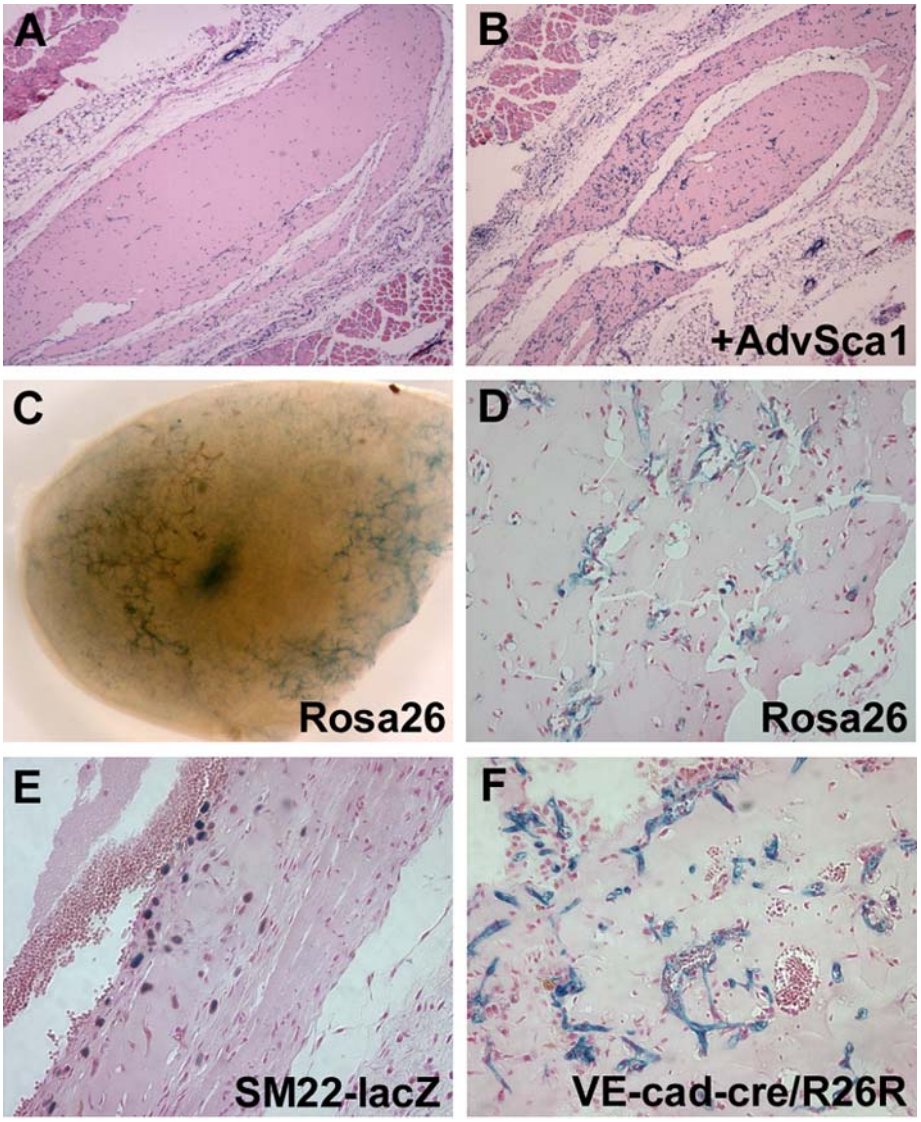


**Figure 21**. *Participation of AdvSca1cells in Matrigel plug angiogenesis*. (A-B) Hematoxylin and eosin staining of 12-day Matrigel plugs with 0.5 μg/mL human FGF2 containing no added cells (A) or freshly isolated AdvSca1 cells (B). The presence of AdvSca1 cells promotes the organization and assembly of cells within the Matrigel plug into vascular structures. (C-D) 10-day Matrigel plug with AdvSca1 cells from Rosa26 mice (constitutive expression of β-galactosidase in all tissues) and 0.2 μg/mL human FGF2. (C) X-gal staining permits visualization of an organized vascular network in a whole-mount view. (D) A cross-section through the plug reveals β-gal$^+$ cells associated with functional, lumenized microvessels. The presence of red blood cells (arrowhead) indicates connection with the host systemic circulation. (E) AdvSca1 cells were isolated from SM22-lacZ mice and incubated in a Matrigel plug with 0.5 μg/mL human FGF2 and 60 U/mL heparin for 14 days. Scattered β-gal$^+$ cells indicate that a subset of AdvSca1 cells differentiate to SM22$^+$ mural cells. (F) AdvSca1 cells were isolated from VE-cadherin-cre/R26R mice and incubated in a Matrigel plug with 0.5 μg/mL human FGF2 and 60 U/mL heparin for 10 days. β-gal$^+$ cells within the newly formed microvessels indicate that a subset of AdvSca1 cells differentiate to endothelial cells.

LacZ expression in SM22α-lacZ transgenic mice recapitulates the arterial smooth muscle expression pattern of SM22α transcripts but does not label visceral and venous SMCs during development (30). From previous reports, it appears that the pattern of SM22α expression in pericytes varies according to developmental stage and anatomic location, with evidence that capillary pericytes at E10.5 do not express SM22α (45) while postnatal retinal capillaries are SM22α-positive (46). When AdvSca1 cells from SM22α-lacZ mice (30) were implanted in Matrigel plugs, we observed scattered β-gal$^+$ cells associated with vascular structures after 14 days of incubation (Figure 21E). These results suggest that AdvSca1 cells have the potential to undergo differentiation to SMC-like cells *in vivo* and reside in a perivascular location. The VE-cadherin-cre transgene targets cre recombinase expression to vascular endothelium, including quiescent adult vasculature (47). In Matrigel plugs containing AdvSca1 cells isolated from VE-cadherin-cre/R26R mice, we observe lacZ$^+$ cells within the newly formed microvascular network (Figure 21F), indicating that AdvSca1 cells also have endothelial differentiation potential.

In order to expand our analysis of AdvSca1 *in vivo* differentiation potential, we isolated cells from mice expressing a membrane-targeted tdTomato fluorescent protein constitutively expressed in all tissues (48). Analysis of Matrigel plugs by confocal microscopy reveals AdvSca1-derived cells contributing to extensive vascular networks after 14 days of incubation (Figure 22A). Importantly, vessels surrounded by tdTomato-positive cells contained Ter119-positive erythroid cells inside the lumen, with no apparent leaking of red blood cells into the perivascular space (Figure 22B), suggesting the tdTomato-positive cells contribute to vascular wall stability and maturation. Immunostaining of cross-sections through the Matrigel plugs showed co-labeling of tdTomato-positive cells with several pericyte markers, including SMα-actin, PDGFR-β, and chondroitin sulfate proteoglycan 4 (Cspg4 or NG2) (Figure 23). Together with the perivascular localization of tdTomato-

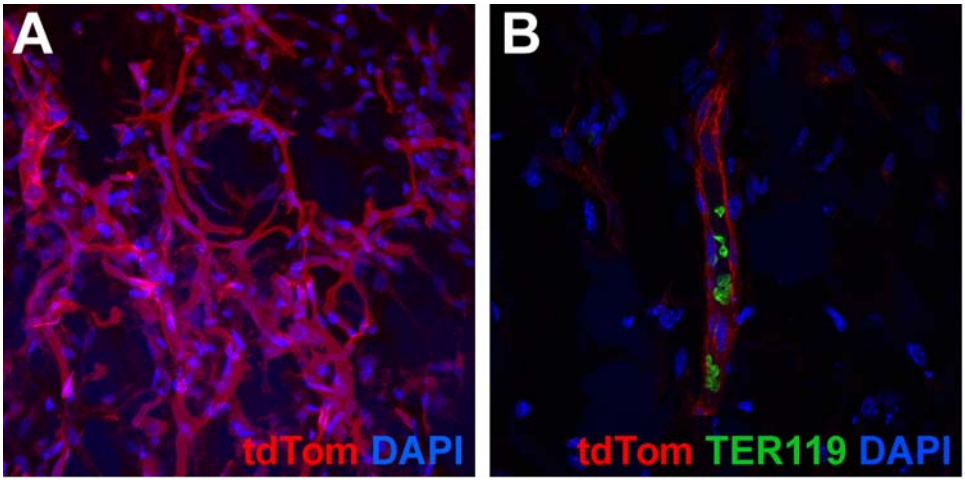


**Figure 22**. *Analysis of a Matrigel plug vascular network derived from AdvSca1 cells.* Freshly isolated AdvSca1 cells from tdTomato mice were incubated with 0.5 μg/mL human FGF2 and 60 U/mL heparin. (A) At 14 days an extensive network is evident. Vessels are functional by 10 days (B) with TER119-positive red blood cells found within the lumens. AdvSca1 cells contribute to an extensive, functional vascular network in Matrigel plugs.

positive cells, these results demonstrate that AdvSca1 cells have the potential to differentiate to functional mural cells in Matrigel plugs *in vivo*.

Based on the mural cell differentiation of AdvSca1 cells *in vivo*, we checked for expression of common pericyte markers. Freshly isolated AdvSca1 cells express RGS5, Cspg4 (NG2), and PDGFR-β but not desmin (Figure 24A). Moreover, when cultured AdvSca1 cells were treated with 50 ng/mL PDGF-BB for 24 hrs, they adopted an elongated morphology reminiscent of cultured tumor-derived perivascular progenitor cells as reported by Song et al. (49). Together, these results suggest that AdvSca1 cells share some features of pericytes, including responsiveness to PDGF-BB signaling, and can differentiate to perivascular mural cells *in vivo*.

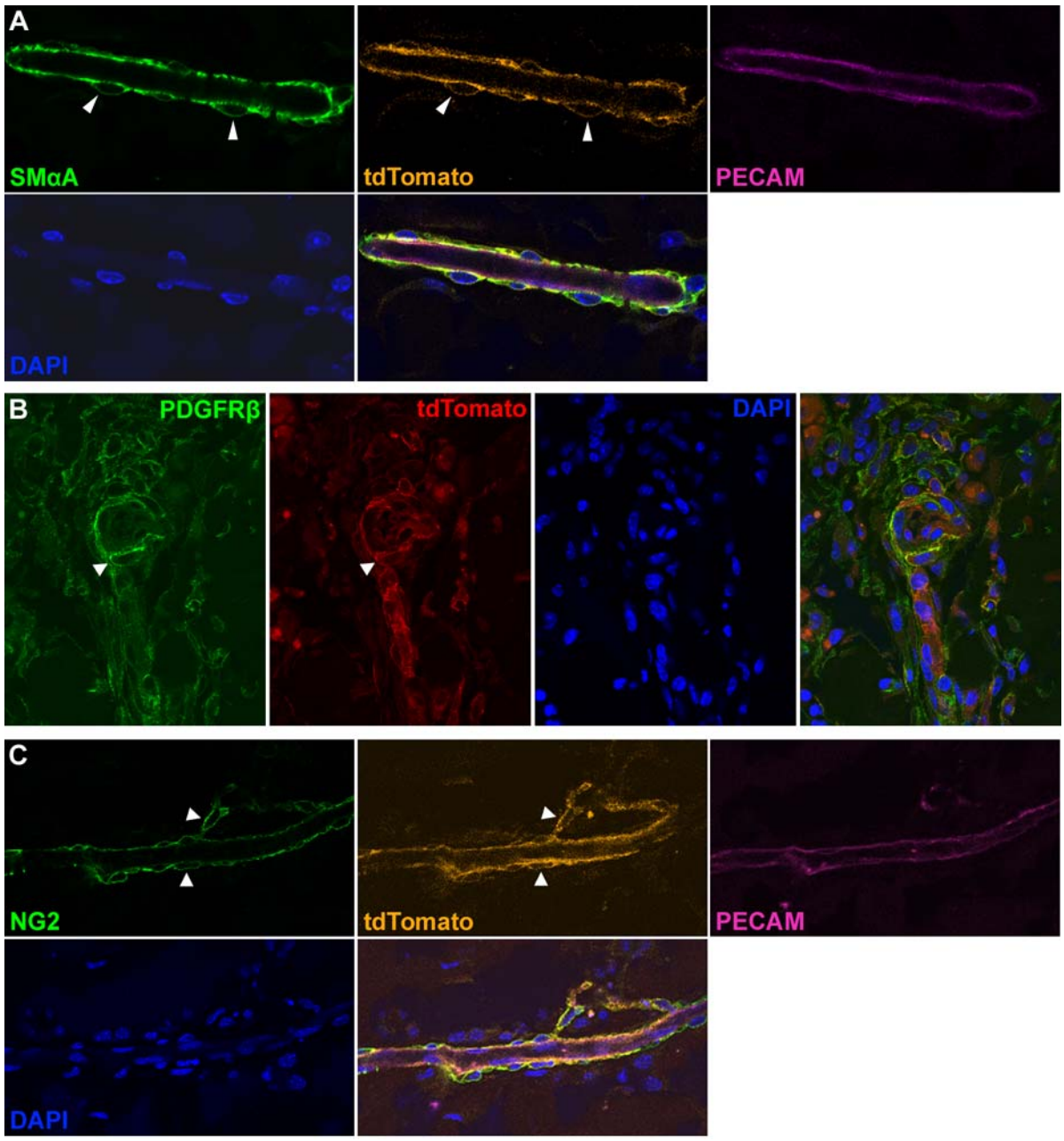


**Figure 23**. *Expression of pericyte markers in perivascular mural cells derived from AdvSca1 cells.* Isolated AdvSca1 cells expressing membrane-localized tdTomato fluorescent protein were incubated in Matrigel plugs with 0.5 μg/mL human FGF2 and 60 U/mL heparin for 10 d. Single confocal Z-sections indicate tdTomato co-localization with SMα-actin (arrowheads, A), PDGFR-β (arrowheads, B), and NG2 (arrowheads, C). PECAM staining (A,C) marks endothelial cells. AdvSca1 cells in Matrigel plugs differentiate to mural cells and express pericyte markers.

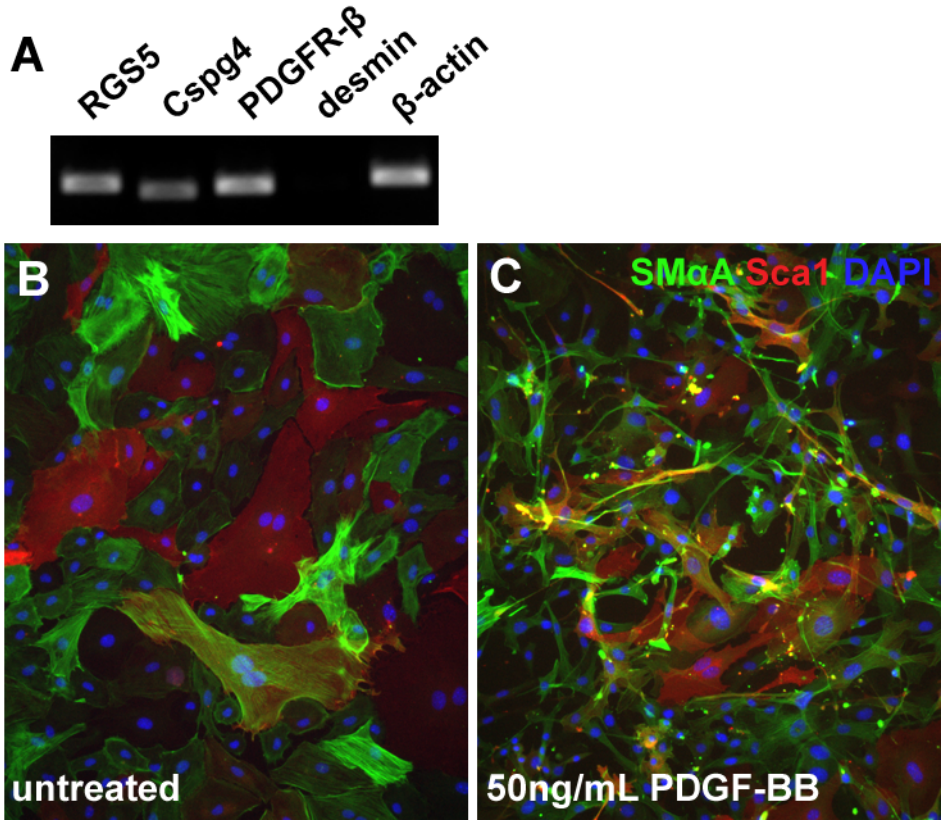


**Figure 24**. *Pericyte marker RT-PCR and PDGF treatment of AdvSca1 cells in vitro*. (A) RNA from freshly isolated AdvSca1 cells was screened for expression of common pericyte markers by RT-PCR. (B-C) Treatment of AdvSca1 cells *in vitro* with 50 ng/mL PDGF-BB for 24 hr induces morphology changes (C) compared to untreated cells (B). AdvSca1 cells share some markers with pericytes and readily adopt an elongated morphology in response to PDGF-BB stimulation.

*AdvSca1 behavior during flow-induced neoadventitia formation*

In the mouse model of flow-induced carotid artery remodeling described by Korshunov and Berk, the left internal and external carotid arteries are ligated, thus restricting blood flow to the occipital artery (Figure 25A). Immediately after ligation, blood flow is reduced by ~90% in the left common carotid artery (LCA) and increased by ~70% in the right carotid artery (RCA). Over the course of 4 weeks following ligation, the LCA exhibits a characteristic sequence of vascular remodeling events, culminating in significant neointima formation and decrease in lumen volume (35).

One of the earliest responses to flow reduction is significant adventitial thickening, which can be observed by one week post-ligation. Indeed, adventitial proliferation is an early response to several forms of vascular injury, as discussed in Chapter 1. However, to our knowledge, no study has investigated which cell types within the adventitia exhibit this early proliferative response. Thus, we examined Sca1 expression in the adventitia of carotid arteries at one and two weeks post-ligation. In the ligated group, the left internal and external carotid arteries of 4 month-old Ptc2-lacZ mice were ligated as described (36) (Figure 25A). In sham controls, the surrounding tissue was gently separated to expose the vessel, but no ligature was placed.

In order to determine whether hedgehog signaling is altered during neoadventitia formation, we stained segments of the common carotid arteries with X-gal. At one week post-ligation, the time at which the greatest adventitial response had previously been observed (35), the number of lacZ-positive cells appears grossly unchanged when ligated LCAs are compared to either sham controls or contralateral RCAs (Figure 25B). Similar results were observed at two weeks post-ligation.

Upon examination of carotid artery cross-sections, we observe an increase in adventitial area and the number of AdvSca1 cells in ligated vessels at one week post-ligation (Figure 25C). However, by two weeks post-ligation there is no observable difference between ligated vessels and sham controls, suggesting that the adventitial response to flow reduction occurs early in the remodeling process, well before neointimal development at 2-4 weeks post-ligation. The increase in AdvSca1 cells in response to flow-reduction could result from the proliferation of resident cells or the recruitment of circulating $Sca1^+$ cells. To begin to address this question, we stained one-week cross-sections with the mitotic marker phospho-histone H3. Mitotic AdvSca1 cells, while not abundant, are present in the adventitia at one week post-ligation (Figure 26). These results suggest that the expansion

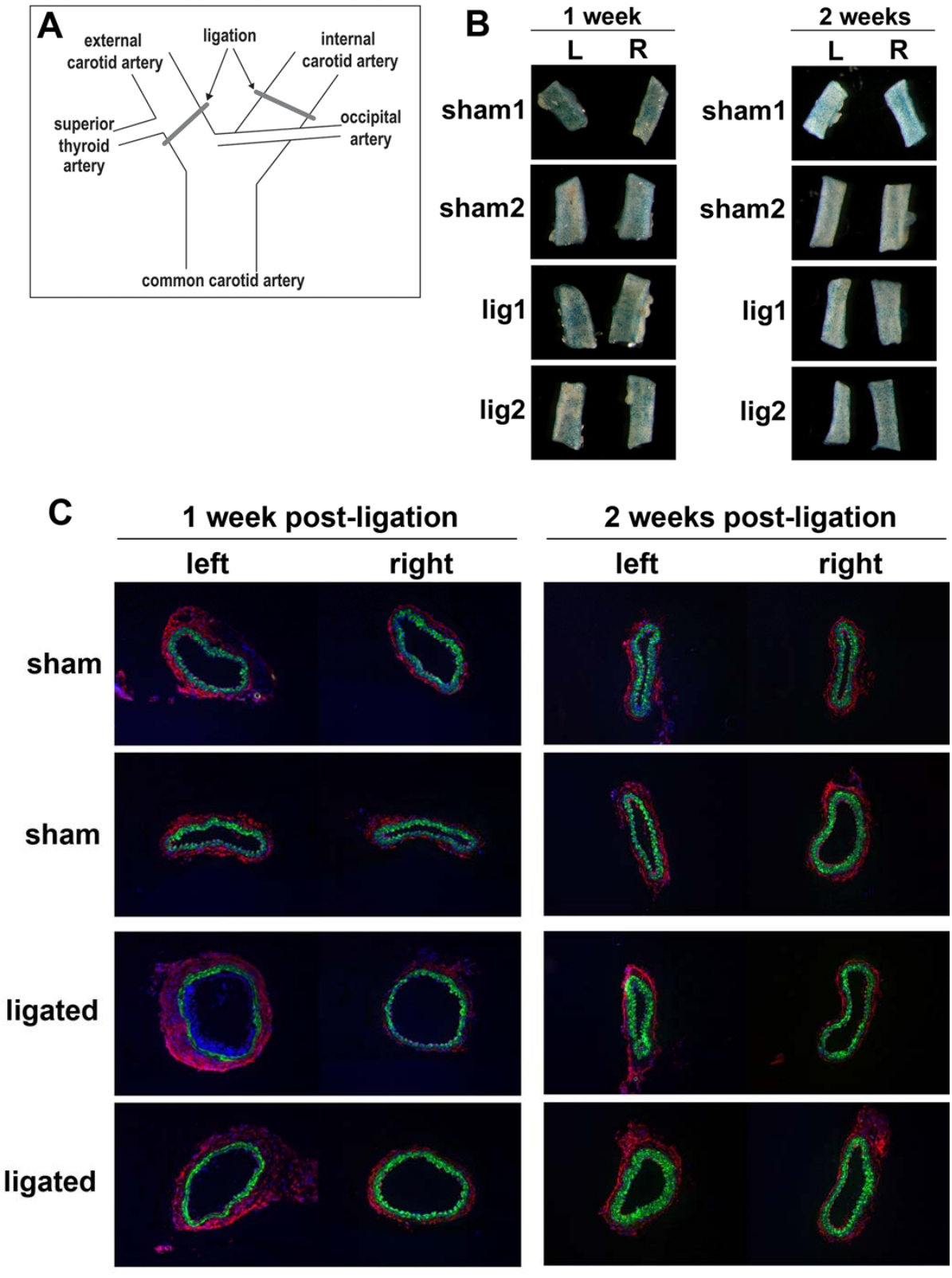


**Figure 25**. *Adventitial response to carotid flow-reduction*. (A) Schematic showing placement of ligations of left internal and external carotid arteries, just distal to the vessel

bifurcation. Adapted from Korshunov and Berk, 2003 (35). (B) Adult Ptc2-lacZ mice were used for surgery. Vessels were isolated at one or two weeks post-ligation, and segments of the common carotid artery were stained with X-gal to reveal hedgehog-responsive cells. (C) Sections of the common carotid artery stained with SM α-actin (green), Sca1 (red), and DAPI.

of AdvSca1 cells results from proliferation of cells already present, as opposed to recruitment of new cells from distal sites. Additional experiments at earlier timepoints will be necessary to thoroughly address both the regulation of Hh signaling during neoadventitial formation and the proliferation of resident AdvSca1 cells (see Future Directions).

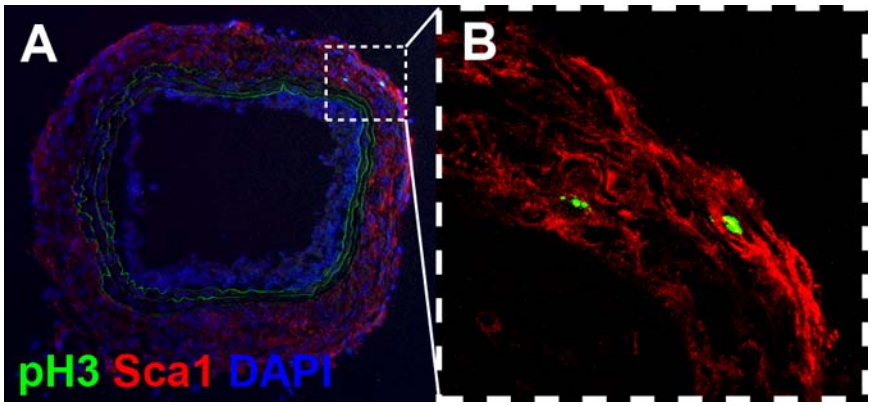


**Figure 26**. *AdvSca1 cells proliferate in response to flow-reduction*. (A) Cross-section of ligated LCA at one week post-ligation, stained for phospho-histone H3 (green), Sca1 (red), and DAPI (blue). Elastin layers in the media are visible by green autofluorescence. (B) Higher magnification confocal image of mitotic (pH3-positive) AdvSca1 cells.

## Discussion

### *Development of the adventitia*

The origins of AdvSca1 cells are unclear. We investigated this question for aortic AdvSca1 cells by using genetically modified mice to mark cells of lineages known to contribute to local SMC populations. Crossing Wnt1-cre transgenic mice to Rosa26-reporter mice generates offspring in which β-gal is permanently expressed in neural crest-derived cells (13). Migrating neural crest cells are responsible for aorticopulmonary septation (12), which occurs at ~E10.5 during mouse development (50). The neural crest population is known to contribute SMCs to the aortic root, a region with numerous AdvSca1 cells in the adventitia. However, our results show that AdvSca1 cells directly adjacent to Wnt1-cre-positive aortic SMCs did not express the neural crest marker when evaluated by either lacZ histochemical staining or by anti-β-galactosidase immunostaining (Figure 15C). Moreover, our developmental time course studies indicate that AdvSca1 cells do not appear in aortic arch arteries until between E16.5 and E17.5 (Figure 9), much later than the migration of neural crest-derived SMC progenitors into the aortic arch complex. Lineage tracing using Nkx3.2-cre mice (paraxial somitic mesoderm labeling) and WT1-cre mice (mesothelial cell labeling) also failed to mark AdvSca1 cells in the aortic root. We did not test the other lineage which contributes SMCs to the aorta and other great vessels, anterior heart field (AHF). Lineage-specific cre mice such as Mef2c-AHF-cre (51, 52) could be used for this purpose. Based on our results with other SMC lineages, it would be very surprising to find that AdvSca1 cells in the aortic root are AHF-derived. Like neural crest cells, AHF-derived cells populate the outflow tract beginning much earlier in embryonic development than the late stage at which we first observe Sca1 signal (52). Furthermore, AHF contribution is localized to the immediate proximal outflow tract region, whereas AdvSca1 cells are found within the adventitia along the entire length of the ascending, transverse, and thoracic descending aorta.

The experimental results from Hu et al. (7) verified that AdvSca1 cells in adult mice do not represent a transient population of bone marrow-derived progenitors that reside within the adventitia. However, the turnover rate of resident AdvSca1 cells within the adult adventitia has not been investigated. Therefore, we can not rule out the possibility that bone marrow-derived cells contribute to the initial AdvSca1 colonization of the adventitia and that the cells self-renew *in situ* thereafter. The primary site of hematopoietic stem cell (HSC) activity in the developing mouse embryo migrates from the fetal liver to the bone marrow and spleen between E15 and E17.5 (53). The idea that AdvSca1 cells may be deposited in the adventitia during this migration is intriguing. However, the lack of labeling in Tie2-cre lineage tracing experiments suggests that AdvSca1 cells are not of HSC origin. It remains possible that AdvSca1 cells are generated *in situ* by Shh signaling within the resident embryonic adventitial cell population beginning ~E16.5.

*Fibroblasts vs multipotent mesenchymal stromal cells vs pericytes: can AdvSca1 cells be categorized?*

Fibroblasts are widely distributed connective tissue cells of mesenchymal origin that synthesize a variety of extracellular matrix components. They are considered to typically show a flat, spindle-shaped morphology with multiple cellular processes (54). Multipotent mesenchymal stromal cells (MSCs, previously termed mesenchymal stem cells but also known variously as mesenchymal stromal cells, bone marrow stromal cells, marrow-isolated adult multipotent inducible cells, and multipotent adult progenitor cells (55)) have been described as multipotent, plastic-adherent cells with a fibroblast-like appearance that can differentiate to various connective tissue lineages (56). Pericytes have traditionally been identified as perivascular cells with an extensively branched morphology that establish intimate contact with underlying endothelial cells in order to provide support to the

microvessels (57, 58). Yet beneath these simplistic definitions lie multiple studies attempting to characterize these cell types, often with overlapping observations.

Unfortunately, one characteristic common to fibroblasts, MSCs, and pericytes is the inability to phenotypically "define" the cell type. Many of the criteria used currently refer to the behavior of isolated cells *in vitro*, which is obviously not ideal for tracing the activity and responses of the cells *in vivo*. Fibroblasts are often identified by the absence of markers for other cell lineages (59), yet they can also adopt a myofibroblast phenotype which shares features of smooth muscle differentiation. Recent reviews have highlighted a more standardized basic set of marker criteria for each cell type, which is listed in Table 1.

| **Myofibroblasts** (60) | **Multipotent mesenchymal stromal cells** (61) | **Pericytes** (58) |
|---|---|---|
| Vimentin positive | $CD73^+$ | Smooth muscle α-actin |
| Smooth muscle α-actin positive | $CD90^+$ (Thy1) | Desmin |
| Smooth muscle myosin negative | $CD105^+$ (endoglin) | NG2 (chondroitin sulfate proteoglycan 4) |
| Non-muscle myosin positive | $CD45^-$ | PDGF receptor β |
| Virtually no desmin | $CD34^-$ | Regulator of G-protein signaling 5 (RGS5) |
| Fibronectin positive | $CD14^-$ or $CD11b^-$ | |
| | $CD19^-$ or $CD79\alpha^-$ | |
| | HLA-$DR^-$ | |

**Table 1**. *Criteria for cellular classification based on marker expression.*

The heterogeneity of the cell populations referred to as fibroblasts, MSCs, or pericytes adds to the difficulty in establishing generally applicable classification criteria. Moreover, there is considerable overlap between the localization and differentiation potential of these cell types. For example, Song et al. characterized the perivascular cells in mouse

pancreatic islet tumors. By sorting first on the basis of PDGFRβ expression, they found that only a subset (14-15%) of the PDGFRβ$^+$ perivascular cells expressed markers of "mature" pericytes, including desmin, NG2, or SMαA. Moreover, some mature pericytes did not express PDGFRβ. The authors suggest that the PDGFRβ$^+$ perivascular cells in the tumor actually represent a population of progenitors with the capacity to differentiate to desmin, NG2, and SMαA-positive pericytes (49). MSCs are increasingly considered to reside within a perivascular niche (reviewed in (57)) and there have been some reports of MSCs that express pericyte markers (62). In addition, at least subsets of pericytes retain mesenchymal multipotentiality. Fibroblasts/myofibroblasts are among the potential differentiation fates that pericytes can adopt *in vitro* (63).

Cells such as AdvSca1 cells or the PDGFRβ$^+$ perivascular cells characterized by Song et al. do not fit neatly into one particular category. Given the considerable overlap between fibroblasts, MSCs, and pericytes, it may be more conceptually straightforward to envision a spectrum of cell identity with multiple intermediate phenotypes. AdvSca1 cells share many characteristics with MSCs and pericytes, including the ability to differentiate along adipogenic and osteogenic lines. Indeed, our results from the Matrigel plugs suggest that differentiation to a mature pericyte is one potential fate of AdvSca1 cells *in vivo*. Are AdvSca1 cells then distinct from the "adventitial fibroblasts" so frequently mentioned in the literature? Lacking a more specific definition of a fibroblast, it is difficult to draw a definitive conclusion. However, several observations argue against the characterization of AdvSca1 cells as typical connective tissue fibroblasts. Sca1$^+$ cells can be identified in the aortic adventitia only at ~E17.5 and later (Figure 9). Yet vessel cross sections from earlier timepoints, as well as sections from Shh-/- vessels, indicate that there are adventitial cells present even in the absence of AdvSca1 cells (Figure 12 and data not shown). When AdvSca1 cells are isolated and cultured, ~20-30% of the cells lost Sca1 expression but did not upregulate SMC markers. Although there is no direct evidence, it is reasonable to

hypothesize that at least a subset of those cells adopt a more typical fibroblast phenotype under *in vitro* culture conditions. Finally, AdvSca1 cells have the potential to become fully differentiated SMCs *in vitro*, as evident from the expression of SM-MHC (Figure 20). Myofibroblasts express some SMC marker genes such as SMαA but do not fully differentiate.

*AdvSca1 growth factor response in vitro and in vivo*

When we treated AdvSca1 cultures with PDGF-BB, we did not observe a robust change in Sca1 or SMαA expression (Figures 18 and 24). These results contrast with data reported by Hu et al. in which PDGF-BB treatment induced large increases in the expression of SMαA, calponin, and SM-MHC (7). There are a couple of methodology differences that could account for the variation. In order to isolate AdvSca1 cells, Hu et al. first explanted adventitial tissue from the aortic arch and root, allowing cellular outgrowth in a stem cell medium containing leukemia inhibitory factor (LIF) and β-mercaptoethanol. Cells grown out from the explant were then dispersed with trypsin and immunomagnetically selected for Sca1 expression. $Sca1^+$ cells were expanded through at least one passage before use in experiments (7).

In contrast, we used immunomagnetic selection to isolate $Sca1^+$ cells from a suspension of aortic adventitial cells. Isolated cells were placed in culture and used without passage. In our hands, culturing AdvSca1 cells in media containing LIF and β-mercaptoethanol did not alter the proportion of cells that lost the $Sca1^+$ phenotype and upregulated SMαA (data not shown). The behavioral differences in the cells isolated by these two methods may indicate an interesting heterogeneity in the population of adventitial cells selected by Sca1 expression. By isolating $Sca1^+$ cells from explant cultures, Hu et al. may have excluded a subset of AdvSca1 cells that are more prone to *in vitro* differentiation,

which we observe as cells that upregulate SMαA without any additional exogenous treatment. The potential heterogeneity of AdvSca1 cells is further discussed below.

One other important difference is that Hu et al. were isolating AdvSca1 cells from ApoE-/- mice, whereas the cells in our studies were from wild-type mice. In follow-up work to the initial characterization of AdvSca1 cells, Mayr et al. compared the proteomes of ApoE+/+ and ApoE-/- SMCs to SMCs derived from adult ApoE-/- AdvSca1 cells. Unlike $Sca1^+$ progenitors derived from embryonic stem cells, ApoE-/- AdvSca1 cells proteomically resembled ApoE-/- SMCs following PDGF-BB treatment. Notably, there were distinct differences from wild-type SMCs (64). However, a functional or proteomic comparison of AdvSca1 cells from wild-type versus ApoE-/- mice has yet to be performed.

Basic fibroblast growth factor (FGF2) is a mitogen for both endothelial cells and mural cells (65) and has been shown to promote angiogenesis in Matrigel plugs as well as other *in vivo* models (66). FGF2 mRNA is found almost ubiquitously throughout tissues, including all cells of the artery wall (67, 68), yet the protein itself is often localized to the extracellular matrix (69, 70). Injury to the vessel wall, however, causes the increase of FGF2 expression (71) and the release of FGF2 protein (72). Significantly, Cuevas et al. found that infusion of FGF2 onto the adventitia of rat carotid arteries induced extensive cell proliferation and a robust (>10-fold) expansion of the vasa vasorum. The expanded microvasculature had capillaries and sinusoids with vessel walls composed of endothelial cells, pericytes, and in some cases multiple layers of SMCs (68). In our evaluation of AdvSca1 differentiation in the *in vivo* Matrigel plug assay, we used FGF2 to create an angiogenic environment. Under these conditions, AdvSca1 cells differentiated to mural cells and contributed to the formation of a vascular network. At the moment, we do not know whether FGF2 acts directly on AdvSca1 cells or if they respond to other signals within the angiogenic environment. It would be very interesting to test whether AdvSca1 cells in the intact adventitia respond to injury or FGF2 infusion by contributing to the expansion of vasa

vasorum microvessels. The release of FGF2 may be one factor that promotes neoadventitia formation following vessel injury. It will therefore be important to evaluate the effect of FGF2 on AdvSca1 cell proliferation as well as mural cell differentiation.

*Transcriptional regulation of the AdvSca1 progenitor phenotype*

AdvSca1 cells do not express SMC marker proteins *in vivo*. It was intriguing, therefore, to find that AdvSca1 cells contained transcripts for SRF and myocardin family members and were immunopositive for SRF when examined immediately after isolation from the artery wall. These transcription factors are strong activators of SMC gene expression (22, 73-75) and are necessary for vascular SMC differentiation *in vivo*. Yet SRF also interacts with potent transcriptional corepressors, and it is the competition between coactivators and corepressors that determines whether SRF-dependent target genes are transcribed (74-78). Indeed, we found that the transcriptional corepressors *Msx1*, *Klf4*, and *FoxO4* were also expressed by AdvSca1 cells. Msx1 forms a ternary complex with SRF and myocardin and inhibits binding of SRF-myocardin to CArG box motifs in SMC target genes (41). Klf4 is a zinc finger-containing protein that binds to a GC-rich element (TCE) and inhibits SRF-dependent transcription of multiple SMC marker genes (77). The forkhead transcription factor FoxO4 interacts with myocardin and inhibits its coactivator function for SRF-dependent SMC gene expression (43).

These results are reminiscent of a report by Matsuura et al. (79), showing that $Sca1^+$ cells from adult mouse hearts express Nkx2.5 and GATA4, two transcription factors important for cardiac myocyte differentiation, yet they do not express markers of differentiated myocardial cells. Heart-derived $Sca1^+$ cells differentiate to beating cardiomyocytes when exposed to oxytocin in vitro, thus confirming their cardiogenic potential (79). These findings in heart and artery wall suggest a model in which $Sca1^+$ progenitor cells in postnatal tissues are specified for certain cell fates by expression of

transcription factors that are required for those fates. The maintenance of a SMC progenitor phenotype may therefore critically depend on expression of transcriptional corepressors that block SRF-dependent transcription and recruit chromatin remodeling complexes to silence gene expression.

*The Matrigel plug assay as a test of AdvSca1 in vivo differentiation potential*

Matrigel is solubilized basement membrane that is prepared from Engelbreth-Holm-Swarm mouse sarcoma. Laminin is the major component, followed by collagen IV, heparin sulfate proteoglycans, and entactin/nidogen (80). In growth factor-reduced preparations, FGF2 (0-0.1 pg/mL), epidermal growth factor (<0.5 ng/mL), insulin-like growth factor 1 (5 ng/mL), PDGF (<5 pg/mL), nerve growth factor (<0.2 ng/mL), and TGF-β (1.7 ng/mL) are also present. The Matrigel plug angiogenesis assay (44) is commonly used to test the angiogenic (or antiangiogenic) potential of growth factors and chemical compounds, as well as to evaluate the behavior of cells suspended in the plug. As an *in vivo* assay to test AdvSca1 behavior and differentiation potential, Matrigel plugs offer both advantages and disadvantages. By isolating AdvSca1 cells for the Matrigel plugs from genetically labeled mice, we can easily differentiate AdvSca1-derived cells in the plug from host-derived cells. The AdvSca1 cells are incubated in an *in vivo* environment, as opposed to the highly artificial conditions in a culture dish. The implantation procedure is relatively non-invasive and does not induce a robust inflammatory response. Finally, the possibility that AdvSca1 cells actively participate in angiogenic events in the adventitia holds great developmental and pathological relevance (see further discussion in Chapter 5). Thus, it is logical to test AdvSca1 behavior in a well-established angiogenesis assay.

There are several factors that are important to keep in mind when interpreting the Matrigel plug results. Foremost, the Matrigel environment is going to differ from the adventitia in multiple ways. The adventitia is generally considered to have a collagen-rich

extracellular matrix (81), while the primary component of Matrigel is laminin. The thickness and subcutaneous location of a Matrigel plug may also result in substantial differences in oxygen levels compared to the arterial adventitia. The availability and concentrations of growth factors are almost certainly going to differ between the adventitia and the Matrigel plug. Finally, the AdvSca1 cells in the Matrigel plug are initially suspended at a concentration that would not promote cell-cell contact or readily allow diffusion of secreted factors between cells. Each individual AdvSca1 cell is thus initially influenced only by autocrine signaling and factors available from the Matrigel itself.

Bearing in mind the potential differences between the adventitia and the Matrigel, we can nonetheless draw valuable insight from the *in vivo* transplant assay. The FGF2 in the plugs provides an angiogenic signal that induces cells from the host tissue to infiltrate the Matrigel and assemble into vascular structures, a process which can not easily be duplicated in a culture dish. AdvSca1-derived cells integrate into the microvessels (Figures 21-23) and may also promote efficient vascular assembly in the early stages by secreting additional proangiogenic factors. We observed differentiation of AdvSca1 cells to mural cells (Figure 23), which supports our *in vitro* data demonstrating differentiation to SMC-like cells. Furthermore, our preliminary data suggest that AdvSca1 cells also have the *in vivo* potential to differentiate to macrophages and adipocytes (Supplemental Figure 1 in Appendix A), two cell types extremely relevant to adventitial and periadventitial tissue. While additional models will certainly be required to fully explore the behavior of AdvSca1 cells *in vivo*, the participation of AdvSca1 cells in Matrigel plug angiogenesis gives us a valuable clue to a potential role for the cells in the adventitia in general and in remodeling of the vasa vasorum microvasculature in particular.

*AdvSca1 self-renewal versus differentiation*

Sca1 is a cell surface marker commonly associated with stem/progenitor populations, but it is expressed by multiple cell types (2). Morphologically, $Sca1^+$ cells in the adventitia *in vivo* appear grossly uniform, having a slightly flattened and elongated shape (see Supplemental Figure 2 in Appendix A). Yet none of our data rules out the possibility that there are intrinsic differences between adventitial cells which both express Sca1. When AdvSca1 cells are directly isolated from the adventitia and placed in culture, a subset of the cells differentiates to SMC-like cells that express SMαA (Figure 16). Whether an individual cell differentiates or retains Sca1 expression under these conditions might be a stochastic choice, or alternatively the fate of each cell may be influenced by the differential expression of factors such as growth factor receptors or SRF cofactors. In support of the latter possibility, culturing AdvSca1 cells in conditions promoting adipogenic or osteogenic differentiation also induces only a fraction of the cells to adopt those fates (Figure 19). An *in vitro* clonal assay would begin to address the question of whether each individual AdvSca1 cell has the potential for self-renewal as well as SMC, adipogenic, and osteogenic differentiation. It will be critical, however, to first gain a better understanding of the environment to which the cells are exposed within the adventitia *in vivo*. When isolated for culture, AdvSca1 cells are removed from the influence of cell-cell contacts, extracellular matrix interactions, growth factor and cytokine exposure, and other potentially important elements of the adventitial environment. Any or all of these components may be critical for regulating AdvSca1 phenotype and the balance between self-renewal and differentiation. Using culture conditions that reproduce important elements of the *in vivo* environment will lead to a better understanding of AdvSca1 behavior in the artery wall.

During flow-induced carotid artery remodeling, one of the earliest responses is proliferation within the adventitial layer, resulting in neoadventitia formation by one week post-ligation. Our results indicate that AdvSca1 cells proliferate during this time period

without undergoing significant differentiation to a myofibroblast or SMC phenotype (Figures 25 and 26). More extreme responses have been observed in other models of arterial injury, particularly balloon overstretch models which mimic angioplasty (82-86). It seems reasonable to draw a correlation between the severity of the arterial injury and the degree of adventitial response. Balloon injuries, which circumferentially distend the vessel and often denude the endothelial layer, are far more damaging to the vessel wall than the initial increase in pulsatile pressure in the flow-reduction. It would be informative to compare the early arterial response to balloon injury versus flow-reduction with regards to changes in signaling factors and adventitial gene expression. Factors that are altered by balloon injury but not flow-reduction may be candidates for provoking AdvSca1 differentiation *in vivo*. Likewise, any differences between flow-reduced vessels and uninjured vessels may play a role in promoting AdvSca1 proliferation.

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# CHAPTER 4

## Krüppel-like factor 4 contributes to repression of smooth muscle genes and helps maintain the capacity for self-renewal in AdvSca1 cells

## Introduction

Krüppel-like factors (Klf) are transcriptional regulators that play diverse roles in differentiation, development, proliferation, and apoptosis (1, 2). Klf family members have three highly homologous zinc fingers at the carboxy terminus of the protein that mediate binding to DNA, and as a result, family members bind very similar consensus DNA sites. In contrast, the non-DNA binding regions of the Klf proteins are highly divergent (3). Many family members can function as both transcriptional repressors and activators.

Murine knockouts of individual Klf family members result in diverse phenotypes (4). Notably, KLF2-/- mice die between E12.5 and E14.5 from severe intra-embryonic and intra-amniotic hemorrhaging. They display normal vasculogenesis and angiogenesis, but fail to properly recruit and organize supporting SMCs and pericytes (5). KLF5 knockout blastocysts fail to implant, resulting in early embryonic lethality (6, 7). Furthermore, Klf5 may play an essential role in the maintenance of pluripotent embryonic stem (ES) cells by suppressing the expression of differentiation-related genes (7). KLF5 heterozygous mice are viable but display vascular defects, including abnormal thinning of the aortic media and adventitia and abrogated response to vessel injury (6).

KLF4 knockout mice die shortly after birth from dehydration as a result of defective skin barrier formation (8). No obvious cardiovascular defects were reported in the germline knockout mice, but several studies have implicated Klf4 in the regulation of SMC gene expression in smooth muscle cells undergoing phenotypic modulation (9-14). Most recently, Yoshida et al reported that KLF4 expression is induced in medial SMCs following carotid artery ligation. Mice with a conditional depletion of Klf4 showed a delay in the repression of SMC marker genes, consistent with the reported role of Klf4 as a co-repressor of SRF-dependent SMC gene expression (13).

Coexpression of Klf4 along with Oct4, Sox2, and c-Myc or UTF1 dramtically reprograms fully differentiated adult somatic cells to pluripotent embryonic stem cells (15,

16). Klf4 has therefore been referred to as a pluripotency factor, although it clearly has a number of important functions in adult cell types, including monocytes (17), sertoli cells (18), and epidermal cells (8). The roles played by Klf4 in cellular reprogramming involve transcriptional control pathways for key pluripotency target genes such as Oct4 and Nanog (19, 20). Jiang et al recently reported that Klf2, 4, and 5 function within a Klf-dependent transcriptional network to regulate self-renewal in ES cells, and that they display some degree of functional redundancy in the control of pluripotency (19).

## Hypothesis

Sca1$^{+}$ smooth muscle progenitor cells within the arterial adventitia self-renew and maintain a "pre-SMC" progenitor phenotype that is poised for signal-responsive SMC differentiation. The transcription factor Klf4 regulates the maintenance of AdvSca1 cells as progenitors within the vessel wall by controlling one or both of these processes.

## Materials and Methods

Animals Used: All protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina. KLF4 mice were a gift of Julie Segre, National Human Genome Research Institute, and are described in Segre et al. (8).

Immunofluorescence Staining: Tissues were fixed for 1 hr in freshly prepared 4% PFA, rinsed in PBS, saturated with 20% sucrose for cryoprotection, embedded in agar, and frozen in OCT. Twelve-micrometer cryosections were fixed in methanol at room temperature for 5 min, permeabilized with 0.05% Tween-20 (in PBS) for 2 min, rinsed in PBS, then blocked in 2% normal goat serum (NGS) or normal donkey serum for 2–8 h. Sections were incubated with primary antibodies at 4°C overnight, rinsed twice in PBS + 0.1% BSA, then incubated with secondary antibodies for 2-8 h at room temperature,

protected from light. Nuclei were counterstained with 10 μg/ml Hoechst 33258 in $H_2O$ and slides were mounted in Mowiol with 2.5% DABCO.

For cells in culture, cells were fixed by incubating in freshly prepared 4% PFA for 15 min at room temperature, then permeabilized with 1:1 methanol/acetone for 1 min. Cells were blocked in 2% normal serum for 1-4 h at 4°C, then incubated with primary antibodies at 4°C overnight. After two rinses in PBS + 0.1% BSA, cells were incubated with secondary antibodies 1-4h at room temperature protected from light. Nuclei were counterstained with 10 μg/ml Hoescht 33258 in $H_20$. For EdU labeling experiments, cells were incubated with 10 μM EdU (Invitrogen C10340) for one hour then fixed and stained with the Click-iT Edu Imaging Kit according to manufacturer instructions. After washing in PBS, standard protocol was followed to stain with additional primary and secondary antibodies.

Primary antibodies used for these studies included mouse anti-SMαA (1:100; Sigma A2547), rat anti-Sca1 (1:100; BD Pharmingen 553333), mouse anti-FLAG (1:500; Sigma F1804), and chicken anti-GFP (1:1000; Abcam ab13970). All secondary antibodies were AlexaFluor-conjugated (Invitrogen) and used at a dilution of 1:500 in PBS. Immunofluorescence staining was visualized with a Leica BM IRB inverted epifluorescence microscope with images captured by a QImaging Retiga 1300 digital camera or a Olympus IX 81-ZDC Inverted Fluorescence Microscope with images captured by a Hammatsu ORCA RC camera. Confocal images were obtained with a Zeiss LSM710 spectral confocal laser scanning microscope and processed with Zeiss LSM Image Browser and Adobe Photoshop.

AdvSca1 Cell Isolation and Culture: Mice were sacrificed by cervical dislocation and perfused through the left ventricle with PBS. The thoracic aorta was cut at the diaphragm and periadventitial fat was cleared from around the vessel. The aorta was gently lifted away from the dorsal body wall, with intercostal arteries trimmed even with aortic wall. In arch region, the superior vena cava and pulmonary artery were cleared and branching arteries and ductus arteriosus were trimmed even with the aortic wall. At the junction with the

myocardium, the aorta was cut flush with the ventricular wall and placed in HBSS at 37ºC. Before digesting, any remaining fat and coagulated blood were cleared from the tissue.

Whole aortas were briefly digested in 0.4 mg/mL collagenase in HBSS at 37°C for 6 min. Tissue was immediately removed and placed in fresh HBSS at 37°C. Adventitia was gently separated from the media of each aorta by peeling the layer up and over the vessel. Adventitias were placed in the second digestion solution of 15 mg/mL collagenase II (Worthington, 4176) and cut into small pieces. Tissue was digested for 2-2.5 hrs at 37°C with constant rocking to achieve a single-cell suspension, which was then passed through a 30 µm pre-separation filter (Miltenyi, 130-041-407). Filtrate was collected and cells were pelleted at 300xg then rinsed in PBS + 0.5% BSA. Aortic $Sca1^+$ cells were isolated using anti-Sca1 immunomagnetic MicroBeads (Miltenyi ) and a MACS cell separation system (Miltenyi ) according to manufacturer's instructions. Cells were passed over two consecutive columns to increase purity of the isolation. Isolated $Sca1^+$ cells were cultured in DMEM (Sigma) plus 10% FBS (HyClone) and 1x antibiotic/antimycotic solution (Gibco) at 37°C, 5% $CO_2$. Cells were seeded in 48-well tissue culture plates at a density of $8x10^3$-$1.5x10^4$ cells per well, depending on the assay.

Adenovirus infection: Purified adenovirus expressing GFP was purchased from the UNC Gene Therapy Center Virus Vector Core Facility. Adenovirus expressing FLAG-KLF4 under control of the CMV promoter was a gift from Gary Owens, University of Virginia. AdvSca1 cells were isolated according to standard procedure and incubated overnight in DMEM +10% FBS + 1x antibiotic/antimycotic. The next day, adenovirus was added to 300 MOI with 1.2 µg/mL polybrene (Sigma H9268). Cells were incubated for 5 d with virus and then fixed for immunostaining.

RT-PCR Analysis: Total cellular RNA was isolated by guanidinium isothiocyanate denaturation and phenol/chloroform extraction as described (21). Two-step RT-PCR was carried out with the GeneAmp RNA PCR kit (Applied Biosystems) according to the

manufacturer's instructions. The sequences for primers used for RT-PCR analysis of gene expression are provided in Appendix B. Unless otherwise indicated, primer sequences were designed for this study based on mouse genomic sequence available through Ensembl (release 45, June 2007; (22)). Primers were designed with the aid of Primer3 software (v. 0.4.0) (23).

siRNA Transfection: AdvSca1 cells were isolated and cultured according to standard protocol until cells reached 60-70% confluency. Medium was replaced with DMEM + FBS (no antibiotics) and cells were incubated overnight. The following day, cells were transfected with Dharmafect4 transfection reagent (0.02 µL per 100 µL transfection medium; Thermo Scientific) and siGENOME SMARTpool siRNAs (100 nM; Thermo Scientific). An siRNA that does not target known mouse genes was used as a negative control (AAUAGAUGAGAGACACACAGCTT, 100 nM; MWG Biotech). Cells were incubated for 48-72 h, as indicated.

## Results

### *Knockdown of Klf4 in vitro causes loss of Sca1 expression*

KLF4 is highly expressed in AdvSca1 cells, downregulated as the cells differentiate in culture, and expressed only at extremely low levels in SMCs of the aortic media (Figure 20). Based on its known functions as a co-repressor of SRF-dependent SMC gene transcription and as a regulator of self-renewal in embryonic stem cells (ESCs), we sought to test whether AdvSca1 cells rely on Klf4 activity to maintain their status as SMC progenitor cells. We used a siRNA-mediated approach *in vitro* to evaluate the effects of loss of Klf4 activity. At 72hrs after siRNA transfection, KLF4 mRNA expression is >90% reduced (Figure 27F). Klf4 knockdown decreases Sca1 mRNA expression (Figure 27F) as well as reducing the number of Sca1-positive cells, as evaluated by immunofluorescence staining (Figure 27 B,D). While there appears to be an increase in SMα-actin mRNA expression

(Figure 27F), the percentage of total cells that exhibit SMα-actin-positive staining in a definite cytoplasmic stress fiber filament pattern did not increase upon Klf4 knockdown (Figure 27E). This may suggest that SMα-actin expression remains repressed in a subset of AdvSca1 cells in spite of Klf4 knockdown, perhaps due to the activity of other SRF co-repressors. Alternatively, the 72 hr time point used for these experiments may be too short to see nascent SMα-actin protein incorporated into cytoplasmic filaments. Increased levels of SMα-actin mRNA (Figure 27F) would argue that Klf4 knockdown produced a corresponding increase in SMC marker expression. However, evaluation of SMα-actin mRNA and protein expression at additional time points will be necessary to solidify these conclusions.

Work by Jiang et al. suggests that Klf4 plays a role in regulating the self-renewal capacity of pluripotent stem cells (19). We used EdU (an analog of BrdU) to examine the proliferation index in siRNA-treated AdvSca1 cultures. At 72 hrs after siRNA transfection, we found ~90% reduction in proliferating $Sca1^+$ cells compared to control samples (Figure 27G). In comparison to the ~73% reduction predicted by the overall decrease in the number of $Sca1^+$ cells, these results are consistent with loss of Klf4 activity causing a defect in the ability of AdvSca1 cells to proliferate while maintaining a $Sca1^+$ progenitor phenotype. Additional experiments will be required to substantiate a role for Klf4 in the regulation of true AdvSca1 self-renewal.

Klf2 and Klf5 can functionally substitute for Klf4 activity in the maintenance of ESC self-renewal (19), therefore we also tested whether knockdown of multiple Klf's would synergistically promote AdvSca1 differentiation or self-renewal defects. While KLF5 expression was also reduced following siRNA treatment, we did not observe any additive effects upon simultaneous knockdown of Klf4 and Klf5 (Figure 27). We were unable to achieve knockdown of Klf2 using these methods. Overall, these results suggest that Klf4 plays an important role in the maintenance of AdvSca1 progenitor phenotype. Based on

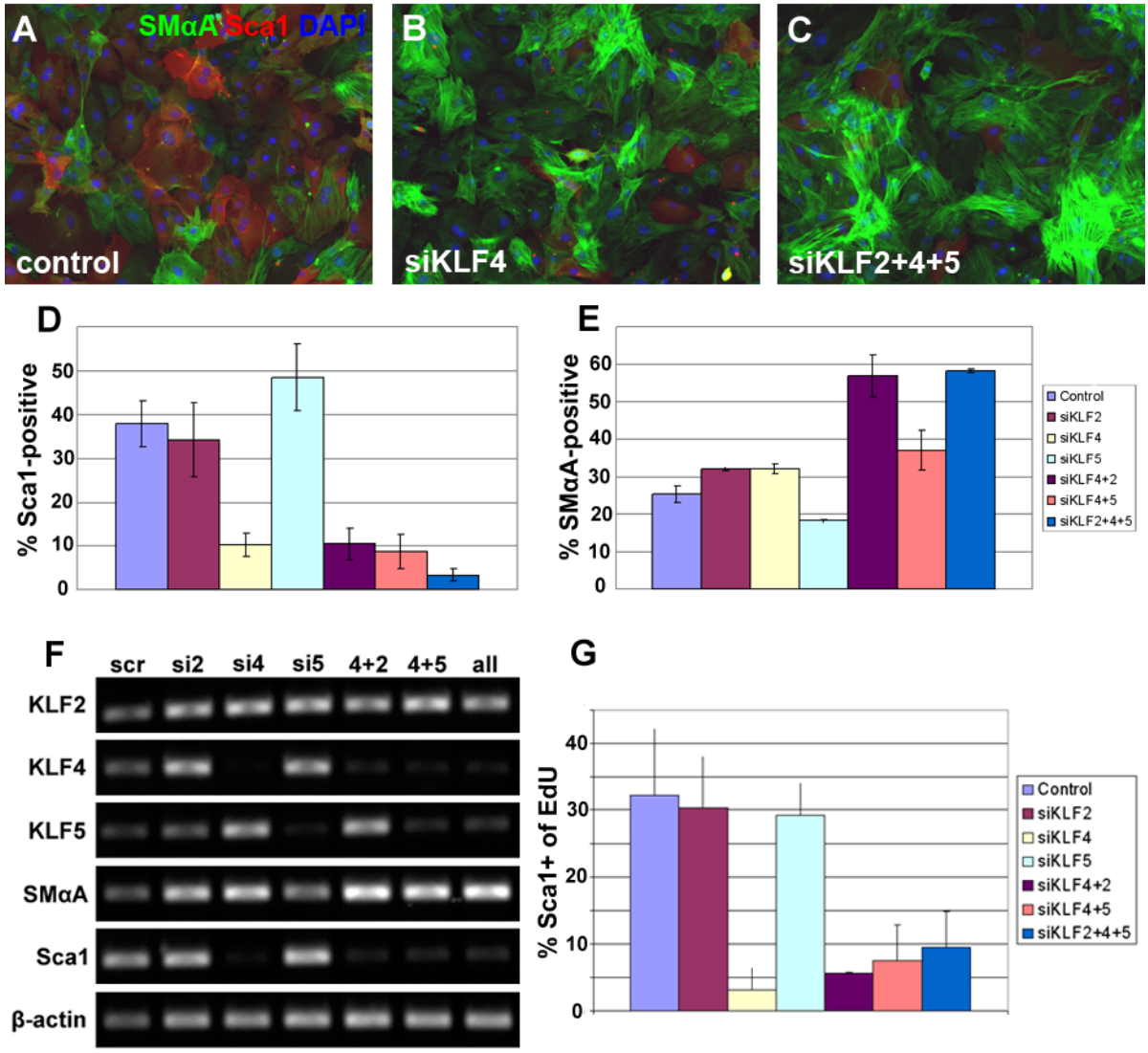


**Figure 27**. *Effects of Klf4 knockdown on AdvSca1 cells in vitro*. Cultured AdvSca1 cells were transfected with siRNAs targeting KLF2, KLF4, or KLF5, as indicated, and evaluated 72 hrs after siRNA transfection. (A-C) Representative images from treated cultures with immunostaining for SMα-actin (green), Sca1 (red), and DAPI (blue). (D) Sca1-positive and (E) SMα-actin-positive cells from each treatment group were counted and normalized to total cell numbers. (F) RNA was collected from treated cells and RT-PCR used to evaluate knockdown efficiency and changes in Sca1 and SMα-actin gene expression. (G) Cell proliferation was evaluated by EdU incorporation and co-staining for Sca1. Values are expressed as percent of total EdU-positive cells expressing Sca1. Overall, Klf4 knockdown decreases Sca1-positive cells in AdvSca1 cultures.

these preliminary findings, these effects are specific to Klf4, as the presence or absence of Klf2 and Klf5 did not impact the observed changes in Sca1 expression or the number of SMα-actin-positive cells.

*Overexpression of Klf4 prevents AdvSca1 differentiation to smooth muscle cells*

AdvSca1 cells differentiate to SMC-like cells *in vitro*, a change accompanied by downregulation of KLF4. Whether the decrease in KLF4 expression during these initial changes in cell phenotype is a cause or effect of AdvSca1 differentiation is unclear. Since knockdown of Klf4 promoted the loss of a $Sca1^+$ progenitor phenotype, we also tested whether overexpression of Klf4 in AdvSca1 cells *in vitro* would prevent their differentiation. Cells overexpressing adenovirus-driven FLAG-KLF4 were co-stained with SMα-actin or Sca1 (Figure 28) as markers of cellular identity. GFP adenovirus was used as a

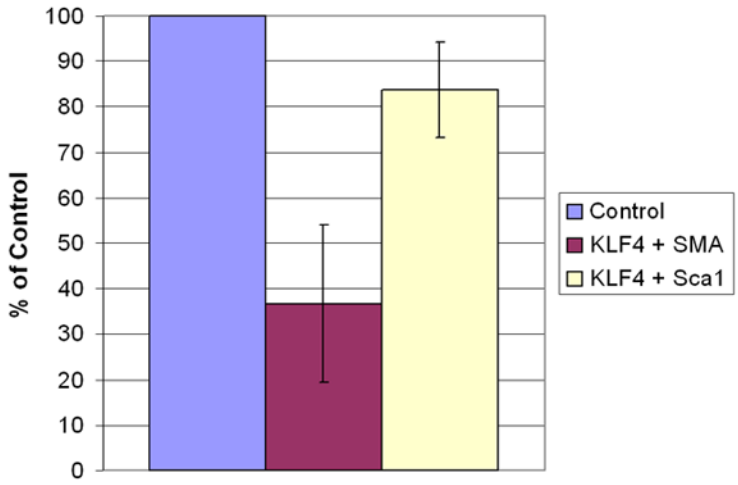


**Figure 28**. *Evaluation of SMα-actin and Sca1 expression in cells overexpressing Klf4*. Cultured AdvSca1 cells were infected with adenovirus expressing GFP (control) or FLAG-KLF4. After 5 d, cells were fixed, stained for SMα-actin (SMA) or Sca1, and evaluated for adenovirus co-expression. Values are expressed as fraction of adenovirus-positive cells expressing either SMα-actin or Sca1, normalized to control. Klf4 overexpression decreases the number of SMα-actin-positive cells but does not prevent or reverse the downregulation of Sca1.

control for comparison. If Klf4 is functioning as a co-repressor of SRF-dependent transcription in AdvSca1 cells, we would predict that the expression of SMC marker genes such as SMα-actin would be blocked by overexpression of Klf4. Indeed, our results indicate that the number of cells co-expressing FLAG-KLF4 and SMα-actin was significantly reduced compared to control virus (Figure 28). Interestingly, however, we did not observe an increase in the number of cells co-expressing FLAG-KLF4 and Sca1, as would be expected if overexpression of Klf4 was sufficient to maintain AdvSca1 cells as $Sca1^+$ progenitors *in vitro*. While not conclusive, these results suggest that Klf4 overexpression in AdvSca1 cells can block the expression of SRF-dependent marker genes but that additional factors are required to maintain a $Sca1^+$ progenitor phenotype when AdvSca1 cells are removed from their native adventitial environment.

## Discussion

### *Role of Klf4 in regulation of smooth muscle gene expression*

KLF4 was initially identified as a gene that is highly expressed in growth-arrested NIH 3T3 cells but nearly undetectable in exponentially proliferating cells (24). Klf4 was first connected to SMC gene transcription in work by Adam et al, where they found that Klf4 bound to a GC-rich transforming growth factor β (TGF-β) control element in the SM22α promoter, repressing its activity (9). Subsequent work suggests that Klf4 may actually have multiple mechanisms for repression of SMC genes. SMCs overexpressing Klf4 *in vitro* displayed decreased expression of myocardin (11) and distinct reductions in histone H4 acetylation at the SMα-actin and SM-MHC promoters (25).

The striking reduction in Sca1 expression upon knockdown of Klf4 in AdvSca1 cells is reminiscent of the effect of treating cultured AdvSca1 cells with TGF-β (Figure 19), which also causes a loss of Sca1 expression. There is a plausible link between these two

observations. The TGF-β control element (TCE) found in the promoters of SMC genes such as SMα-actin and SM22α is critical for expression of these genes *in vitro* and *in vivo* (9, 26). Klf4 has been shown to bind to the TCE and exert repressive effects on TGF-β-stimulated gene expression (9, 10, 26). Initially, TGF-β treatment was observed to decrease KLF4 gene expression (9) but recently, Kawai-Kowase et al. reported that the inductive effects of TGF-β on SMα-actin gene expression are mediated through the SUMO E3-ligase PIAS1. The authors went on to show that PIAS1 promotes the degradation of Klf4, which requires the SUMO E3-ligase activity (27). Klf4 can activate its own promoter (28), potentially explaining the observed reduction in KLF4 gene expression following TGF-β stimulation. It will be interesting to further investigate a possible link between TGF-β, Klf4, and differentiation of AdvSca1 cells.

*AdvSca1 cells depend on Klf4 to maintain a progenitor phenotype*

Drawing a distinction between loss of the capacity for self-renewal and promotion of differentiation can be conceptually challenging. EdU incorporation reveals fewer proliferating $Sca1^+$ cells in Klf4 knockdown cultures, consistent with a decreased ability of dividing AdvSca1 cells to maintain their progenitor phenotype. Yet the possibility remains that loss of Klf4 may simply remove a block preventing differentiation rather than representing the loss of a factor that actively promotes the expression of genes that maintain the progenitor phenotype. Knockdown of Klf4 followed by challenging the cells with factors that promote the adoption of cell fates other than smooth muscle may be useful in determining if loss of the progenitor phenotype makes AdvSca1 cells vulnerable to other instructive cues or whether Klf4 knockdown promotes only the smooth muscle fate.

Studies into the mechanisms controlling self-renewal of other adult progenitor cells such as hematopoietic stem cells and myogenic satellite cells have illustrated the critical interplay between the external niche environment and transcription factors controlling cell-

type-specific gene expression (reviewed in (29-31)). In muscle satellite cells, the paired box 7 (Pax7) gene appears to be a critical regulator of both cell fate and self-renewal. Quiescent satellite cells express Pax7 (32), and levels of the myogenic regulatory factor MyoD are very low or undetectable (33). When satellite cells are activated, MyoD is rapidly induced and Pax 7 levels begin to decrease. The activity of MyoD is regulated at multiple levels (34), including repression by Pax7 itself (35). Thus, one could consider Pax7 as a central factor in a network of muscle satellite cell transcriptional regulation, with roles in both repression of differentiation and control of self-renewal. None of our results rule out such a dual role for Klf4 in AdvSca1 cells, with functions in the regulation of both self-renewal and repression of SMC gene expression.

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# CHAPTER 5

## Summary and future directions

**Summary of results**

1) Beginning at E15.5 and persisting into adulthood, active Hh signaling in the artery wall is restricted to the adventitial compartment, as demonstrated by Ptc1-, Ptc2-, and Gli1-lacZ reporter mice. Hh-responsive adventitia is found throughout the arterial system, including the aorta, pulmonary trunk, coronary, femoral, mesenteric, and intercostals arteries. Shh protein is concentrated at the border between the media and adventitia.
2) $Sca1^+$ vascular progenitor cells (AdvSca1 cells) reside within the adventitia beginning between E16.5 and E17.5 and persisting through adulthood. RT-PCR analysis demonstrates that AdvSca1 cells express multiple Hh signaling pathway components. In Shh-/- mice, AdvSca1 cells are drastically reduced in number, and *in vitro* experiments using cyclopamine to inhibit Hh pathway activity suggest that Shh signaling plays an important role in promoting the proliferation of AdvSca1 cells.
3) Lineage tracing analyses demonstrate that AdvSca1 cells are derived from different embryonic origins than their neighboring medial SMCs. AdvSca1 cells were also not labeled by Tie2-cre expression, indicating that they are not of hematopoietic stem cell origin.
4) AdvSca1 cells do not express SMC markers *in vivo*, yet a subset of isolated AdvSca1 cells in culture loses Sca1 expression and upregulates SMC marker genes. RT-PCR analysis indicates that AdvSca1 cells express SRF and SRF cofactors thought to be sufficient for SMC differentiation, but the presence of SRF co-repressors, which are also expressed *in vivo*, may prevent the expression of SMC marker genes. As AdvSca1 cells differentiate *in vitro*, the expression levels of co-repressors are decreased and levels of co-activators are increased.
5) *In vitro* and *in vivo* experiments indicate that AdvSca1 cells have SMC and mural cell differentiation potential. In addition, evidence suggests that at least some AdvSca1

cells can produce osteogenic cells *in vitro*, endothelial cells and possibly macrophages *in vivo*, and adipogenic cells both *in vitro* and *in vivo*. In Matrigel plugs *in vivo*, AdvSca1 cells localize to the perivascular region of newly formed microvessels and express markers commonly associated with perictyes.

6) Knockdown of Klf4 *in vitro* drastically decreases the number of AdvSca1 cells that retain a $Sca1^+$ progenitor phenotype, causes defects in $Sca1^+$ cell proliferation, and increases SMα-actin mRNA levels. Conversely, overexpression of KLF4 blocks SMα-actin expression.

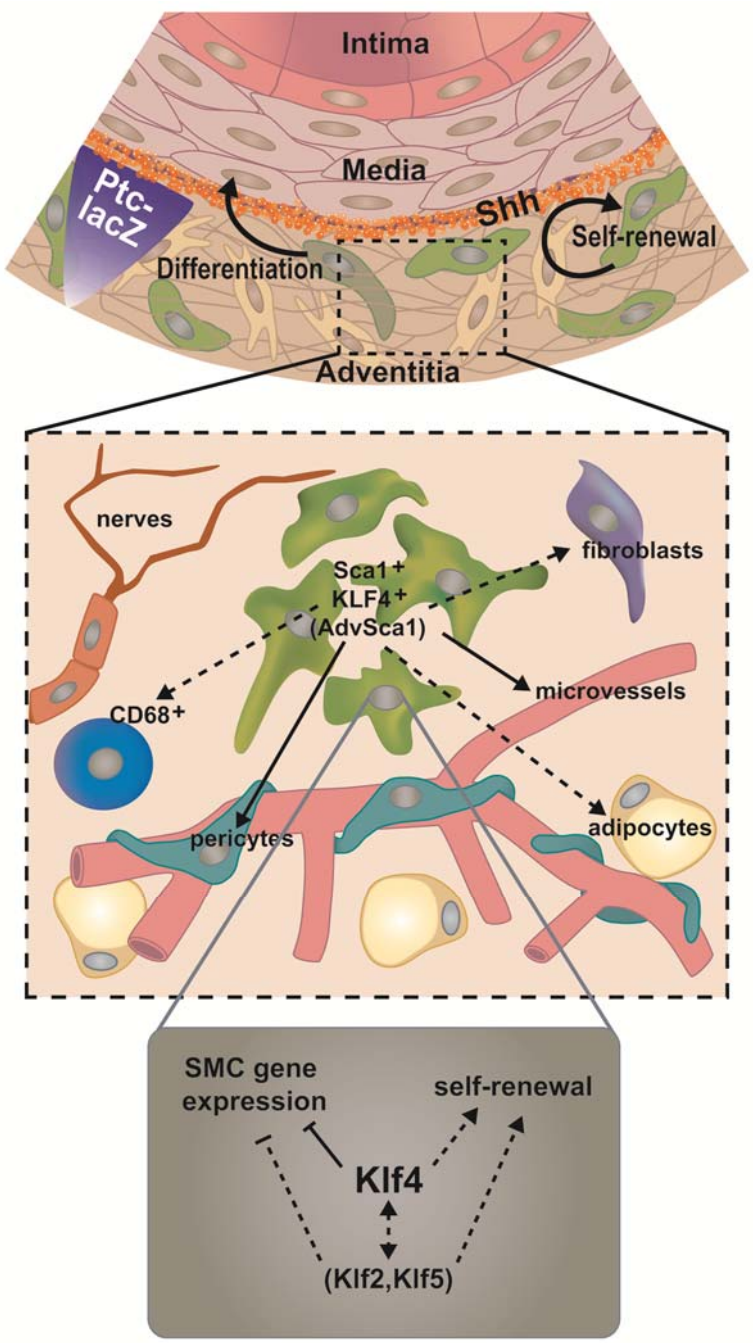


**Figure 29.** *Working model of AdvSca1 regulation*. Shh protein concentrated at the border between the arterial media and adventitia signals to multiple cell types within the adventitia. Resident within this Hh-responsive zone are $Sca1^{+}$ vascular progenitor cells with the capacity for self-renewal and differentiation to multiple vascular cell types. The transcription factor Klf4 is highly expressed in AdvSca1 cells and may serve as a central factor in a regulatory network controlling the expression of cell-type specific genes and progenitor cell self-renewal.

## Future directions

*Hedgehog signaling and development of the artery wall*

The developmental localization of Hh signaling and Shh protein to the adventitia is striking. While Shh is clearly important for the proper establishment of AdvSca1 cells, this role is likely to be secondary to other functions for Shh signaling in the vasculature. Indeed, other data from our lab demonstrate that multiple adventitial cell types are Hh-responsive at early postnatal timepoints, including macrophages, adipocytes, and perineural cells. Experiments are currently underway to evaluate the production of Shh ligand by different adventitial cell types to determine if Hh-responsive cells also synthesize ligand. These studies use Shh-EGFP mice in which enhanced green fluorescent protein is knocked into the endogenous Shh gene locus, replacing the first 35 base pairs of exon one following the ATG (1), and results will be verified by *in situ* hybridization for Shh mRNA.

Hedgehog signaling could be critical for the development and/or function of any of the responsive cell types. In addition to its roles in progenitor cell maintenance, proliferation, and survival, Shh has been demonstrated to have chemoattractive qualities for neural progenitor cells (2) and monocytes (3) and influences the migration of several other cell types (4). Therefore, one possible role of Shh expression in the adventitia during development is to recruit and assemble the various cell types that are integral to a functional adventitial compartment. Genetic tools will be useful in targeting Hh pathway activation or knockout in specific cell types such as SMCs, macrophages, and adipocytes. Cell type-specific cre mice used in combination with floxed-Smo (5) or mice that conditionally express a constitutively active form of Smo (6) will allow evaluation of the effects of Hh signaling deletion or constitutive activation, respectively.

The role of Hh signaling in SMC differentiation is intriguing. Preliminary data from our lab suggest that multiple Hh pathway components are downregulated as cells undergo

SMC differentiation (see Supplementary Figure 3 in Appendix A), indicating that mature vascular SMCs may have limited or no ability to respond to Hh ligands. This parallels the observations of Yu et al. in the kidney and ureter, where SMC progenitors, but not differentiated SMCs, respond to paracrine Shh signaling (7).

Another possible role for Shh signaling in the adventitia is to promote and regulate the development of the vasa vasorum. Angiogenic effects of Shh are mediated through interstitial fibroblasts, which upregulate VEGF, angiopoietin-1, and angiopoietin-2 in response to Shh treatment (8). The development of the vasa vasorum has not been well characterized in mice; therefore it will be very interesting to conduct a detailed analysis of the timing and construction of the vascular plexus in both wild-type and Shh-/- embryos. These studies could then be further extended to test for a link between the formation of the vasa vasorum and the appearance of AdvSca1 cells in the adventitia. Pericytes depend on endothelial-derived PDGF-B for proper recruitment and function (9, 10) and given the strong expression of PDGFR-β by AdvSca1 cells, it is logical to speculate that PDGF signaling is also important in their regulation. Colonization of the adventitia by AdvSca1 cells only after vasa vasorum establishment may also provide an explanation for the appearance of AdvSca1 cells relatively late in embryonic development.

*Genetic targeting of AdvSca1 cells*

Further investigation into the mechanisms and factors that regulate AdvSca1 self-renewal and differentiation will have important implications for vascular disease and development. Examination of AdvSca1 phenotype in global knockout mice provides useful information regarding the development of the cells when the factor of interest is constitutively absent. However, these models are often limited by other phenotypes that cause severe defects or embryonic lethality, complicating the interpretation of the effect on AdvSca1 cells and limiting the time window in which they can be examined.

One strategy that has been used to more specifically target adventitial cells is the direct application of adenovirus or plasmid vector to the adventitial surface of the artery wall (11-13). Control experiments using reporter genes confirmed that exogenous gene delivery was limited to adventitial cells and indicated that gene expression persists for at least 14d following application. This method is best suited to gene overexpression rather than removal of gene function, although it could be adapted for delivery of chemical inhibitors such as cyclopamine or TGF-β receptor kinase inhibitors. Due to the inherently invasive nature of exposing the arterial adventitia for direct application of factors, it is a good choice for use in combination with surgical vascular injury models such as carotid artery flow-reduction (see below).

For gene deletion, a genetic approach using mice with conditionally expressed cre recombinase in combination with a floxed gene of interest would be optimal. This will require the identification of an appropriate promoter that can be used to drive cre expression in AdvSca1 cells. Sca1 itself is a risky choice because it is expressed in multiple different cell types (14). For example, Sca1 expression by hematopoietic cells and adipose stromal cells could complicate labeling experiments and increase the possibility of deleterious side-effects of targeted gene knockout that are unrelated to AdvSca1 functions. An unbiased screen for genes expressed by AdvSca1 cells would reveal candidates. The ideal factor will be as specific as possible to AdvSca1 cells and should not be expressed in SMCs or other arterial cells. A transgenic construct in which cre expression is driven by the promoter of interest in an inducible fashion will further help to minimize effects on non-AdvSca1 cells. A transgenic mouse with cre expression in AdvSca1 cells would be a valuable tool that could be used in combination with existing flox lines, giving us the ability to evaluate the effects of Hh pathway deletion, constitutive activation, and deletion of one or more Krüppel family members without the complications of embryonic lethality and effects on other cell types.

Furthermore, inducible cre expression would allow us to genetically mark AdvSca1 cells and follow their fate during postnatal development and adventitial response to vascular injury.

*AdvSca1 cell differentiation potential*

In the course of these studies, we have observed isolated AdvSca1 cells differentiate to SMCs and osteogenic cells *in vitro*, pericytes, endothelial cells, and macrophages *in vivo*, and adipocytes both *in vitro* and *in vivo*. As discussed above, all of these cell types are relevant to the adventitia and artery wall. Whether an individual AdvSca1 cell is capable of differentiating to all the cell types we observed or if the AdvSca1 population is heterogeneous with respect to differentiation potentials is not yet entirely clear. In addition, the extent to which the assay environment determines the cell types that are produced by AdvSca1 cells needs to be carefully considered. It will be important to expand our *in vivo* analysis of AdvSca1 potential using models that complement the Matrigel plug assay. To this end, the methods used for the Matrigel plug assay could easily be adapted to test the behavior of AdvSca1 cells in other matrices. Collagen is an abundant component of the adventitial extracellular matrix, so testing the differentiation behavior of AdvSca1 cells suspended in purified collagen may be a relevant alternative to Matrigel. Fibrin, which has been used in *ex vivo* aortic ring assays (15) and *in vitro* endothelial tube formation (16), is another possibility. It would be interesting to extend these studies by analyzing the actual ECM composition in the adventitia of normal and diseased arteries. Knowing the relative levels of different matrix components, one could tailor an assay to better mimic the *in vivo* adventitial environment where AdvSca1 cells normally reside.

Other angiogenesis assays such as the corneal micropocket assay, the chick chorioallantoic membrane assay, and the rodent mesentery angiogenesis assay have both advantages and disadvantages (17, 18). Given the range of mesenchymal cell potentials observed from AdvSca1 cells, it makes sense to move beyond an angiogenic environment

for additional *in vivo* assays. The kidney capsule transplantation assay and denatured collagen sponge implant would be appropriate choices for testing AdvSca1 behavior in different environments. In the kidney capsule transplantation model, cells or tissues are implanted in the subcapsular region of the kidney and allowed to differentiate. The kidney is highly vascularized, and the capsule can physically accommodate a variety of tissue and cell types (19). This system has the advantage of being unbiased towards a particular cell fate, as cells are not treated with exogenous factors. Differentiation to tissues as diverse as bone and bone marrow (20), pancreatic beta-cells (21), and neural tissue (22) have been found in different uses of kidney capsule transplantation. In contrast, the collagen sponge implant assay would be most useful for directed differentiation of AdvSca1 cells towards an osteogenic fate, which we have not yet observed *in vivo*. In this model, isolated AdvSca1 cells would be treated with BMP2 and mounted on a collagen sponge, which is implanted subcutaneously into a host mouse (23). Both the kidney capsule and collagen sponge assays can be used with $10^6$ or fewer cells and do not require prior cell culture, which is necessary for AdvSca1 cell assays.

*Hedgehog signaling and AdvSca1 regulation in vascular injury models*

In our preliminary studies using the carotid artery flow reduction model of vascular injury and remodeling, we observed an expanded adventitial compartment at one week post-ligation, as has been previously reported (24). The neoadventitia included expanded numbers of AdvSca1 cells, some of which were positive for the mitotic marker phospho-histone H3 (pH3). Hh pathway activity, as evaluated by X-gal staining for Ptc2-lacZ expression, was unchanged in the adventitia of flow-reduced vessels compared to sham controls. These experiments will need to be expanded and refined, particularly to include earlier timepoints. Godin et al. performed complete ligation of the common carotid artery and evaluated the proliferation index by BrdU incorporation at 1, 3, 7, 14, and 28 days. At

one day post-ligation, they observed >20% BrdU-positive cells in the adventitia. The rate of proliferation decreased sharply thereafter and reached baseline by one week (25). It is likely, therefore, that we have missed the peak of adventitial cell proliferation by conducting our examination at one week post-ligation. Furthermore, our data suggest that Shh signaling plays an important role in regulating AdvSca1 proliferation. Analyzing gene expression changes at early timepoints, particularly with respect to Hh pathway components, will be a more reliable method of evaluating Hh pathway activation.

One area that has not been addressed in prior studies is whether the development of neoadventitia in vascular injury models is accompanied by a concomitant expansion of the vasa vasorum. Non-diseased arteries in mice are generally thought to have an extremely limited vasa vasorum (26) yet our lab has been able to visualize adventitial microvessels using immunostaining and whole-mount confocal microscopy. Several studies have reported that the vasa vasorum is greatly expanded in mouse models of atherosclerosis (26). The vasa vasorum network functions to supply oxygen and nutrients to cells in the outer layers of the artery wall (27). Expansion of the vasa vasorum following vascular injury could be a response to angiogenic factors released as a result of mechanical stress in the vessel wall or a reaction to hypoxia induced by increased artery wall thickness. While an extensive investigation of these questions is not directly relevant to the concepts presented in this work, it is worth considering the possibility that any increases in adventitial Hh signaling following vascular injury may also promote vasa vasorum angiogenesis. Therefore, if Hh pathway activation is observed following carotid flow reduction or balloon injury, an evaluation of vasa vasorum density should also be conducted. These experiments could be further expanded to include adventitial treatment with cyclopamine suspended in pluronic gel, in order to observe alterations in adventitial response to vascular injury when Hh pathway activation is blocked.

*Regulation of the progenitor phenotype by Klf4*

The results of our experiments involving Klf4 knockdown in AdvSca1 cells strongly suggest that Klf4 plays a critical role in regulating the progenitor phenotype. However, it remains unresolved whether Klf4 actively promotes progenitor cell self-renewal, blocks the expression of smooth muscle genes, or both. As we expand the scope of these experiments, gaining a better understanding of what constitutes the "progenitor phenotype" of AdvSca1 cells will be critical for interpreting experimental results. Sca1 is an extremely useful marker for AdvSca1 cells, as it is a cell-surface protein not expressed by other adventitial cell types. We currently isolate and evaluate the progenitor status of AdvSca1 cells based on the presence or absence of Sca1 protein and gene expression. Nevertheless, the validity of this approach needs to be verified by examining other aspects of the AdvSca1 phenotype. For example, as Sca1 expression is lost in response to Klf4 knockdown, are other progenitor cell markers such as CD34 also downregulated? A more extensive analysis of gene expression changes following Klf4 knockdown will not only be informative in giving us a wider view of global changes in the phenotype of AdvSca1 cells, but may provide clues as to the particular function of Klf4.

An evaluation of the AdvSca1 phenotype in KLF4 mutant mice will be a critical supplement to our *in vitro* experiments. KLF4 knockout mice have been generated and characterized, and no obvious vascular defects were detected. KLF4-/- mice are born in normal Mendelian ratios, but die within 15 hours after birth due to loss of skin barrier function (28). These mice will be useful for basic analyses of AdvSca1 phenotype to identify abnormalities such as delayed appearance, reduced numbers, or altered distribution. If AdvSca1 cells are present, they can be isolated for gene expression analysis and tested for *in vitro* and *in vivo* differentiation behavior.

The siRNA experiments represent an acute loss of Klf4 expression, while the KLF4-/- mutant mice exhibit a constitutive absence of Klf4 function. It will be interesting to compare

the effects of these two models on AdvSca1 cell phenotype. In addition, evaluation of acute loss of Klf4 *in vivo* can be achieved through the use of floxed-KLF4 mice (29). Until the development of an appropriate cre mouse to genetically target AdvSca1 cells, experiments would rely on conditional expression of globally-expressed cre. Nonetheless, it would be informative to investigate the behavior of Klf4-deficient AdvSca1 cells *in vivo* without removing them from the native adventitial environment. These experiments could be further expanded to analyze the response of Klf4-deficient AdvSca1 cells to various forms of vascular insult, including vascular injury models or genetic susceptibility to atherosclerosis.

To our knowledge, there have been no reports directly connecting Klf4 activity and signaling through the Hh pathway, although both have been linked to adipogenesis. Through a screen in *Drosophila*, Pospisilik et al. identified the Hh signaling pathway as an important regulator of adipocyte differentiation. When suppressor of fused (Sufu) was deleted in adipose tissue, thus activating the Hh pathway, mutant mice displayed defective white adipocyte differentiation and a significant reduction in white adipose tissue (30). In contrast, Klf4 has been reported to function as an immediate early regulator of adipogenesis through the direct transcriptional activation of C/EBPβ (31). It will be interesting to evaluate any direct relationship between the Hh pathway and Klf4 in AdvSca1 cells, although it is important to consider that reciprocal regulatory effects may be mediated by cross-talk between multiple other signaling pathways.

*Additional modes of progenitor cell regulation*

It is important to keep in mind that multiple other factors are likely to play significant roles in regulating the progenitor phenotype of AdvSca1 cells *in situ*. Therefore, I will conclude with a brief discussion of two additional candidate regulators.

Hypoxia: Little has been reported regarding oxygen levels in normal arterial adventitia. In animal models, hypoxic exposure induces rapid and dramatic changes in

adventitial architecture of pulmonary vessels. These include adventitial fibroblast proliferation and changes in ECM protein synthesis (32). Fibroblasts isolated from the main pulmonary artery adventitia upregulate SMα-actin when cultured in a hypoxic environment (33). As with all previous literature addressing the behavior of adventitial "fibroblasts", we question whether the observed cellular response is attributable to AdvSca1 cells, fibroblasts, or both.

There have been numerous reports in the literature of environmental oxygen content modulating the behavior of different progenitor cell types. Notably, Berthelemy et al. observed differential fates of circulating mononuclear cells when cultured under normoxic or hypoxic conditions. Under normoxia, the cells displayed the morphology and markers of endothelial cells, while hypoxia induced expression of SMC markers (34). Adipose stromal cells also modulate various aspects of their phenotype in response to hypoxia (35-37). Interestingly, Sims et al. reported that Shh expression is upregulated following ischemic/hypoxic insult the brain, inducing neural progenitor cell proliferation (38). Given these precedents, it would be very interesting to compare AdvSca1 behavior when cultured under standard conditions versus hypoxia. Our preliminary data indicates AdvSca1 cells express key members of the hypoxia-inducible factor family, including HIF1α, HIF2α, and ARNT (HIF1β) (data not shown). Observing changes in AdvSca1 proliferation or differentiation at different oxygen concentrations would suggest that oxygen levels are an important regulator of progenitor cell behavior in the adventitia.

Chromatin conformation: Previous studies have suggested that histone modifications such as acetylation play a role in the regulation of SMC gene transcription (39-43). The ability of SRF to activate transcription of SMC marker genes depends on its ability to recognize and bind CArG box elements found in promoter and intronic locations. Histone tail modifications can change the structure of chromatin, thus impacting the ability of transcription factors to recognize and bind target sites. Chromatin structure and accessibility

of target genes are regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Chromatin immunoprecipitation (ChIP) analysis in cultured SMCs has shown that acetylated histones H3 and H4 are associated with the SMαA, SM22α, and SM-MHC gene regulatory regions (39, 40). McDonald et al showed enrichment of the activating modifications H3K4dMe, H3K79dMe, H3K9Ac, and H4Ac at CArG box chromatin in SMCs versus other cells types, including embryonic stem cells, endothelial cells, and undifferentiated A404 cells (42). Currently, however, little is known about the epigenetic regulation of SMC progenitors *in vivo* and how histone modifications may be altered as these cells differentiate and express SMC marker genes for the first time. Our preliminary data indicates that AdvSca1 cells express HDACs 1, 2, and 5 (data not shown), histone modifying enzymes which are known to interact with Klf4 (42, 44, 45). Given that AdvSca1 cells express SRF and known co-activators of SMC marker gene transcription, it will be very interesting to evaluate the status of CArG elements in AdvSca1 cells. Coimmunoprecipitation and ChIP techniques can be used to address the status of SRF-CArG occupancy, SRF-Klf4 interaction, Klf4-HDAC interaction, and CArG element histone modifications in AdvSca1 cells before and after *in vitro* differentiation.

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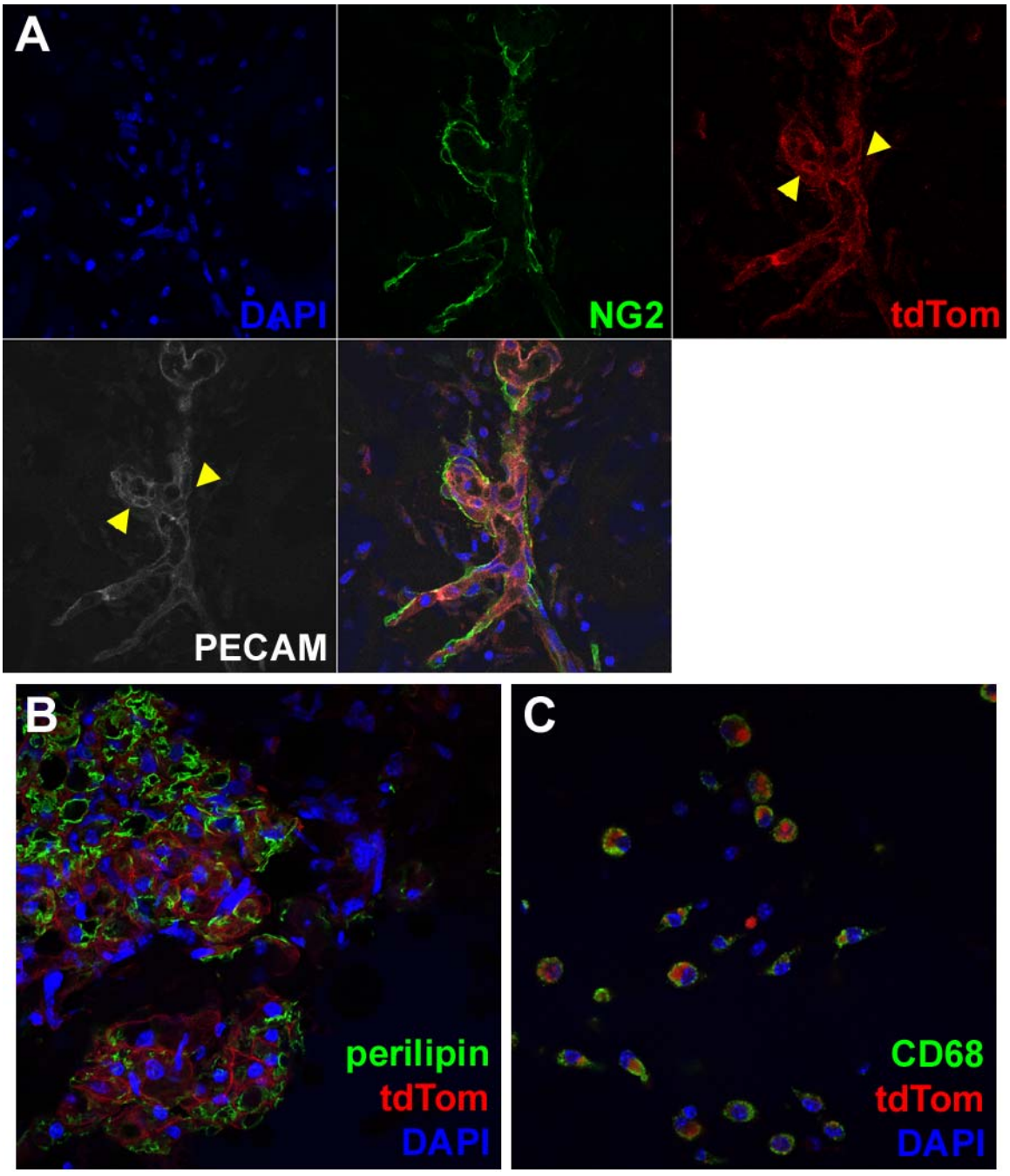
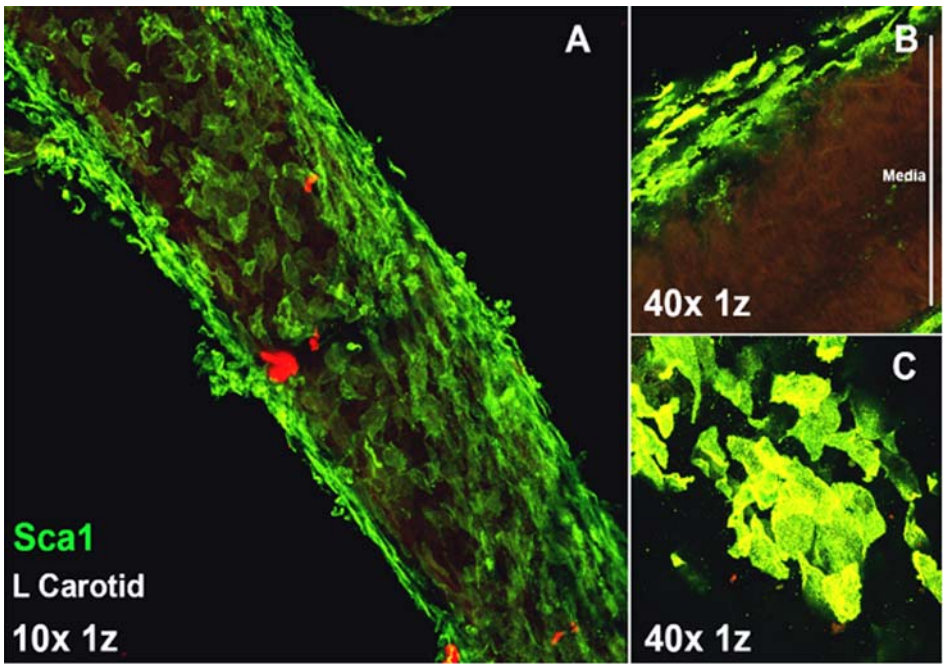
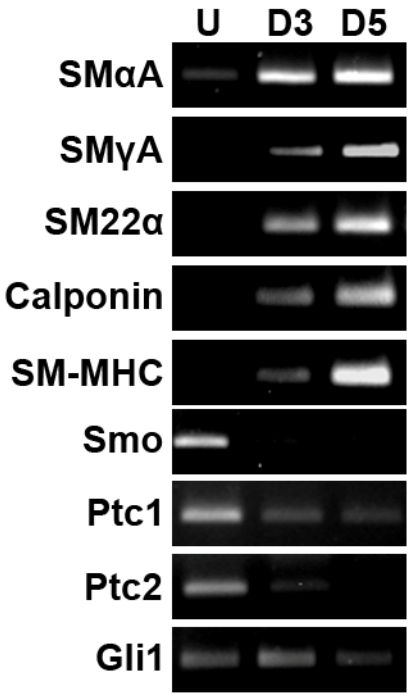
## Appendix A: Supplementary Figures




**Supplemental Figure 1**. *Other potential fates of AdvSca1 cells in vivo.* Isolated AdvSca1 cells expressing membrane-localized tdTomato fluorescent protein were incubated in Matrigel plugs with 0.5 µg/mL human FGF2 and 60 U/mL heparin for 10 d. Single confocal Z-sections indicate tdTomato co-localization with PECAM (arrowheads, A), perilipin, a marker of adipocytes (B), and CD68, a marker of macrophages (C).




**Supplemental Figure 2**. *Whole-mount views of AdvSca1 cells in the artery wall.* The left carotid artery of a wild-type mouse was stained for Sca1 expression and evaluated by confocal microscopy. Views from different angles (A-C) show AdvSca1 cells localized to the adventitia, where they display a slightly flattened morphology. Data shown was generated by Virginia Hoglund.




**Supplemental Figure 3**. *Downregulation of Hh pathway components during SMC differentiation.* The A404 cell line was developed from P19 mouse embryonal carcinoma cells as described by Blank et al. (1). Treatment with retinoic acid (RA) induces SMC differentiation. RNA was isolated from undifferentiated (U) cells, cells at day 3 (D3), and cells at day 5 (D5) of RA-induced SMC differentiation and the expression of SMC marker genes and Hh pathway components was evaluated by RT-PCR. As A404 cells undergo SMC differentiation, multiple Hh pathway components are downregulated. A similar trend was observed during the *ex vivo* differentiation of proepicardial cells (data not shown). Data shown was generated by Chang Su.

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## Appendix B: List of Primer Sequences

| Gene | Forward Primer | Reverse Primer | Product Size | Reference |
|---|---|---|---|---|
| β-actin | TGTTACCAACTGGGACGACA | CTCTCAGCTGTGGTGGTGAA | 393 | (1) |
| Boc | CCCAGAAGCTCCAGACAGAC | TGTCCCCTTCTACCATGTCC | 500 | |
| Calponin | CACCAACAAGTTTGCCAG | TGTGTCGCAGTGTTCCAT | 213 | (2) |
| CD140b (PDGFR-β) | AGCTACATGGCCCCTTATGA | GGATCCCAAAAGACCAGACA | 367 | |
| CD34 | TTGACTTCTGCAACCACGGA | TAGATGGCAGGCTGGACTTC | 300 | (3) |
| Cdo | AGGGGAGAGTGAGTTCAGCA | CCTGGTCAGGGAGTTTGTGT | 493 | |
| Csrp1 | AGTCTCTGGGCATCAAGCAT | CCGCTGATGAAAAGCTTAGG | 376 | |
| Csrp2 | GCATGGTTTGCAGGAAAAAT | CCCACACCTGGAACACTTCT | 260 | |
| Dhh | ATGCCCAATTGACAGGAGAG | GGCCTTCGTAGTGGAGTGAA | 500 | |
| Disp1 | CAAGAGGGACCACGATAGGA | GTGCCGTTTTGGTAGTGCTT | 505 | |
| Flk1 | TCTGTGGTTCTGCGTGGAGA | GTATCATTTCCAACCACCCT | 270 | (3) |
| Foxo4 | TGTAACAGGTCCTCGGAAGG | GACAGACGGCTTCTTCTTGG | 394 | |
| Gli1 | TGGAAGGGGACATGTCTAGC | ATGGCTTCTCATTGGAGTGG | 501 | |
| Gli2 | CCTCCAACCTCAACAAGAGC | CTGCAGGAGGGAGAAAACTG | 403 | |
| Gli3 | CCGTTCAAAGCCCAGTACAT | TCTTCACCTGGAGGCACTCT | 505 | |
| Hhip | CCGTGGATCGACATCCTACT | GGGCAGGTTGAACTGTGACT | 498 | |
| Ihh | GGCCATCACTCAGAGGAGTC | ATATTGGCCTGGTTGCACAT | 501 | |
| Kit | GGCTCATAAATGGCATGCTC | CTTCCATTGTACTTCATACATG | 400 | (3) |
| Klf2 | ACCAAGAGCTCGCACCTAAA | CAGAACTGGTGGCAGAGTCA | | |
| Klf4 | ATTAATGAGGCAGCCACCTG | GGAAGACGAGGATGAAGCTG | 400 | |
| Klf5 | GTCTGCGGTTTAAAGGATGG | GCCAGTTAATTCGCCAACTC | | |
| MRTF-A | CCAGGCTGGCTGATGACCTCAATG | CTGTGATTTCTCGCTGGCAGACTTG | 473 | (2) |
| MRTF-B | ACCCCAGCAGTTTGTTGTTC | TGGAATGACTCAGCAAGTCG | 488 | |
| Msx1 | GCTGGAGAAGCTGAAGATGG | AGGGGTCAGATGAGGAAGGT | 391 | |
| Msx2 | AGACATATGAGCCCCACCAC | GGGAGCACAGGTCTATGGAA | 365 | |
| Myocardin | ATGCACCAAACACACCTCAA | GCTGCCAAAGTGGTAGAAGC | 384 | |

| | | | | |
|---|---|---|---|---|
| Ptc1 | CATTGGCAGGAGGAGTTGAT | CCTGAGTTGTCGCAGCATTA | 499 | |
| Ptc2 | TCCCCCAGAGCTCTTCTACA | GGGATGGCACTCAGTTTGAT | 501 | |
| Sca1 | CTCGAGGATGGACACTTCT | GGTCTGCAGGAGGACTGAGC | 400 | |
| Shh | TCTGTGATGAACCAGTGGCC | GCCACGGAGTTCTCTGCTTT | 241 | (4) |
| SM $\alpha$-Actin | ACGGCCGCCTCCTCTTCCTC | GCCCAGCTTCGTCGTATTCC | 415 | |
| SM-MHC | GACAACTCCTCTCGCTTTGG | GCTCTCCAAAAGCAGGTCAC | 201 | (5) |
| SM22α | TCCAGTCCACAAACGACCAAGC | GAATTGAGCCACCTGTTCCATCTG | 328 | (6) |
| Smo | GGCTGGAGTAGTCTGGTTCG | TGGCTTGGCATAGCACATAG | 500 | |
| SRF | CTACCAGGTGTCGGAATCTGA | CCAGACGGTGCTGTCAGGAACA | 651 | (2) |
| Sufu | CAAAGGCATTGAGACAGACG | GCATACGGGTGTTCCTCAGT | 507 | |

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## Appendix C: List of Antibodies

| Antigen | Supplier (Catalog #) | Method Notes (as differ from text) |
|---|---|---|
| β-galactosidase | MP Biomedical (55976) | |
| Shh | R&D Systems (AF445) | |
| SM-MHC | Biomedical Technologies (BT-562) | |
| SMαA | Sigma (A2547) | best with methanol or methanol/acetone fixation/permeabilization |
| Sca1 | BD Pharmingen (553333) | does not require permeabilization, not compatible with Triton X-100 treatment |
| PECAM-1 | BD Pharmingen (550274) | |
| BrdU | BD Pharmingen (555627) | |
| SRF | Santa Cruz Biotechnology (sc335) | |
| FLAG | Sigma (F1804) | requires permeabilization |
| GFP | Abcam (ab13970) | |