CHARACTERIZATION OF THE ARTERIAL ADVENTITIA AS A SONIC HEDGEHOG RESPONSIVE NICHE

Virginia Jean Hoglund

A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Curriculum in Genetics and Molecular Biology

Chapel Hill
2012

Approved by:

Mark Majesky, PhD

Nobuyo Maeda, PhD

James Faber, PhD

Monte Willis, MD, PhD

Scott Bultman, PhD

ABSTRACT

VIRGINIA JEAN HOGLUND: Characterization of the Arterial Adventitia as a Sonic Hedgehog Responsive Niche
(Under the direction of Mark W. Majesky)

While formerly viewed as merely connective tissue, the emerging view of the arterial adventitia is that of a complex and organized vascular progenitor cell niche. Previously, our lab has identified a domain of Sonic Hedgehog (Shh) signaling restricted to the adventitia. This domain is locally produced and maintained, with the peak of activity being during embryonic and neonatal development of the vessel from embryonic day 14.5 (e14.5) to postnatal day 14 in the mouse. Developmental studies suggest that the mesenchyme continuous with the perichondrium of the axial skeleton contains Shh responsive cells that may contribute to adventitial development around e14.5. The cell types that participate in this Sonic Hedgehog adventitial signaling community are CD68-positive macrophages, Sca1-positive progenitor cells, and Perilipin A-positive adipocytes that make and/or respond to Shh protein as shown using transgenic reporter mice. Thus, we characterize the adventitia as a Shh responsive vascular niche.

ACKNOWLEDGEMENTS

First I would like to acknowledge and thank Jesus Christ for His constant faithfulness and purpose for my life. (Jeremiah 29:11, Philippians 4:13)

Second, I would like to acknowledge and thank my mentors, Dr. Mark Majesky and Dr. Xiu Rong Dong. Mark, I most sincerely thank you for bringing me into the Majesky lab family, for your teaching and guidance, for your infectious love of science, and for bringing me along to Seattle. It has been an honor, and I am truly blessed to be a part of the lab. Xiu Rong, thank you for being a constant source of loving help and a fount of practical knowledge. You are a delight and a blessing to work with.

I'd also like to thank Drs. Jenna Regan and Colin Maguire for all of their help getting the project started, training me in all of the basics, and laying a foundation for me and my work. Thanks to other Majesky lab members as well (Laurel, Liz, Woan Nu) for your helpful discussions and support.

The UNC Microscopy Services Lab deserves much credit for help with the confocal images, and training me in the love of microscopy. Thanks Bob, Vicki, and Steve. You guys are the best. Also, thanks to Leica and Olympus for teaching me more about the art of confocal and for allowing me to play with their machines during the confocal shopping process.

Thank you to my committee members, Drs. Nobuyo Maeda, Jim Faber, Scott Bultman, and Monte Willis for your helpful input. I'm only sorry I didn't take more advantage of your wisdom and knowledge.

Of course, a huge thank you to all of my family (especially Mom, Dad, and Christy), friends (in Florida, Carolina, and Seattle), and church family for your support, love, prayers, and willingness to put up with me no matter what time zone I'm in.

Lastly, I'd like to thank UNC Chapel Hill, the Curriculum in Genetics and Molecular Biology, Seattle Children's Research Institute, and all of their wonderful and supportive staff (Sausyty, Cara, Bob, Mackenzie, and Wendy; to name a few) for giving me a place to work and learn and grow, and of course the NIH and the American Heart Association for funding this journey.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ad	Adipocyte
Adv	Adventitia
AdvSca1	Adventitial stem cell antigen 1-positive progenitor cell
Ang	Angiotensin
Ao	Aorta
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BOC	Brother of CDO
BSA	Bovine serum albumin
CDO	Cell adhesion molecule-related/downregulated by oncogenes
ChIP	Chromatin Immunoprecipitation
Ci	Cubitus interruptus
CK1	Casein kinase 1
CNS	Central nervous system
Cul	Cullin
DAPI	4',6-diamidino-2-phenylindole
DBH	Dopamine β-hydroxylase
Dhh	Desert hedgehog
Disp	Dispatched
E	Endothelium
EC	Endothelial cell
ECM	Extracellular matrix

EEL	External elastic lamina
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
Eln	Elastin
F	Fibroblast
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSC	Follicle stem cell
Gas	Growth arrest specific
GFP	Green fluorescent protein
Gli-A	Gli activator form
Gli-FL	Gli full length
Gli-R	Gli repressor form
GRK	G protein-coupled receptor kinase
GSC	Germline stem cell
GSK	Glycogen synthase kinase
HA	Hyaluronic acid
Hh	Hedgehog
Hhip	Hedgehog inhibiting protein
HSC	Hematopoietic stem cell
HSPG	Heparin sulfate proteoglycan
IEL	Internal elastic lamina

IFT	Intraflagellar transport
Ihh	Indian hedgehog
iNOS	inducible form of nitric oxide synthase
Int	Intima
IP	Intraperitoneal
KLF4	Krüppel-like factor 4
LysM	Lysozyme M
M	Media
MCP	Monocyte chemoattractant protein
Med	Media
MMP	Matrix metalloproteinase
MΦ	Macrophage
NDS	Normal donkey serum
NGS	Normal goat serum
OCT	Optimal cutting temperature, (reagent used for frozen tissue sections)
P	Pericyte
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PFA	paraformaldehyde
Ptc	Patched
R26R	Rosa 26 reporter
RT-PCR	Reverse transcription polymerase chain reaction
Sca1	Stem cell antigen 1

Shh	Sonic hedgehog
Shh-C	Sonic hedgehog C-terminal domain
Shh-N	Sonic hedgehog N-terminal domain
Ski	Skinny hedgehog
SMC	Smooth muscle cell
SM-MHC	Smooth muscle myosin heavy chain
Smo	Smoothened
SMαA	Smooth muscle α actin
SPOP	Speckle-type POZ protein
SRF	Serum response factor
Stk	Serine/threonine kinase
Sufu	Suppressor of fused
SVZ	Subventricular zone
TGFβ	Transforming growth factor β
twhh	Tiggy-Winkle Hedgehog
Ulk3	Uncoordinated51 like kinase 3
VEGF	Vascular endothelial growth factor
WT	Wild type
YFP	Yellow fluorescent protein
βTrCP	Beta-transducin repeat containing protein

CHAPTER 1

INTRODUCTION AND BACKGROUND

Background

Niche Environments

Classic and Flexible Niche Models

Stem cell biology and regenerative medicine are frontiers of research that are rapidly gaining momentum and showing promise as future therapeutics for many diseases including Alzheimer's, muscular dystrophy, and repair of ischemic injury after myocardial infarction. Important in understanding the nature of stem cells is characterizing the specialized microenvironments that prevent their differentiation and promote their survival called niches. A niche is defined to be a "subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production *in vivo"* (Figure 1)[1]. There are two types of niches: a classic niche and a "flexible" niche. The Drosophila ovary is a model system that contains both types of niches for the three types of stem cells involved in oogenesis. The germline stem cells (GSCs) and escort stem cells are maintained in classic niches, while the follicle stem cells (FSCs) reside in a flexible niche [2, 3].

Classical niches consist of differentiated cells that directly contact stem cells and direct their self-renewal and behavior (Figure 1A)[2, 4]. In the fruit fly *Drosophila*

melanogaster, the ovary, known as the “germarium” was first described as a classic niche for the germline stem cells (GSCs) by Xie and Spradling in 2000[5]. In the last decade of research, data suggest that there are two key mechanisms that promote functionality of this classical niche: adhesive interactions between the niche cells and stem cells and asymmetric signaling[6].

In contrast, follicle stem cells (FSC) reside in a “flexible” niche where the architecture and position of the niche is less clearly defined (Figure 1B)[3]. In such niches, there are no obvious differentiated support cells to provide an anchoring function. Instead the stem cells generate some of the components necessary for their self-renewal and maintenance[2, 3, 7]. Specifically, FSCs in the germarium produce the essential local niche component, Laminin A, which activates integrin signaling in the FSC, promoting anchoring and proliferation of the cell[3]. Additionally required, differentiated niche resident cells (red and blue, Figure 1B) secrete paracrine signals to the FSCs (green, Figure 1B) to stimulate proliferation. In one example, niche resident cells produce the ligand Hedgehog (Hh) to signal to FSCs that express its receptor Patched (Ptc) and effector proteins Smoothened (Smo) and the Drosophila homolog of transcription factor Gli, Cubitus Interruptus (Ci)[8, 9]. Mutants for Hh, Smo, or Ptc all have defects in FSC proliferation rates, indicating an important role for the pathway in control of FSC proliferation[8, 10].

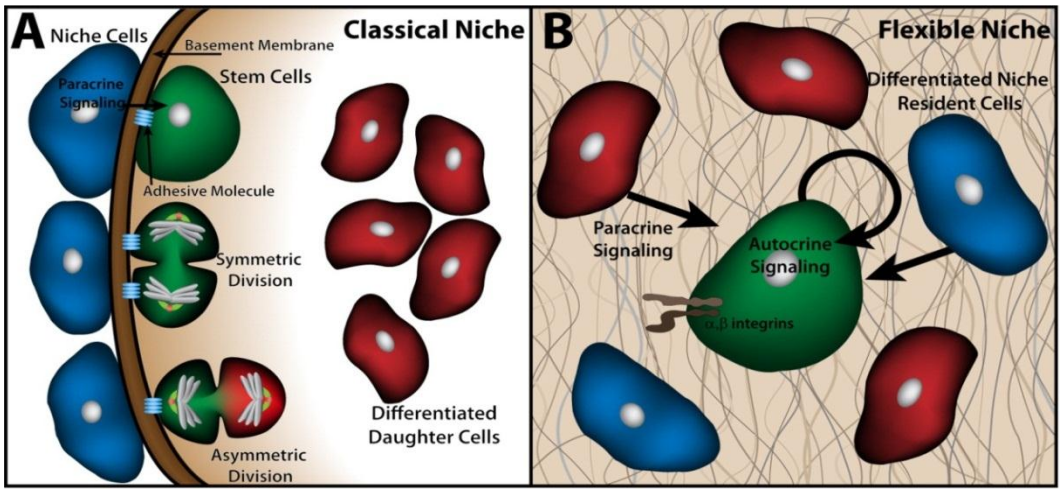


Figure 1: *Schematic of two types of niches*. A) A classical niche: Adhesive factors (light blue) keep the stem cells (green) inside the niche to reinforce paracrine signals from niche cells (blue) that block differentiation. When stem cells divide, they can either divide symmetrically to make 2 stem cells (upper mitotic cell) or they can divide asymmetrically (lower mitotic cell) and produce a stem cell and a differentiated daughter (red). Figure adapted from Spradling et al.[1] B) A flexible niche: Stem cells (green) receive paracrine factors from niche resident cells (blue, red) but also produce autocrine factors for self-maintenance. Integrin signaling is one mechanism stem cells use to adhere to the ECM (brown and grey lines) and maintain position in the niche.

The Perivascular Niche

In vertebrates, there are several examples of perivascular spaces as being important for nurturing stem cells. One example is the neural progenitor niche in the brain. Neurons, astrocytes, and blood vessels are organized in functional "neurovascular units" in which the vasculature can impact neuronal activity and, in

turn, dynamically adjust to its change[11]. Recent work by Licht et al. shows that vascular endothelial growth factor (VEGF) is important for neurogenesis. Transgenic mice with tet inducible expression of VEGF in the brain increased angiogenesis and neurogenesis in the hippocampus and improved memory. Once tetracycline was removed and the overexpression was turned off, neurogenesis was still improved over controls, although memory regressed back to control levels[11]. The authors speculate that the expanded vasculature provided an expanded niche for neuronal stem cells in the VEGF on>off mice[11]. Additionally, Kokovay and colleagues show that proliferative subventricular zone (SVZ) neural progenitor cells home to endothelial cells in a stromal-derived factor 1 and CXC chemokine receptor 4 (CXCR4)-dependent manner, making cell-cell contact with the vessel, and laying down long processes along its length[12]. These studies suggest that the vasculature provides an important niche environment for neuronal precursors.

Hematopoietic stem cells have also been shown to be maintained in a perivascular niche. Rafii et al. showed that bone marrow derived endothelial cells (ECs) co-cultured with hematopoietic stem cells (HSCs) produced several hematopoietic cytokines that promoted proliferation and differentiation of the HSCs[13, 14]. Davis et al. went on to show that ECs from the porcine brain microvasculature were capable of expanding HSCs in culture, suggesting that ECs from non-hematopoietic tissues could also support HSC growth *in vitro*[15]. Subsequent studies confirmed that human brain endothelial cells produced soluble factors that induced a 10 fold expansion of HSCs from cord blood and bone marrow *in vitro*[16-18]. Collectively, these studies suggest an important function for the bone marrow

vasculature, particularly the ECs, in regulating hematopoiesis and hematopoietic regeneration.

In the mammalian testis, it has recently been shown that spermatogenic stem cells are localized to the vasculature. Yoshida and colleagues performed experiments using time lapsed confocal imaging of GFP tagged spermatogenic stem cells that showed that the stem cells stayed close to the vessels and migrated away as they differentiated[19]. Furthermore, experimental alteration of the vasculature resulted in rearrangement of the stem cells[19]. While the stem cells did not directly touch the vasculature, they were in close proximity, and clustered with macrophages and Leydig cells, a testis support cell thought to be derived from pericytes. The researchers speculate that these perivascular cells take part in producing this niche by production of androgens and other necessary factors[19]. It is also possible that the vessels secrete soluble factors that the spermatogenic stem cells require for maintenance, and unlike the gonads in flies, tight junctions with the niche cells are not required.

The Arterial Adventitia

Structure and Function

The tunica adventitia has been long thought of as a simple layer of collagen-rich connective tissue matrix embedded with a few fibroblasts and perivascular nerves (reviewed in Majesky, 2011 [20] and Majesky, 2012 [21]). This definition is fast becoming outdated as research is now discovering that the adventitia is a complex,

organized, and diverse community of cell types that serve important functions in the wall, and support a progenitor cell niche[20-31].

Functionally, the adventitia serves as a dynamic compartment for cell trafficking into and out of the vessel wall, and adventitial cells play key roles in artery wall growth, control of lumen size by remodeling the extracellular matrix, opposing the transmural pressure to prevent overdistension of vessel, and defense against infection[32-34]. This layer of artery wall also contains a microvasculature, called the *vasa vasorum*, which penetrates and nourishes the medial and intimal layers of the artery wall[32, 35]. Adventitial microvessels from the *vasa vasorum* extend through the media and into atherosclerotic plaques where they serve as important channels for leukocyte trafficking into and out of the lesion[36, 37]. Recent studies describe novel and interesting roles for the adventitia insofar as it supports resident stem cell antigen-1 (Sca1)-positive progenitor cells that can adopt both vascular and non-vascular cell fates[20-31]. The molecular pathways that maintain vascular progenitor cells in the adventitia, that promote the growth of adventitial microvessels, and that coordinate overall growth and remodeling of the adventitia during development and the postnatal period have not been described.

The structure of the adventitial matrix has recently been reported in the porcine coronary artery[34]. The researchers found that both the collagen and elastin fibers formed alternating layers in the inner adventitia. In the outer adventitia, elastin fibers were largely absent, and collagen fibers were randomly arranged compared to the inner adventitia. This is consistent with an earlier report examining the elastin composition of the adventitia in the canine aorta that found alternating lamellae of

collagen and elastin fibers in the adventitia[38]. These reports suggest a level of organization to the adventitia and a role in structural support of the wall.

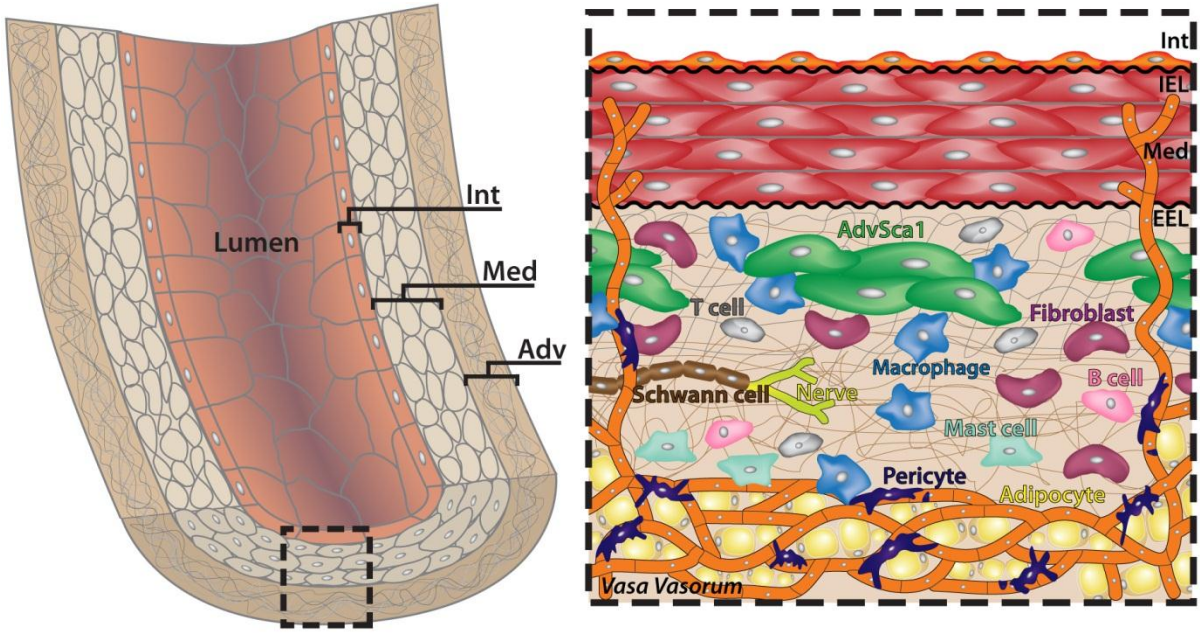


Figure 2: *Schematic of the artery wall*. The adventitia is home to progenitor cells (AdvSca1, green), a diverse array of leukocytes (blue, pink, grey, light green), perivascular nerves and their support cells (yellow-green and brown), fibroblasts (purple), the *vasa vasorum* (orange), and its supporting adipose tissue and pericytes (yellow, dark purple, respectively) all embedded in a collagen and elastin rich extracellular matrix (grey and brown lines). Int = intima, Med = media, Adv = adventitia, IEL = internal elastic lamina, EEL = external elastic lamina

Adventitial Progenitor Cells

Adventitial progenitor cells were first described in work by Hu et al.[23]. They sought to find a source of progenitor cells in ApoE knockout mice. By staining many types of non-bone marrow tissues for a panel of stem cell markers, they found that the arterial adventitia was the only location where significant numbers of cells expressing these markers could be found. These cells expressed Sca1, c-kit, CD34, and Flk1, but lacked embryonic stem cell marker SSEA-1[23]. To test the *in vivo* potential of these cells, they isolated genetically labeled cells from Rosa26 donor mice and transplanted them into wild type mice on the outside of an experimental vein graft. After 4 weeks, the genetically labeled cells were found to have migrated through the media into the graft neointima, and had become smooth muscle marker positive and Sca1-negative. In the control experiment, Sca1-negative adventitial fibroblasts from Rosa26 donor mice did not leave the graft adventitia, and were only rarely found in the neointima[23]*. In vitro* experiments confirmed the ability of Sca1 cells to differentiate into smooth muscle in response to PDGF-BB[23]. In this model, adventitial Sca1 cells were shown to be able to differentiate into SMCs and contribute to neointimal formation[20].

There have been several reports since then that have found similar results. Work by Campagnolo et al. isolated CD34-positive cells from human saphenous veins of patients undergoing cardiac bypass surgery[39]. These cells expressed the stem cell marker Sox2 and exhibited multilineage potential. Furthermore, the when transplanted into a mouse hindlimb after ischemic injury, these human CD34-positive cells differentiated into pericytes that formed N-cadherin mediated contacts

with endothelial cells and enhanced angiogenesis that promoted recovery in the ischemic limb[39].

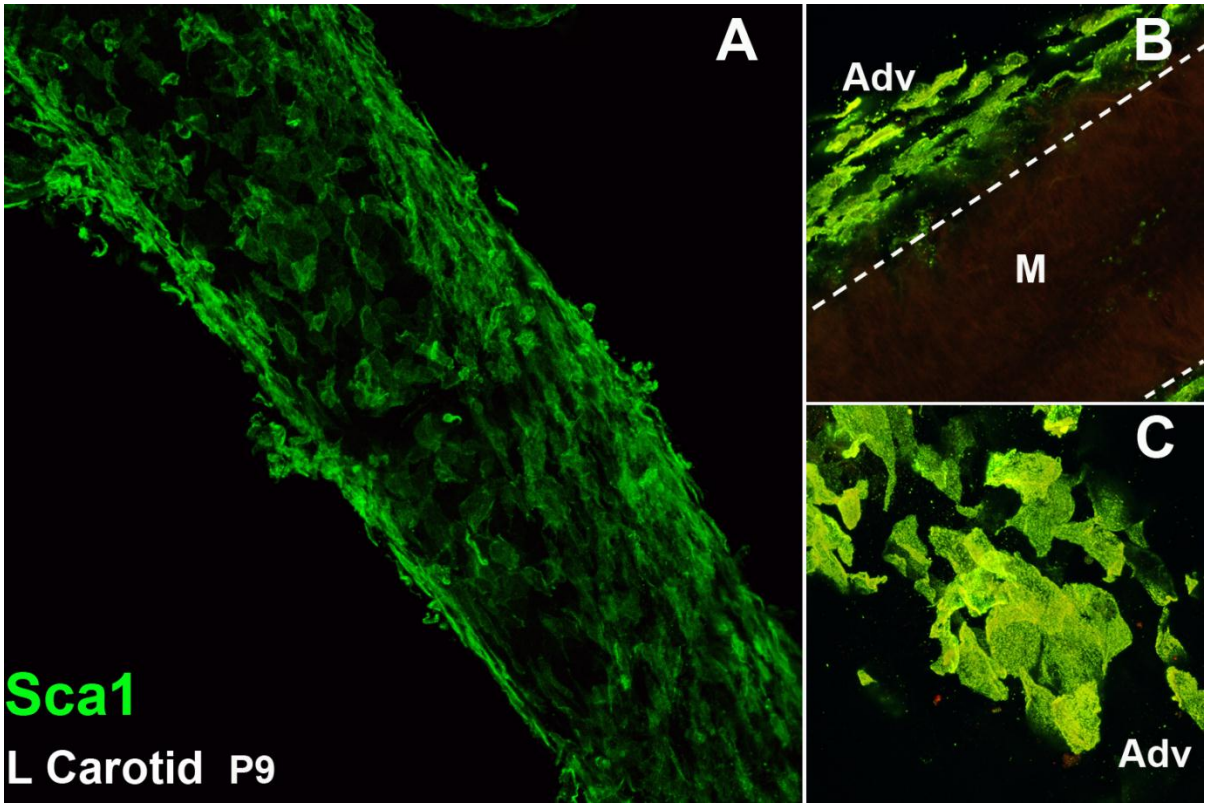


Figure 3: *Vascular progenitor cells*. Sca1-positive progenitor cells reside in the adventitia, and form highly organized layers and clusters with each other[20].

Work by Passman and colleagues further characterized the adventitial niche and AdvSca1 cells by describing a novel domain of Sonic Hedgehog (Shh) signaling restricted to the adventitia[25]. Passman and colleagues concluded that Shh was involved in survival and proliferation of the AdvSca1 cells. *In vivo*, Shh knockout mice had significantly reduced, but not absent, numbers of AdvSca1 cells. *In vitro*, treatment of AdvSca1 cells with the Hh signaling inhibitor cyclopamine showed

reduced proliferation and survival with no increase in apoptosis[20, 25, 40]. They confirmed results by Hu et al. that AdvSca1 cells differentiated into SMCs *in vitro*, and further showed that roughly half of these cells readily differentiated into SMCs in culture after removal from the adventitial niche with only the addition of serum to the culture medium and no additional growth factors[23, 25]. This observation that AdvSca1 cells differentiate into SMCs in response to serum was also made by Howson et al. examining spheroid forming rat adventitial cells[41].

Passman et al. went on to confirm that this SMC population occurred because of differentiation and not proliferation of contaminating SMCs by treatment with aphidicolin, an inhibitor of cell division. Further examination of these cultures revealed roughly ¼ of the cells retain their Sca1 phenotype, and the remaining ¼ lose Sca1 expression but do not detectably express smooth muscle markers[20, 25, 40]. These data suggest that AdvSca1 cells are a heterogeneous population of cells with different potentials (See figure 19 for model). Indeed, culturing isolated AdvSca1 cells from Rosa26 mice in Matrigel plugs containing recombinant FGF2 and heparin injected subcutaneously in the mouse showed these cells can differentiate into pericytes, adipocytes, and macrophages[40] (Supplementary figure 1).

Molecular Control of Progenitor Cells

Much current research is being conducted on the molecular and epigenetic control of stem and progenitor cells and their self-renewal and differentiation. Smooth muscle progenitors in particular are governed by the actions of key transcription factors including serum response factor (SRF), co-activators like

myocardin, and co-repressors like Krüppel-like factor 4 (Klf4) (reviewed in Xiao, 2010 [42]).

SRF is a MADS-box containing transcription factor, comprised of a DNA binding element, a dimerization domain, and an interface for protein-protein interaction[43]. Smooth muscle genes contain one or more of the binding sites for SRF called CArG elements, with a CC $[A/T]_2$ A $[A/T]_3$ GG motif, in the promoter or intronic sequence[44]. Because SRF is not specific for smooth muscle, interactions with co-activators and co-repressors provide regulation and control over target genes. Some influence SRF binding to DNA[45], while others form a complex with SRF to either stimulate (myocardin related factors, Csrp1) or inhibit (Klf4 [46], Msx1 and 2 [47], Elk1 [48]) transcriptional activity. Also important to the regulation of SRF-mediated transcription is the actin cytoskeleton. Myocardin related factors bind to G-actin and are released into the nucleus to interact with SRF during Rho-activated actin polymerization and treadmilling[49]. Upstream activators of SRF and myocardin activity include TGFβ[50], PDGF[42], and Wnt2[51].

Repressors of SRF-mediated transcription include factors like Foxo4: a transcription factor that represses the activity myocardin[52]; and Klf4: a transcription factor that inhibits the expression of myocardin and possibly recruits histone deacetylases to modify chromatin structure at smooth muscle genes[44, 46]. Klf4 has recently come on stage as one of the four transcription factors that reprogram fibroblasts into pluripotent stem cells[53, 54]. Passman and colleagues showed that AdvSca1 cells express both Foxo4 and Klf4, and upon removal from the adventitial niche, AdvSca1 cells downregulated Klf4 expression and upregulated smooth

muscle marker genes[25]. Knockdown of Klf4 by siRNA enhanced differentiation of AdvSca1 cells into SMCs, and overexpression of Klf4 by adenoviral infection inhibited differentiation in response to serum[40]. The factors released by the niche to promote and regulate expression of Klf4 to maintain AdvSca1 cells have not yet been determined.

Chromatin remodeling is an important aspect of transcriptional regulation in all cell types, especially stem/progenitor cells. Studies of differentiation of HSCs into erythrocytes showed the importance of higher order chromatin structure and chromatin looping of the β-globin locus control region to interact with a downstream promoter by Brg1, a component of the SWI-SNF chromatin remodeling complex[55]. Also, it has been shown that various stimuli known to alter SMC phenotype, such as TGF-β, PDGF, and retinoic acid, appear to act in part through effects upon SMC chromatin structure[56]. Also, as stated above, Klf4 has been implicated in recruiting deacetylases to modify acetylated histone H4, a histone modification that is associated with open chromatin[44]. Understanding the chromatin landscape of AdvSca1 cells may be important in deciphering the mechanisms for self-renewal and regulation of differentiation. As it is likely that AdvSca1 cells are a heterogeneous population of progenitors, chromatin marks and higher order structure may differ from cell to cell in the overall population, and identification of markers of subpopulations within the AdvSca1 total population would benefit an analysis of the chromatin landscape of these cells.

Aging and Progenitor Cells: Dysregulation of the Niche

Progenitors from old and young mice do have intrinsic differences. For example, telomerase is less active in “old progenitors” than “young progenitors”, leading to shortened telomeres and eventually proliferative senescence. Old satellite cells have reduced notch signaling, which reduces their capacity to self-renew[57]. Hepatic progenitors gradually increase expression of cEBPα and chromatin-remodeling factor brahma over time and are less capable of regeneration of the aged liver[58]. However, recent studies suggest that loss of function of niche-dependent signaling may be a contributing factor of various pathologies associated with aging, including greying and loss of hair, fibrosis, kyphosis, mal-absorption of nutrients, and osteoporosis[59-64].

Studies show that exposure of “old” progenitors to a “young” niche enhances proliferative capacity and increases their ability to regenerate. Transplanting muscle from a two year old mouse into a two month old mouse and then injuring the tissue 5 weeks later resulted in increased expression of embryonic myosin heavy chain, BrdU incorporation, and regeneration of damaged muscle than in two year old muscle transplanted into another two year old mouse[65]. This phenomenon has also been seen in liver parabiosis experiments[65].

In a mouse model of premature aging, Fox et al. reported that there was a ten-fold increase in apoptosis in the intestinal crypts of 3-7 month old mice, primarily in the progenitor cell zones, resulting in impaired ability to absorb excess fat when placed on a high fat western diet chow[64]. Stem cell-derived organoids from these mutant mice failed to develop fully in culture and exhibited fewer crypt units,

indicating a dysregulation of intestinal progenitor cell maintenance[64]. While these mice do carry a mitochondrial mutation that impairs editing function of mitochondrial DNA synthesis, it would be interesting to see how much of this phenotype is due to the mutant prematurely aged niche and how much is inherent in the mutant stem cells by transplanting intestinal crypt cells from mutant mice into age matched wild type mice and vice versa.

Reduction of adventitial progenitor cell pools with aging or mutation has not been directly tested, but may be a contributing factor to medial dissection and aneurysm formation in the aorta. It may also predispose patients with diseases like scleroderma or the premature aging syndrome progeria to loss of microvasculature in the *vasa vasorum* and adventitial fibrosis[61, 66]. As AdvSca1 progenitor cells and the critical signaling provided by the adventitial niche are better characterized, age related degeneration and pathological changes to the artery wall due to aging can be targeted for therapeutic intervention[21].

The Adventitia in Disease and Injury

In addition to fibroblasts and the above mentioned progenitor cells, the adventitia is home to several leukocyte cell types including tissue resident CD68-positive macrophages, T-cells, B-cells, and mast cells[67-70]. Although the functions of these cells have not been described in non-pathogenic settings, several studies have implicated adventitial lymphocytes participating in inflammation and remodeling in injury and disease models. George Tellides's lab showed that depletion of macrophages prevented flow-dependent inward remodeling in the mouse carotid[71].

Their lab went on to show that inward remodeling of the mouse carotid artery in response to reduction in blood flow required early adventitial accumulation of CXCR3-positive macrophages. They found evidence that a subset of macrophages with high levels of CXCR3 accumulated in the adventitia undergoing remodeling, suggesting an expansion of a population already resident, homing of monocytes with this gene expression profile, or both[72].

In another study of flow mediated remodeling, Erami and colleagues found that catecholamines had a significant effect on the inward and outward remodeling in response to changes in flow[73]. Ligation of the left carotid artery branches causes a reduction in flow in the left carotid and an increase in flow in the right carotid. In wild type mice, these changes in flow mediate an inward remodeling of the left carotid and outward remodeling in the right carotid. In the left carotid, this is accompanied with increases in the size of the adventitia, with increased proliferation, increased apoptosis, and as stated above, increase in macrophage accumulation[73]. These effects are inhibited in knockout mice of dopamine β-hydroxylase (DBH), the enzyme that synthesizes epinephrine and norepinephrine. Catecholamines have been shown to regulate Shh signal in lung morphogenesis[74]. Knockout mice of α_{2B}-AR, an adrenoceptor, die right after birth of respiratory failure. These mice show strong upregulation of Shh, leading to an overproliferation of mesenchymal cells in the lung, reducing alveolar space[74]. It is possible that Shh is also increased in the adventitia of the DBH knockout mice with the reduction of catecholamine signaling. It would be interesting to test this hypothesis, and determine if an increase in Shh signal

reduces the hyperproliferative and inflammatory effects of flow dependent remodeling of the artery wall.

Additionally, adventitial macrophages have been shown to respond to infusion angiotensin II (Ang II), a vasopressor known to promote vascular inflammation. Infusion of Ang II into 7 to 12 month old C57Bl6/J mice caused adventitial expansion and recruitment of macrophages to the adventitia. Adventitial cells in these mice produced inflammatory cytokines IL-6 and MCP-1, leading to the development of aortic dissection in some mice, a condition where the inner layers of the aorta tear, and blood begins to force the layers apart; a frequently fatal condition if not surgically repaired[75]. The researchers went on to show that the IL-6 and MCP-1 produced in the adventitia promoted differentiation of monocytes into macrophages, and stimulated production of matrix metalloproteinase 9 (MMP9) and MCP1 by adventitial fibroblasts, creating an amplification loop that accelerated inflammation[75]. The production of these cytokines was 2.3 fold greater *in vitro* when adventitial fibroblasts were cocultured with monocytes over either cell type alone[75, 76]. These data suggest there is a paracrine signaling relationship between different adventitial cell types and provide further support for the importance of the adventitia as a mediator of inflammatory cell interactions that can initiate physiological flow-dependent remodeling as well as the pathological remodeling of the artery wall[20].

Another example of adventitial leukocytes playing a role in vascular remodeling comes from a study examining the role of T-cells and B-cells in hypertension. The researchers found that RAG knockout mice that lack both T-cells and B-cells have a blunted response to infusion of Ang II: a factor that produces

hypertension in wild type mice. Adoptive transfer of T-cells, but not B-cells, restored the wild type hypertensive response[77]. Infusion of low doses of Ang II stimulated production of chemokines by the vessel wall that recruited T-cells to the adventitia and peri-adventitial adipose tissue[77]. These T-cells became activated and produced inflammatory cytokines like TNFα, INFγ, and p47phox. These cytokines stimulate superoxide production that reduces the amount of available nitric oxide in the vessel wall, and increases smooth muscle contractile tone[20, 77]. These studies highlight the important role of the adventitia as a site of T-cell homing and activation in disease.

Other arterial injury models have reported to have an effect on the adventitia. It has been shown that endothelial denudation by balloon injury not only induced neointimal formation, but also increased the fibroblast number and collagen deposition in the adventitial layer[78]. Furthermore, removal of the adventitia from rabbit carotid arteries results in formation of a hyperplastic intimal lesion[79]. This lesion regresses when a “neoadventitia” is grafted over the site of injury unless the animal is fed a high cholesterol diet[79]. Similarly, removal of rat abdominal aortic adventitia shows an initial severe trauma of the endothelial surface leading to complete de-endothelial areas as early as six hours after injury and intimal hyperplasia as early as one month after injury[80]. The researchers in both of these studies suggested that this response was likely due to the removal of adventitial microvessels and the ensuing hypoxia in the artery wall.

The Hedgehog Signaling Pathway

Drosophila *hedgehog (Hh)* was discovered in 1980 by Nusslein-Volhard and Wieschaus[81]. Since then, the hedgehog pathway has come to the forefront as a major player both in embryonic development and disease. Hh signaling has been implicated as a mitogen controlling proliferation and promoting cell survival[82-84], a repressor of cell differentiation in progenitor cells[85], an angiogenic factor[86-90], and as a morphogen controlling cell fate in a dose dependent manner[91-94]. Research into blocking Hh signaling is a promising therapeutic for many cancers[95, 96], as well as some anti-psychotic medicines[97], so new data are constantly being added to the picture and evolving our understanding of this highly regulated and complicated signaling network. Key components of the pathway and regulation are discussed below.

The Vertebrate Hedgehog Signaling Pathway

The vertebrate hedgehog family consists of three secreted ligands- sonic (Shh), indian (Ihh), and desert (Dhh) hedgehog[94]. While all three bind the receptor Patched (Ptc) with similar affinity, these three hedgehog proteins have differing expression patterns and exert functional effects with different potencies.

Knockout mice of each of these genes reveal very different phenotypes. $Shh^{-/-}$ is embryonic lethal with about half of the expected number of embryos surviving to term, and none surviving beyond birth[98]. These mice have defects in numerous systems including craniofacial, spinal cord, limb, lung, skeletal, and patterning of the outflow tract of the heart[98]. $Ihh^{-/-}$ is also embryonic lethal, with similar survival rates

as $Shh^{-/-}$. $Ihh^{-/-}$ embryos primarily have skeletal defects[92]. Mutants display markedly reduced chondrocyte proliferation, maturation of chondrocytes at inappropriate positions, forelimb and rib shortening, and failed development of osteoblasts in endochondral bones[92]. Unlike the other two, $Dhh^{-/-}$ is not lethal. Mice are viable, but with fertility defects, as spermatogenesis is impaired[99].

All three hedgehog ligands are produced in a similar fashion. As a specific example, Shh is synthesized as a ~45 kDa pre-protein that is autocatalytically processed into a ~19 kDa N-terminal domain (Shh-N) and a ~25kDa C-terminal domain (Shh-C) by activity intrinsic to the C-terminal domain[100]. Shh-C further processes Shh-N by adding a cholesterol residue to its C-terminus. Additional lipid modification of Shh-N results in a palmitoyl moiety added to the N terminal Hedgehog domain by the acetyltransferase skinny hedgehog (Ski)[101-103]. These lipid modifications facilitate the integration of Hh into multimeric lipoprotein particles[94, 104]. The release of this complex from the producing cell is dependent on the activity of Dispatched 1 (Disp1)[105]. Harfe and colleagues created a knockin mouse that is able to genetically label cells that are actively producing Shh using an EGFP-cre fusion protein under the control of the Shh locus (Shh^{EGFP}) by examining localization of EGFP[91]; an important tool used in the work described in chapter 2.

Hh signaling is initiated through binding to its receptors Patched-1(Ptc1) and Patched-2 (Ptc2) and to their accessory proteins CDO, Gas1, and BOC, which potentiate signaling[106-108]. In the absence of ligand, patched represses the activity of a signal transducing protein called smoothened (smo). Smo proteins contain a conserved sterol sensing domain and current evidence suggests that ptc represses

Hh signaling by transporting smo-activating sterols out of the cell[84]. In the absence of activated smo, the gli family of transcriptional mediators of Hh signaling is processed to a repressive form. This occurs when full length Gli (Gli-FL), bound to the inhibitor suppressor of fused (sufu)[109], is phosphorylated by protein kinase A (PKA), glycogen synthase kinase 3β, (GSK3β), and casein kinase 1 (CK1)[110-114]. This multi-phosphorylated Gli-FL is then recognized by βTrCP, in complex with an E3 ubiquitin ligase[114]. Gli-FL is polyubiquitinated and marked for partial degradation by the proteasome to the truncated and repressive form (Gli-R).

The gli family of zinc finger transcription factors includes members *gli1*, *gli2*, and *gli3*. Gli1 primarily has an activator function, and no reported repressive ability. Gli3 is primarily a repressor, with weak activator function. In an unusual mechanism, the proteasome processes Gli3 in the absence of Hedgehog pathway stimulation while Gli1 is not processed but degraded completely, despite considerable homology with Gli3[115]. These differences in processing can be described by defining a processing signal that is composed of three parts: the zinc finger domain, an adjacent linker sequence, and a degron. Gli1 is not processed because two components of the processing signal, the linker sequence and the degron, are ineffective[115]. Gli2 is a composite of positive and negative regulatory domains and functions as both an activator and repressor. In cultured cells, truncation of the activation domain in the C-terminal half results in a protein with repressor activity, while removal of the repression domain at the N-terminus converts Gli2 into a strong activator[116]. Studies have described a “Gli code”, where the cell’s response to Hh

signal is determined by the combination and relative levels of the three Gli factors, and the balance of their full length active forms and truncated repressor forms[117, 118].

Shh binding to Ptc1 inhibits sterol transporter activity, thereby allowing activating sterols to accumulate to levels that activate Smo[84]. Activated Smo then traffics to the primary cilium where critical signal mediators (Gli 1-3 and Sufu) are concentrated[119, 120]. Activated Smo antagonizes the effects of Sufu, an inhibitor of Gli. Data currently indicate this occurs by way of two kinases. Uncoordinated51-like Kinase 3 (Ulk3) phosphorylates Gli-FL, and STK36 phosphorylates Sufu[121-123]. This results in processing of Gli-FL proteins (Gli1-3) to their transcriptionally-active forms and Shh-dependent gene expression is stimulated. Importantly, several Hh pathway elements are also targets of Shh signaling including ptc1, ptc2, and gli1, so transgenic mice with the LacZ gene under the control of these promoters are used as sensitive reporters of Shh signaling *in vivo.(Ptc1$^{LacZ/+}$, Ptc2$^{LacZ/+}$, and Gli1$^{LacZ/+}$* respectively*)* These reporter mice as used as tools in specific aims 1 and 2.

Regulation of Hh signaling is mediated by several factors. SPOP is an important regulator of activated Gli. SPOP complexes with Cul3 ubiquitin ligase to recognize Gli-A in the nucleus and mark for proteosomal degradation[109, 114, 124]. As activated Gli 1-3 have a wide variety of targets like growth factors and morphogenic factors that could potentially be dangerous in the event of prolonged activity, it is essential to keep tight control over the half-life of Gli-A, and thus SPOP provides a necessary mechanism of regulation over hedgehog signaling.

Heparin sulfate proteoglycans are components of the ECM that are also involved in regulation of Hh signaling. HSPGs can bind to the secreted Hh ligand

and have been shown to be involved in setting up gradients of Hh, and propagating the Hh signal over long distances. Recent work suggests that the HSPG perlecan is also required for maximal activation of Shh, especially in the brain[125].

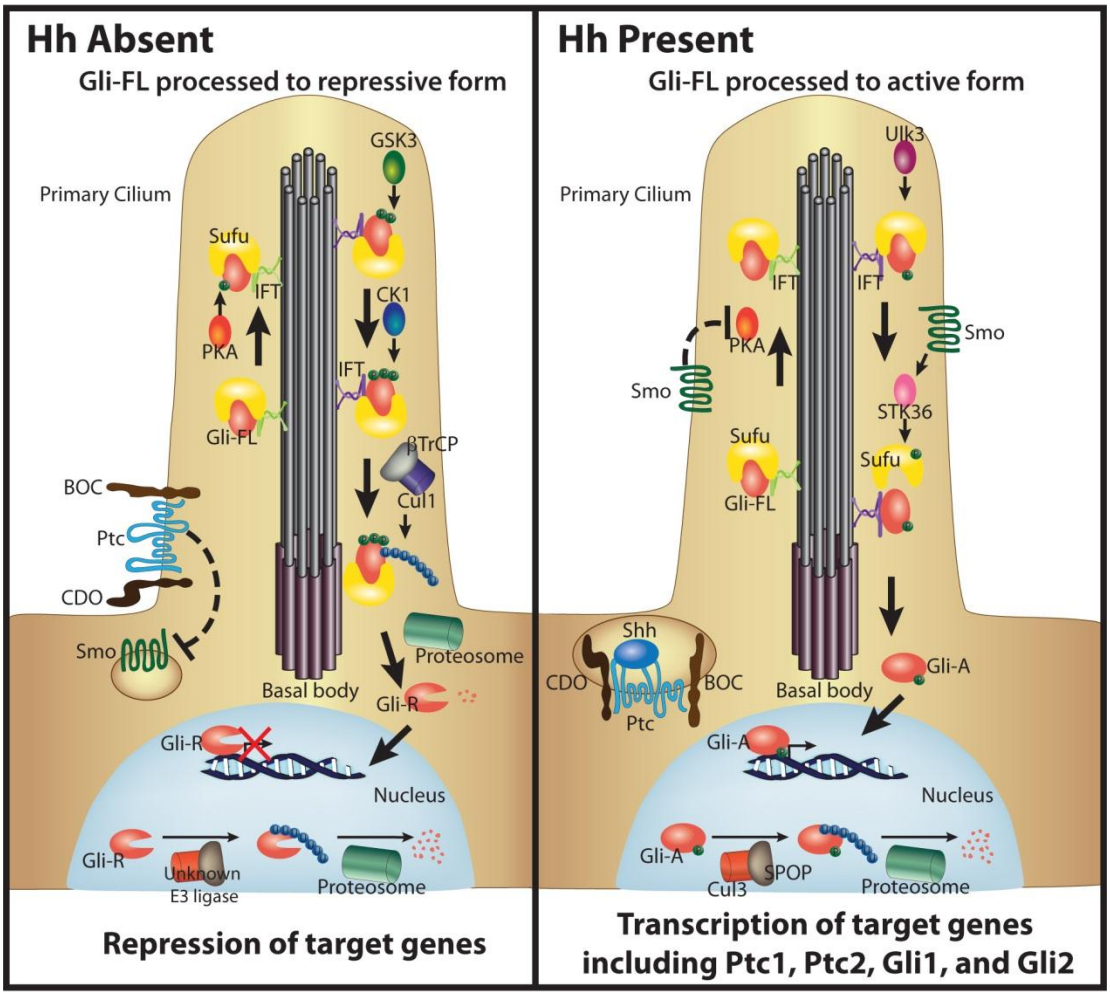


Figure 4: *Schematic of mammalian hedgehog signaling*. In the absence of Hh ligand, Gli transcription factor (Gli 2,3) is processed to a repressor form (Gli-R) by successive phosphorylation by PKA, GSK3β, and CK1. This phosphorylated form is recognized by βTrCP as part of an E3 ubiquitin ligase complex with Cul1. Conversely, Gli (Gli 1-3) is

activated when Hh is present to promote transcription of target genes, including Hh pathway elements *ptc1*, *ptc2*, *gli1*, and *gli2*.

The Primary Cilium

The primary cilium is a crucial component of transmission of vertebrate Hh signal, unlike in *Drosophila*. Once regarded as a mere curiosity, the primary cilium is widely becoming known as an organelle implicated in coordinating and regulating many different signaling pathways, including Hh, Wnt, and PDGF[126-130]. Inappropriate assembly of the primary cilium results in a whole class of disorders called ciliopathies, examples being Bardet-Biedl syndrome, Meckel syndrome, Joubert syndrome, and both the recessive and dominant forms polycystic kidney diseases[131].

Primary cilia consist of an axoneme of nine doublet microtubules that extends from a basal body, which is derived from the centriole[132]. In contrast to those of motile 9+2 cilia, axonemes of primary cilia lack the key elements involved in motility, including the central pair of microtubules, the proteins that surround them, and the dynein arms that power microtubule sliding to produce motility[133, 134]. The axoneme itself is assembled by the intraflagellar transport proteins, including the kinesin and dynein motors that power transport[132]. Knockouts of many of the IFT proteins result in absent or blunted primary cilia, leading to aberrant hedgehog signaling[130, 135-137]. Genetic studies show that IFT proteins act somewhere downstream of Ptc and Smo and upstream of the Gli transcription factors 1-3, and are thought to be involved in Gli processing[114, 138].

The first evidence that vertebrate Hh signaling depends on primary cilia came from a screen for ENU-induced mutations that alter the patterning of the mouse embryo[119, 130]. This screen identified several mutants showing morphological and patterning defects that were phenotypically consistent with disrupted Hh signaling. The genes disrupted in these mutants encode several components of the IFT machinery, including the IFTB complex components IFT122, IFT172 and IFT88, and subunits of the retrograde and anterograde motors DynC2H1 and Kif3a[136, 139].

Despite the evolutionary conservation of the Hh pathway and the importance of primary cilia in vertebrate Hh signaling, cilia are not required for Hh signaling in *Drosophila*. This raises the question of why cilia are required for vertebrate Hh signaling. Recent data suggest that Kif7, a kinesin that is the vertebrate homologue of *Drosophila* Costal 2, may link the vertebrate Hh pathway to cilia[130, 140-142]. Cos2 is a kinesin-related protein that serves as a scaffold for Hh signaling complexes. Cos2 is required for formation of the repressor form of the *Drosophila* Gli homologue Ci by recruiting the kinases that mark Ci for processing. Cos2 is also required after pathway activation to promote maximal pathway activation by antagonizing Sufu[130]. Although Cos2 can bind microtubules, its motor domain has diverged from other kinesins such that its motor function is disabled[143]. The vertebrate homolog Kif7 has similar function as Cos2. It is required for the processing of Gli3 to its repressor form in the absence of Hh and also required for the efficient localization of Gli3 to cilia in response to Hh signaling activation. However, unlike Cos2 the Kif7 motor domain has the motifs that are typical of kinesin motors. In the absence of ligand, Kif7 localizes to the base of the primary cilium and moves to the tip in response to

pathway activation[130, 142]. This translocation depends on the motor domain, which suggests that Kif7 acts as an anterograde motor[141, 142]. These results cumulatively suggest that Cos2/Kif7 are an evolutionary link between Hh and primary cilia.

Furthermore, recent work in planaria suggests that some conserved components of the Hh pathway were associated with cilia before they were associated with Hh. RNAi knockdown of Fused, Cos2, and Iguana, known Hh signaling components in flies and zebrafish, resulted in worms with a reduction in cilia, but intact Hh signaling[144]. Knockdown of known planarian IFT components had similar results[144]. Based on these findings, the authors concluded that cilia are not required for Hh signaling in planarians, and that the ancestral function of Fused, Cos2 and Iguana is in the regulation of ciliogenesis[132, 144]. A related study, which focused on the planarian iguana gene, also arrived at similar conclusions[145]. These data provide insights as to why Hh signaling requires primary cilia in vertebrates but not in lower animals.

Hedgehog Signaling and the Vasculature

In vascular development, Hh is required for arterial-venous specification of endothelial cells[146, 147], angiogenesis in the murine yolk sac[148], as well as the remodeling of branchial arch blood vessels[90]. It is also a potent angiogenic factor[87, 89, 149]. Blocking hedgehog signaling drastically impairs angiogenesis in both the neural tube[150] and retina[151] while overexpression in the neuroectoderm causes hypervascularization[85]. Mechanistically, it has been shown that Shh regulates the

expression of FGF, VEGF, and angiopoietin 1 and 2 in fibroblasts[88, 89, 152, 153]: all potent angiogenic factors.

Other studies suggest a direct effect of Shh on endothelial cells[154, 155]. Renault et al. found that non-canonical Hh signaling in endothelial cells through the Rho pathway led to EC migration, capillary morphogenesis, and upregulated expression of matrix metalloproteinase 9 and osteopontin, important matrix remodeling factors required for angiogenesis[155]. A similar study also found that Shh induced capillary morphogenesis of ECs *in vitro*. The researchers found that Shh upregulated phosphoinositide 3 kinase (PI3K) and Shh induced morphogenesis was abolished when cells were treated with a PI3K inhibitor[154].

Hedgehog Signaling and Progenitor Cells

One important function of Shh is promoting maintenance and survival of progenitor cells. Several studies have shown that Hh signaling is required in various regions of the brain to stimulate proliferation of progenitors and promote their survival. Conditional deletion of Shh or Smo in the telencephalon showed reduced progenitor cell numbers in the hippocampus and subventricular zone in the postnatal mouse brain[156]. Conversely, reactivation of Shh signaling in the mature brain resulted in an increase in proliferation of telencephalic progenitors[156]. In the cerebellum, Shh is required for proliferation of granule cell progenitors[157-159]. Ectopic expression of Shh in the spinal cord expanded neural progenitor cells and prevented their differentiation, reminiscent of tumors of the CNS[85]. Fate mapping Shh responsive cells using an inducible cre under the control of the Gli1 promoter shows

that Hedgehog responsive cells in the brain self-renew and while normally quiescent become mitotically active in response to injury[160].

Shh has also been implicated in the maintenance and proliferation of progenitors in several other niches. Liver progenitor cells are marked by *Ptc*$^{LacZ/+}$ activity. When treated *in vitro* with Hh inhibitor cyclopamine, these cells become apoptotic[161]. In the hair follicle there is a niche for stem cells that contribute to the cycling hair follicle as well as epidermal cells upon injury. These cells are *Gli1*$^{LacZ/+}$ positive, and receive Shh from nearby nerves. Upon denervation, the stem cells in the upper bulge region of the niche are unable to make epithelial stem cells that contribute to wound healing. Their ability to contribute to the hair follicle was unaltered, possibly due to a source of Dhh at the base of the follicle[162]. A similar study found roles for Shh and Dhh in epidermal stem cell maintenance[163]. Overexpression of either in the epidermis produced embryos that either had hyperplastic stem cells or a loss of self-renewal, suggesting an importance for balance of Hh signal in stem cell maintenance[163]. Smooth muscle progenitor cells in the developing ureter have also been shown to rely on Shh signal. Shh promotes mesenchymal cell proliferation, regulates timing of differentiation into SMCs, and sets the pattern of differentiation through dose-dependent inhibition of smooth muscle formation[82]. In the absence of Shh signal, smooth muscle mass is greatly reduced due to a proliferation defect, and the progenitor cells are largely absent[82].

Macrophages and the Vasculature

Macrophages have been implicated in angiogenesis, especially in hypoxic environments. Recent studies have correlated angiogenesis with HIF2α expression in tumor associated macrophages in human breast cancer[164, 165]. These tumor associated macrophages release proangiogenic growth factors including VEGF and fibroblast growth factor (FGF)[166]. Microarray data also implicates the upregulation of more than 30 proangiogenic genes in primary macrophages exposed to hypoxia, including angiopoietin, IL-8, VEGF receptor 1, and inducible form of nitric oxide synthase (iNOS)[167].

Furthermore, recent studies show that ablation of macrophages pharmacologically from aortic explants blocked angiogenesis *in vitro*. This ability was restored when exogenous macrophages were added to the culture[168]. Enhanced capillary sprouting in aortic rings by co-culture with macrophages was not dependent on cell-cell contact between the ECs and macrophages, as conditioned medium from macrophages had the same stimulatory effect[169].

Interestingly, a recent study provided evidence that macrophages played a key role in the development of the vascular plexus in the mouse brain, retina, and zebrafish trunk. Their data suggest that macrophages guide tip cells together to form an anastomosis[170]: an important process in increasing the complexity of the plexus.

Hedgehog Signaling In Macrophages and Inflammatory Cells

Macrophages, mast cells, T cells, and other lymphocytes are all residents of the adventitia of normal vessels[67, 171]. Adventitial macrophages may play important

roles in the progression of intimal lesion formation in atherosclerosis. One study found that upon progression of atherosclerosis in apolipoprotein-E-deficient mice, there was a significant increase in the total number of macrophages, T-cells, and dendritic cells in the adventitia, suggesting that atherosclerosis induces the recruitment of inflammatory cells[67].

Cells of the immune system show evidence of Hedgehog signaling. Myeloid cells (CD33-positive), B cells (CD19-positive), T cells (CD3-positive) and primitive progenitors (CD34-positive CD38-) express *ptc*, *smo*, and *shh* mRNA[83, 172-174]. Monocyte derived macrophages express the hedgehog pathway components *ptc, gli1, gli2*, and *smo.* It has also been shown that macrophages upregulate expression of *ptc* in response to stimulation by commercially available recombinant Shh[175]. Recent evidence shows that macrophages use Shh as a chemoattractant to a gastric infection[176]. Deletion of Shh from the parietal cells or deletion of Smo from macrophages resulted in a decrease of inflammation and macrophage recruitment to the stomach in response to infection with the bacterium *H pylori*[176].

Adipocytes and the Vasculature

Adipose tissue is one of the most highly vascularized tissues in the body, with each adipocyte encircled by microvessels[177]. Indeed, emerging data show that angiogenesis modulators affect the expansion of fat mass by regulating the growth and remodeling of the adipose associated vasculature[178]. Vascular beds can also influence the growth of adipose tissue by a number of other mechanisms. For one, activated endothelial cells in angiogenic vessels produce growth factors that

communicate to the adipocytes in a paracrine manner to produce mitogenic effects[177]. Additionally, a recent study has shown that vascular pericytes can be differentiated into preadipocytes and adipocytes[179].

Conversely, adipocytes can influence the growth and remodeling of the vasculature. Many adipokines (cytokines produced by adipocytes) are also angiogenic; an example being Leptin[180]. More than 40 adipokines have been identified so far.

Hedgehog Signaling and Adipocytes

Hedgehog has been implicated in adipocyte biology as a negative regulator of white adipose differentiation[181]. However, this study further showed that brown adipocytes are unaffected by overexpression of Hh signal and develop normally. Adipocytes surrounding the thoracic aorta are largely brown adipocytes[182]. It is yet to be determined whether Hh is required for brown adipocyte formation.

Specific Aims

This research was conducted towards the overall goal of characterizing the arterial adventitia as a Shh responsive vascular niche. We hypothesize that Shh is a key factor maintaining and/or developing this niche, and so we sought to answer the questions of what other cell types participate in Shh signaling in this environment that may be key niche cells, and when this niche forms in development.

Specific aim 1: Determine the cell types involved in Shh signaling in the adventitia that contribute to making the adventitial niche.

Specific aim 2: Determine the time the adventitia first appears in the embryo and characterize its spatial development, using responsiveness to Shh as a marker.

CHAPTER 2

SONIC HEDGEHOG SIGNALING IN THE NEONATAL ARTERIAL ADVENTITIA

Introduction

The adventitial layer of the artery wall has long been considered merely inert connective tissue. Recent evidence suggests that it is an active participant in flow dependent wall remodeling[71, 76, 80, 183, 184], a site for immune cell accumulation and activation in injury and disease[67-69, 72, 75], as well as an active niche for progenitor cells that can adopt both vascular and non-vascular fates[22, 24, 25, 27, 28, 30].

Our previous work identified a novel Shh signaling domain restricted to the adventitial layer of the artery wall[25]. Our data suggest that a paracrine Shh signaling network is involved in maintaining a progenitor cell niche in the adventitia. To begin to identify roles that Shh signaling plays within the artery wall, it is first necessary to identify the cell types that produce Shh in the adventitia, and then to determine what cell types are actively responding to it.

Shh signaling is an essential morphogen, mitogen, and survival factor in many contexts including development and cancer[94, 185]. Shh has been shown to be required in survival and proliferation of progenitor cells in the brain[156, 160], liver[161], hair follicle[162, 163], and ureteral smooth muscle[82].

In vascular development, Shh is required for arterial-venous specification of endothelial cells through the repression of venous cell fate[146, 147], angiogenesis in

the murine yolk sac[148], as well as the remodeling of branchial arch blood vessels[90]. It is also a potent angiogenic factor[87, 89, 149]. Blocking hedgehog signaling drastically impairs angiogenesis in both the neural tube[150] and retina[151] while overexpression in the neuroectoderm causes hypervascularization[85].

Hypothesis

Shh signaling plays a role in developing and maintaining the adventitial niche through paracrine interactions between adventitial progenitors and other differentiated adventitia resident cells.

Materials and Methods

All antibody and reagent product numbers and sources are listed in Appendix B.

Mice

All animal protocols were approved for use by the Institutional Animal Care and Use Committees of the University of North Carolina and Seattle Children's Research Institute. All mouse strains used were obtained from the Jackson Laboratories. Noon on the day of vaginal plug was designated as day 0.5. The day of birth was designated as P0. Mice used include *Ptc1*$^{LacZ/+}$ (*Ptch1*^{tm1Mps}, Jackson stock number: 003081)[186], *Ptc2*^{LacZ}(*Ptch2*^{tm1Dgen}, Jackson stock number: 005827)[187], *Gli1*$^{LacZ/+}$ (*Gli1*^{tm2Alj}, Jackson stock number: 008211)[188], *Gli2*$^{LacZ/+}$ (*Gli2*$^{tm2.1Alj}$, Jackson stock number: 007922)[189], and *Shh*$^{EGFP/+}$ (*B6.Cg-Shh*$^{tm1(EGFP/cre)Cjt}$ Jackson stock number: 005622)[91]. See Appendix C for strain specific genotyping information. See Appendix D for mouse strain information.

Whole Mount Immunostaining

Shh$^{EGFP/+}$, *Ptc1*$^{LacZ/+}$, and *Gli1*$^{LacZ/+}$ mice were sacrificed at postnatal day 2, perfused with PBS to clear the blood, and arterial tissues were then fixed at physiologic pressures with 4% PFA at 100 mmHg. Thoracic aortas and carotid arteries were dissected, post fixed in 4% PFA for 30 minutes at room temperature, and then rinsed with PBS for 15 minutes. Antigen retrieval methods vary based on the primary antibody used. Antibodies against intracellular proteins like SMαA are retrieved with a 15 second wash in methanol. Antibodies against nuclear proteins and primary cilia are retrieved by adding 0.1% Triton X 100 to the blocking solution (2% normal donkey serum (NDS) + 2% normal goat serum (NGS) + 0.1% bovine serum albumin (BSA) in PBS). No antigen retrieval is used when staining for cell surface proteins like Sca1. See appendix B for list of antibodies and dilutions and notes on antigen retrieval conditions.

Samples were blocked at room temperature with blocking solution composed of 2% NDS + 2% NGS + 0.1% BSA in PBS for one hour. Primary antibody was diluted in blocking solution overnight and incubated at 4^0C. Samples were rinsed in PBS for 15 minutes, and blocked again at room temperature for 1 hour in recycled blocking solution. Secondary antibody diluted in 0.1% BSA in PBS was applied to the tissue for 2 hours at room temperature. Samples were then rinsed in PBS for 15 minutes. Hoechst dye (Molecular Probes) was diluted to 1µg/ml in H_2O, and applied for 1 minute. Tissue samples were washed briefly in H_2O, followed by a brief wash in PBS before storage in a 1:1 glycerol: PBS solution at 4^0C. Samples can be stored in

these conditions for several months to a year protected from light with no loss of signal. Samples were imaged on a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope, an Olympus FV500 Confocal Laser Scanning Microscope, an Olympus FV1000 Confocal Laser Scanning Microscope, or a Leica SP5 Confocal Laser Scanning Microscope.

Sections immunohistochemically stained for ECM components were processed by the histology core facility at Benaroya Research Institute at Virginia Mason, Seattle, WA.

FITC Dextran Injection

Mouse pups were anesthetized with 5% isoflurane in 1000cc oxygen, and anesthesia was maintained using 2% isoflurane in full oxygen. A midline incision was made in the chest, and pectoral muscles were reflected to visualize the heart through the intercostal muscles. Pups were injected through the left ventricle with 50µl of 20mg/ml solution of high molecular weight FITC-Dextran (2000kDa) (Sigma). Dextran was allowed to circulate for 5 minutes before euthanizing the animal.

EdU Injection

Mouse pups were given 100µl of 0.3mg/ml EdU in sterile 0.9% NaCl by IP injection (50mg/kg). After 2 hours, mice were sacrificed and fixed with 4% PFA. Staining was done according to the manufacturer's instructions (Invitrogen).

Xgal Staining

Tissues were fixed using 0.2% gluteraldehyde solution in a 0.1 M sodium phosphate buffer pH 7.3 containing 2 mM $MgCl_2$ and 5 mM EGTA for 1 hour at room temperature. Samples were then washed three times for 30 minutes at room temperature with rinse buffer pH 7.3 containing 0.1 M sodium phosphate, 2 mM $MgCl_2$, and 0.1% Triton X100. Finally, tissue samples were stained overnight in a shaking incubator at 37°C using X-gal staining buffer pH 7.3, composed of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.02 M Tris-Cl, and 1 mg/ml Xgal (Bioline) diluted in the rinse buffer described above. Tissue samples were post-fixed in 4% PFA for 30 minutes at room temperature and stored in PBS. Whole mount X-gal stained tissues were imaged on a Leica MZFLIII stereomicroscope with the Leica DFC310FX camera.

Results

Shh Responsive Cell Types in the Arterial Adventitia

Our studies using *Ptc1*$^{LacZ/+}$, *Ptc2*$^{LacZ/+}$, and *Gli1*$^{LacZ/+}$ transgenic reporter mice and cell type-specific antibodies have identified several key players involved in Shh signaling in the adventitia. These reporter mice are a useful because Ptc1, Ptc2, and Gli1 are not only mediators of hedgehog signaling, but also direct Shh target genes, and serve as reporters of active hedgehog signaling[186-188]. We were initially surprised to find that the expression of the reporters was entirely restricted to the adventitia[25](Figure 5). No other signaling pathway that we are aware of has been shown to be confined exclusively to the arterial adventitia. When using cell type

specific antibodies, we observe that cells expressing β-galactosidase (β-gal) in the Hedgehog reporter mice included CD68-positive macrophages and Perilipin A-positive adipocytes in the aorta or carotid arteries from newborn mice. These cells cluster with AdvSca1 progenitor cells, indicating possible paracrine signaling between these cell types.

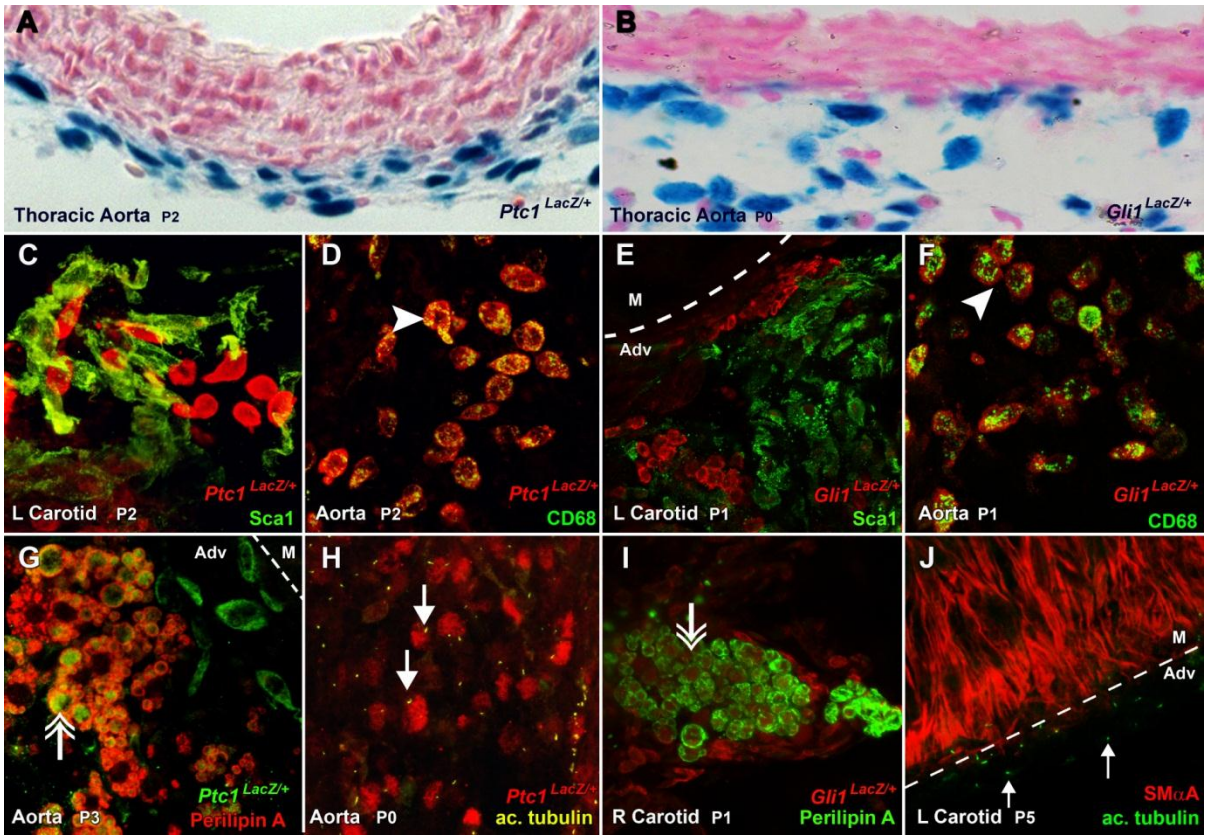


Figure 5: *Hedgehog responsive cells in the adventitia*. *Ptc*$^{LacZ/+}$ and *Gli1*$^{LacZ/+}$ expression is restricted to the adventitia. X-gal stained P0-P2 aorta sections, counterstained with nuclear fast red (A, B). CD68-positive macrophages (D, F, arrowheads), and adipocytes (G, I, double arrows) express both *Ptc1*$^{LacZ/+}$ and *Gli1*$^{LacZ/+}$. AdvSca1 cells cluster with cells strongly positive for *Ptc1*$^{LacZ/+}$ and *Gli1*$^{LacZ/+}$ (C, E). Primary

cilia are also localized solely to the adventitia, marked by acetylated α tubulin (H, J, arrows).

We also detect that these β-gal-positive cells have primary cilia, which are marked by acetylated α-tubulin (Figure 5 H, J). Primary cilia are required proper processing and activation of Gli transcriptional mediators 1-3[130]. We also observe that these β-gal-positive cells express the co-receptor BOC (Figure 6B). Previous work has shown that the overall population of AdvSca1 cells expresses RNA transcripts for Smo, BOC, CDO, Ptc1, Ptc2, Gli1, and Gli2[25]. While AdvSca1 cells do not appear to express *Ptc1*$^{LacZ/+}$ or *Gli1*$^{LacZ/+}$, a couple of them appear to express BOC. These data support and confirm the findings from reporter mouse data.

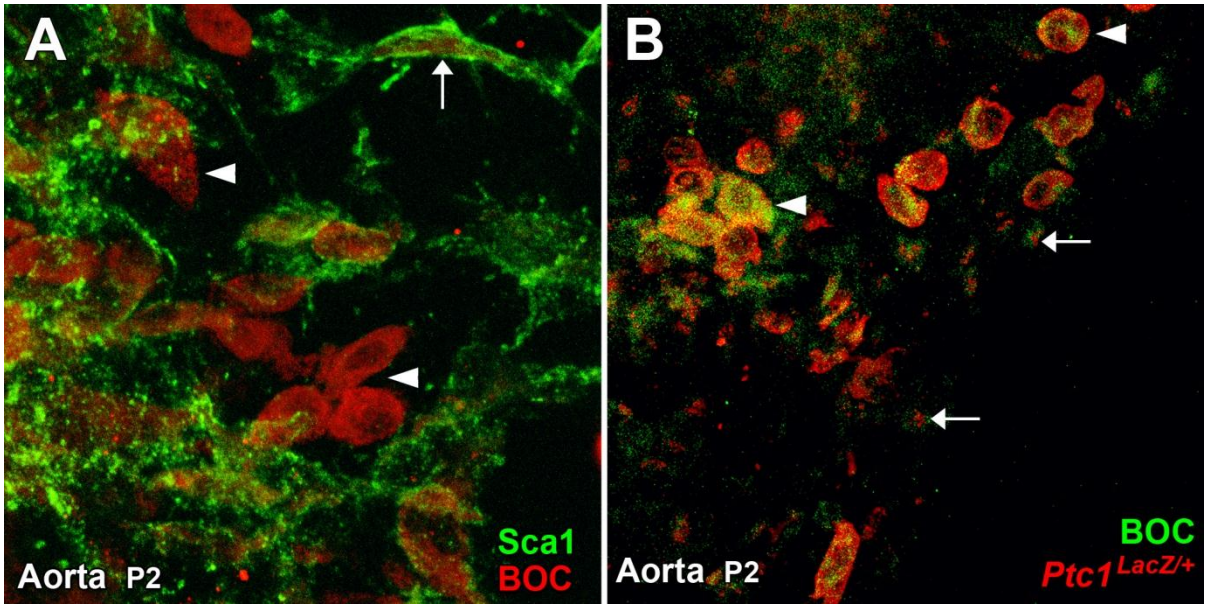


Figure 6: *Immunostaining for Shh pathway elements confirms reporter mouse data*. A small subset of AdvSca1 cells (arrow in A) and *Ptc1*$^{LacZ/+}$-positive cells co-localize staining with Hedgehog co-receptor

BOC. Arrowheads in A: BOC strongly positive cells that morphologically resemble tissue resident macrophages. Arrowheads in B: BOC-positive and *Ptc1*$^{LacZ/+}$-positive cells that morphologically resemble macrophages. Arrow in A: colocalization of Sca1 and BOC. Not all AdvSca1 cells also express BOC. Arrows in B: cells with nuclear localized LacZ that also co-express BOC. These cells cannot be identified by morphology.

We also observe that Hedgehog responsive cells are organized in the adventitia into layers and clusters (Figure 7). This architecture and arrangement of cells is similar to the organization we observe for AdvSca1 cells (Figure 3 B, C) and may reflect the overall structure of the adventitial ECM as described in porcine and canine vessels[34, 38]. We hypothesize that this clustering behavior is important for AdvSca1 maintenance. Adherens junctions and cell-cell mediated contacts have been shown to be important in maintaining stemness in niches[7, 190, 191]. This clustering effect may also be important in regulating the level of Shh signal. Shh has been shown to induce different fates in a dose dependent manner, so clustering and layering may be in part to ensure the cells participating in the paracrine signaling loop are receiving the proper amount of Hh ligand[91, 192, 193]. It has been shown that the inner adventitia is composed of alternating layers of collagen and elastin fibers in the pig and dog[34, 38]. These reports suggest a level of organization to the adventitia, and this ECM organization likely mediates the layering of cells in the adventitia.

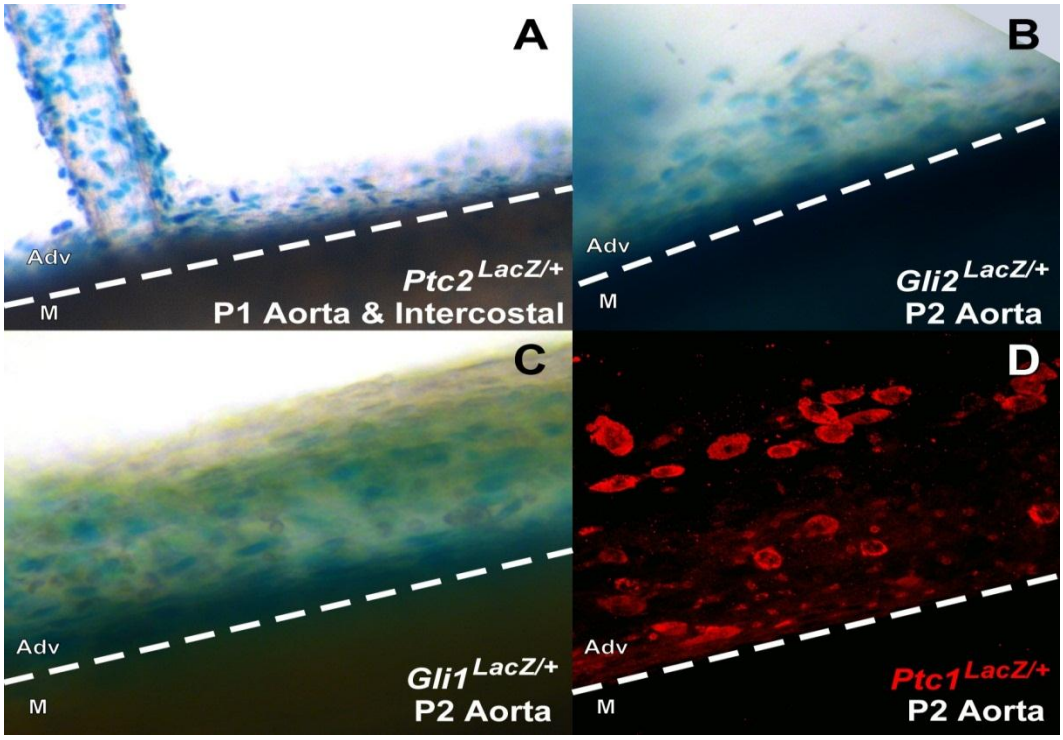


Figure 7: *Hedgehog responsive cells in the neonatal adventitia are organized into clusters and layers*. In the early postnatal aorta, Hedgehog responsive cells are distributed throughout the adventitia in layers and clusters. Images were taken of a side profile of whole mount vessels.

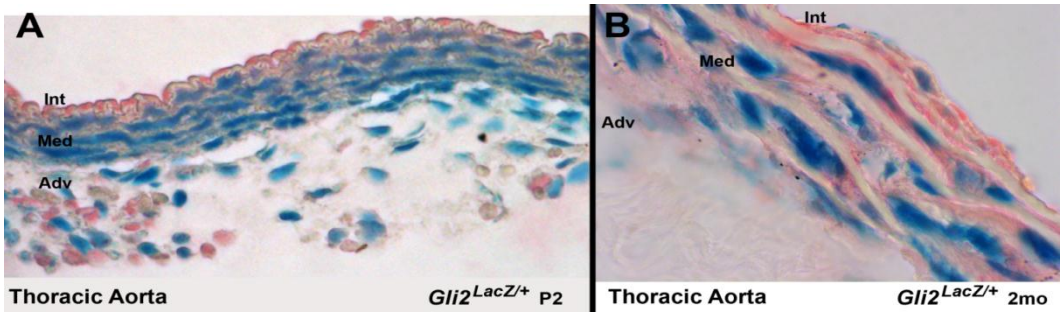


Figure 8: Gli2$^{LacZ/+}$ *expression in the artery wall*. *Gli2*$^{LacZ/+}$ is expressed in both the media and the adventitia in the early postnatal period, and medial expression persists into adulthood.

One curious observation we note is that *Gli2*$^{LacZ/+}$ does not have a similar staining pattern to the Hedgehog reporters shown in Figure 5. In *Ptc1*$^{LacZ/+}$, *Ptc2*$^{LacZ/+}$, and *Gli1*$^{LacZ/+}$, we repeatedly observe that Hedgehog signaling is restricted entirely to the adventitia, and never have we observed any staining in the media, in embryogenesis, perinatal development, or adulthood. *Gli2*$^{LacZ/+}$ mice have abundant and strong expression both in the media and adventitia. This suggests that *Gli2*$^{LacZ/+}$ expression is uncoupled from a dependency on Shh signaling in the artery wall. It is likely that Gli2 is being transcribed in response to a pathway other than Shh. A recent study found that Gli2 and Gli3 are transcribed in response to Notch1 signaling in neural progenitors[194]. It has also been shown that Gli2 is a transcriptional target of TGF-β signaling[195, 196]. Both of these pathways have been shown to be active in vascular SMCs to promote differentiation[50, 197, 198]. Further study will be required to determine the mechanism for Gli2 transcription in the medial smooth muscle cells.

Shh Production in the Arterial Adventitia

Expression of the Hedgehog reporters Ptc1, Ptc2, and Gli1 in the adventitia led to the next obvious question, is Shh protein produced locally, and if so, from which cell types? To address this question, we studied early postnatal mice from another reporter strain, *Shh*$^{EGFP/+}$*.* This mouse has EGFP knocked into the endogenous Shh locus, and serves as a faithful reporter of cells actively producing Shh[91]. Previous work from our lab demonstrated that Shh protein concentrated along the border between the media and adventitia (Figure 10D)[25]. The source of this Shh

protein was unclear. In the analysis shown in Figure 9, adventitial cells producing Shh are identified by anti-EGFP using whole mount confocal microscopy. The data suggest that Shh is synthesized locally and that the protein we detected between the medial and adventitial layers was made by adventitial cells themselves. We observed that EGFP-positive cells are numerous in the postnatal day 1 (P1) adventitia (Figure 9) and like $Ptc^{LacZ/+}$, EGFP expression also was restricted exclusively to the adventitia, with no smooth muscle cells in the media expressing either EGFP or Shh protein. Cell types that we observed to be strongly EGFP positive include CD68-positive macrophages, nerves marked by β-tubulin III, and Sca1-positive adventitial progenitor cells (Figure 9). About half of the Perilipin A-positive adipocytes stain with Shh-antibody, and weakly stain with $Shh^{EGFP/+}$. We confirmed these results by immunostaining with anti-Shh (DSHB) (for antibody information, see Appendix B). The 5E1 Shh antibody developed by Thomas Jessel and Susan Brenner-Morton was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Some of these results are consistent with previous reports on Hedgehog signaling in similar cell types, but not all. Shh has been shown to be expressed in cells of the immune system. Myeloid cells (CD33-positive), B cells (CD19-positive), T cells (CD3-positive) and primitive progenitors (CD34-positive CD38-) express *ptc*, *smo*, and *shh* mRNA[83, 172-174]. Additionally, nerves have been shown to express Shh. Nerve derived Shh supports stem cells in hair follicles. Upon denervation, the stem cells in the upper bulge region of the hair follicle niche are unable to make epithelial

stem cells that contribute to wound healing[162]. Retinal ganglion cells also express and secrete Shh. Interference with Shh signaling in the retinal ganglion cells themselves causes abnormal growth and navigation of contralateral projecting axons, indicating cell autonomous signaling[199]. Additionally, genetic cell fate mapping results show that Shh is exclusively expressed in all spiral ganglion neurons in the cochlea and not in surrounding glia cells[200].

Cell types that have not been reported to secrete Shh that we have identified include the Sca1-positive progenitors and adipocytes. Several studies have shown that stem cells respond to Shh[156, 163, 201], but there is very little literature on progenitor cells expressing Shh. Passman et al. showed that AdvSca1 cells express *shh* mRNA[25], but no other reports that we know of have stated this. Additionally, it has been shown that Hedgehog acts as a negative regulator of white adipose differentiation[181]. Brown adipocytes are unaffected by overexpression of Hh signal and develop normally[181]. Adipocytes surrounding the thoracic aorta are largely brown adipocytes[182]. It is yet to be determined whether Hh is required for brown adipocyte formation, or if brown adipocytes make Shh.

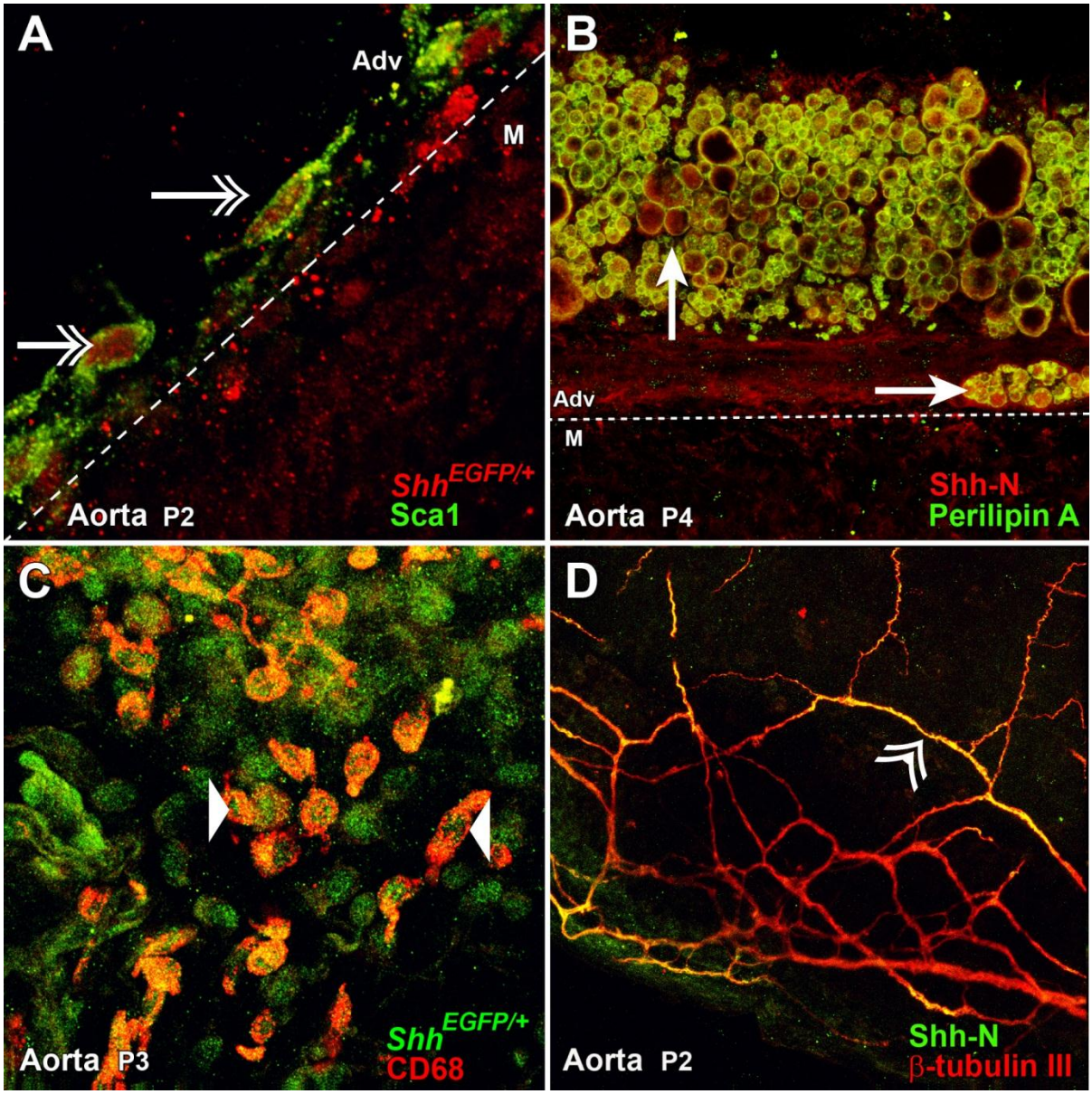


Figure 9: *Shh is locally produced in the adventitia*. AdvSca1 cells (A, double arrows), some Perilipin A-positive adipocytes (B, arrows), CD68-positive macrophages (C, arrowheads), and β-tubulin III-positive nerves (D, double arrowhead) all make Shh as seen by reporter activity. Locally produced Shh protein is localized along the adventitia/media border (B).

Because there are Shh producing cells throughout the adventitia and yet Shh protein is concentrated along the media-adventitia border (Figure 9B, Figure 10A), we hypothesized that the locally produced protein is sequestered along this border due to heparin sulfate proteoglycans (HSPG) and other matrix proteins in the ECM that Shh binds to. Perlecan is one such HSPG reported to bind Shh protein[125]. Perlecan knockout cells have an impaired ability to respond to Shh compared to wild type[125]. Indeed, we see that Perlecan is robustly expressed in the media in adult mice, and in the matrix in the innermost layer of the adventitia (Figure 10B). Note the distribution of Shh (Figure 10D) resembles the distribution of Perlecan in the inner adventitia (Figure 10B).

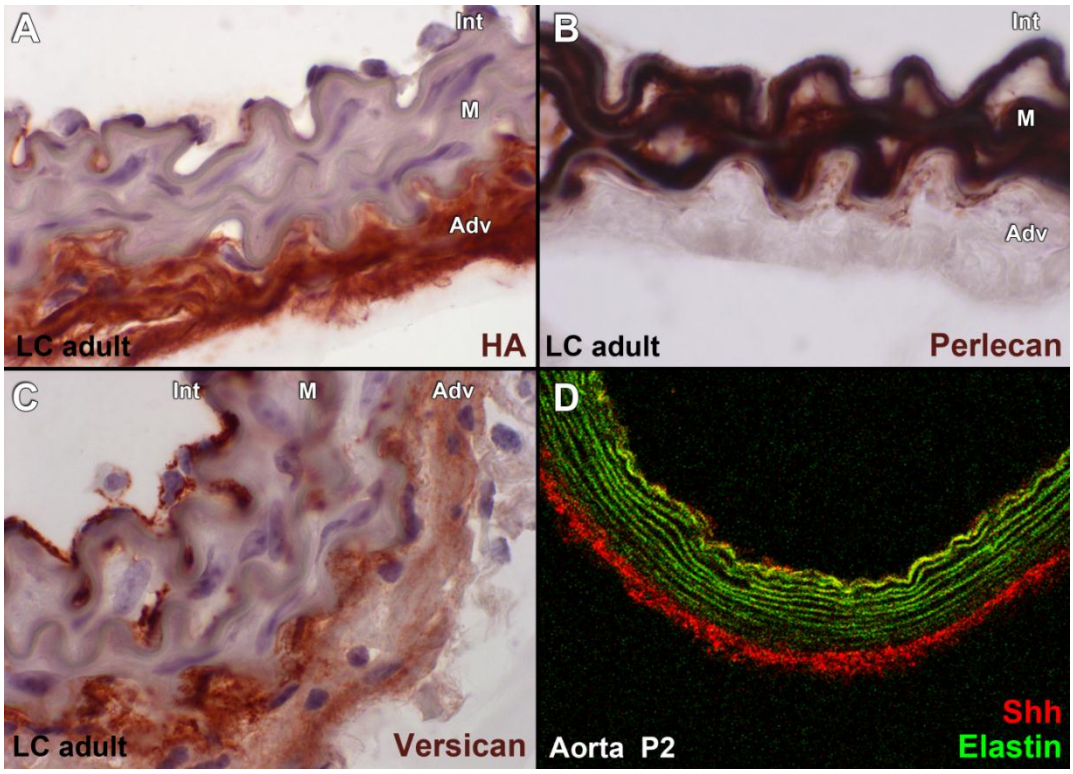


Figure 10: *ECM expression in the adult artery wall*. Shh is distributed along the media/adventitia border (D). We hypothesize that the

distribution pattern is controlled by the localization of ECM proteins that bind Shh protein. The HSPG Perlecan is robustly expressed in the media and the innermost layer of the adventitia (B). Hyaluronic Acid (A) and Versican (C) are expressed in the adventitia and intima. Panel D is modified from Passman et al.[25].

Shh and the *Vasa Vasorum*

The adventitia and particularly the periadventitial adipose tissue contains a microvascular network called the *vasa vasorum*. In the perinatal period of development, these microvessels are rapidly expanding, particularly within the rapidly growing perivascular adipose tissue. Immunostaining for anti-phospho-histone H3 or Ki67 show a large number of dividing endothelial cells. Many endothelial cells in the adventitial microvasculature also incorporate EdU, a thymidine analog, into their DNA within 2 hours, indicating active DNA synthesis (Figure 11F).

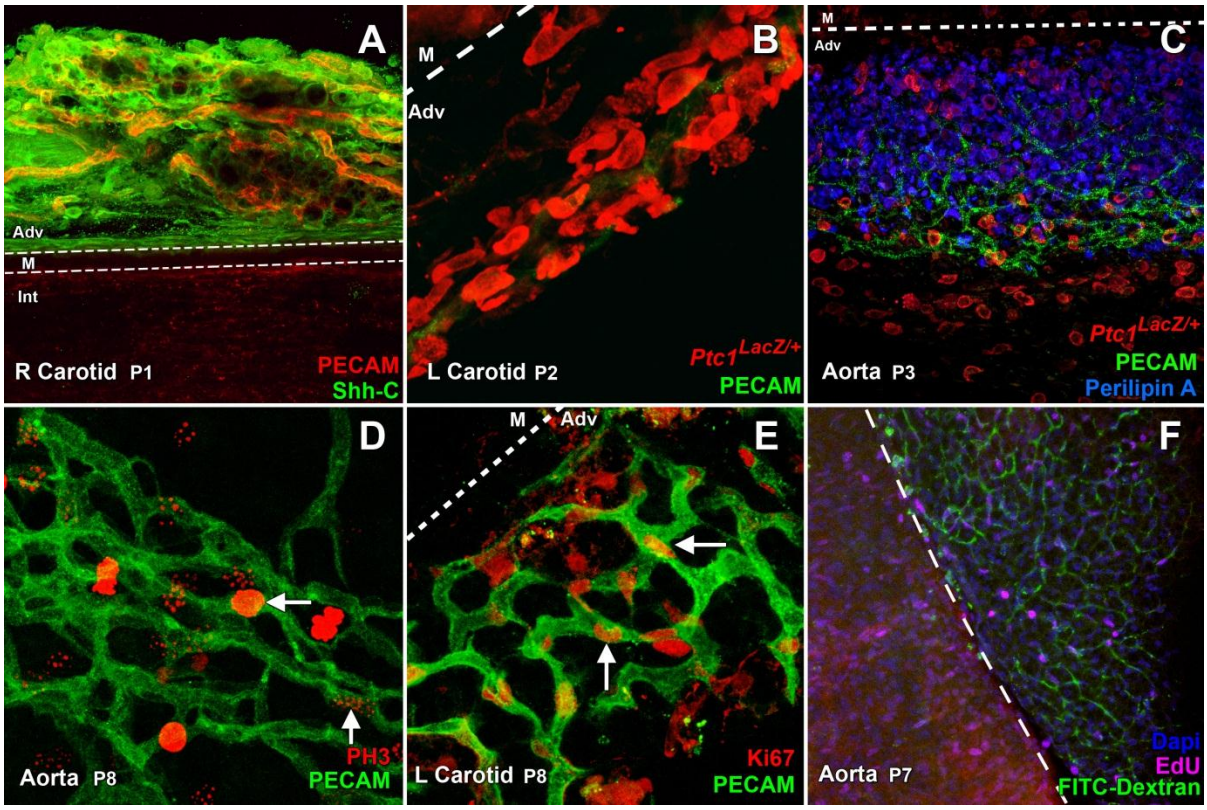


Figure 11: *The vasa vasorum*. Adventitial microvessels are rapidly growing during the perinatal time period as shown by phospho-histoneH3 and Ki67 staining, and EdU incorporation in a 2 hour pulse. Microvessels also closely associate with Shh responsive cells.

We also observe that microvessels reside in a Shh rich matrix (Figure 11A) and closely associated with hedgehog responsive cells, as seen in the *Ptc1*$^{LacZ/+}$ and *Gli1*$^{LacZ/+}$ reporter mice (Figure 11B, Figure 12). We identify these hedgehog responsive cells to be CD68-positive and *LysM*$^{cre/+}$-positive tissue resident macrophages (Figure 12). Macrophages have been shown to play a role in anastomosis in the cerebral vasculature[170]. Macrophages have also been implicated in angiogenesis in hypoxic environments, specifically tumors. Tumor associated

macrophages release proangiogenic growth factors including VEGF and fibroblast growth factor (FGF)[166]. Microarray data also implicates the upregulation of more than 30 proangiogenic genes in primary macrophages exposed to hypoxia, including angiopoietin, IL-8, and iNOS[167]. We hypothesize that macrophages are playing a role in angiogenesis of the *vasa vasorum,* and that hedgehog signaling in macrophages may be important in this process.

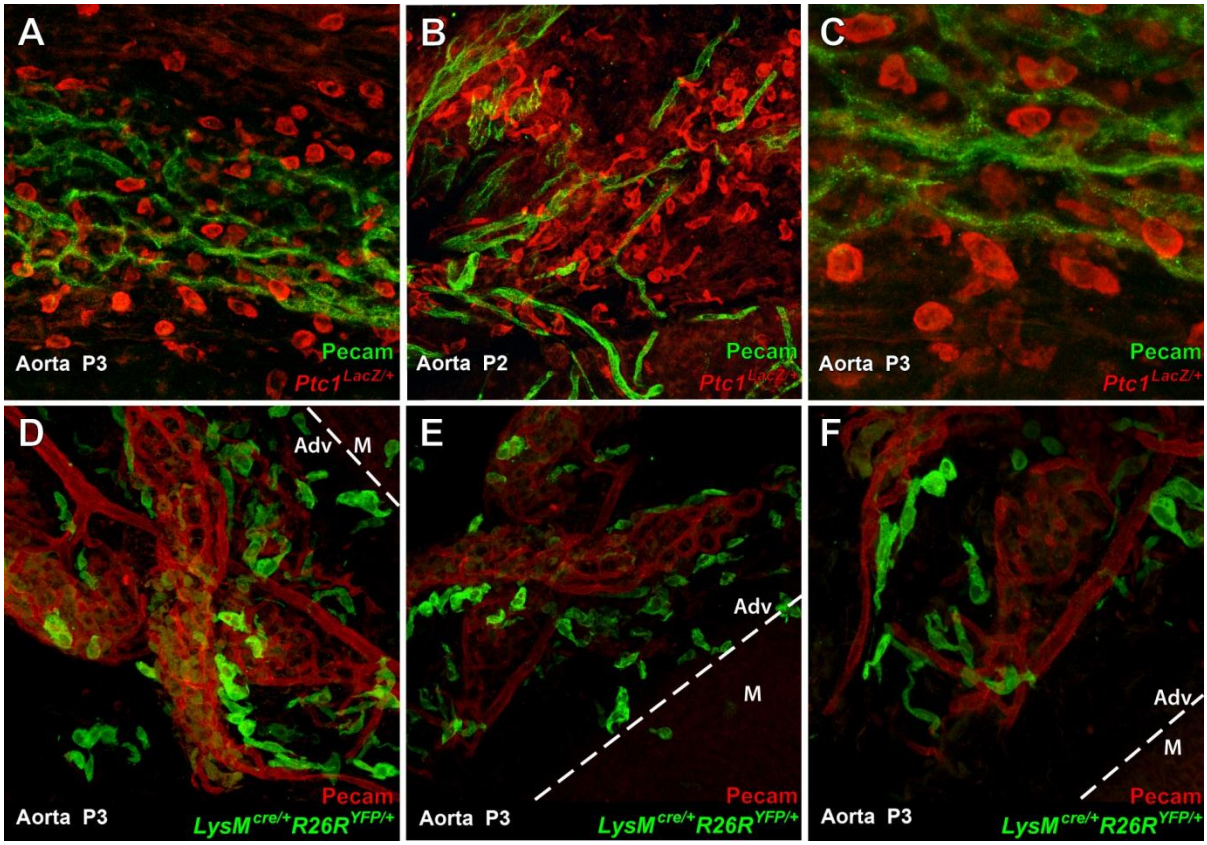


Figure 12: *Macrophages associate with the vasa vasorum*. A-C: postnatal day 3 aortas (P3) were whole mount immunostained for PECAM and β-galactosidase in *Ptc1*$^{LacZ/+}$ mice. Cells strongly positive for β-gal (most likely CD68-positive macrophages) are closely associated with adventitial microvessels (See Figure 11B). D-F:

postnatal day 3 aortas were whole mount immunostained for PECAM and anti-GFP in *LysM*$^{cre/+}$*/R26R*$^{YFP/+}$ mice. Macrophages positive for YFP immunostaining are closely associated with PECAM positive microvessels in the adventitia.

Discussion

Hedgehog Reporter Signaling in the Adventitia

The basic structure of the mammalian artery wall is well established at the time of birth. However, a period of very rapid growth and structural remodeling of major elastic arteries occurs during the first two weeks after birth. For example, the number of SMCs in aorta of the rat increases 2.5 fold, the thickness of the wall doubles, there is a 3 fold increase in collagen and elastin, and smooth muscle protein mass amasses correspondingly in the first month after birth[202, 203]. These changes in wall structure are adaptations to large increases in blood flow and blood pressures that occur in tandem with the growth of the animal during the first two to four weeks after birth. After three months of age, growth of the artery wall is completed, cell proliferation rates are low, and wall structure has reached an adult state. Passman et al. showed *Ptc1*$^{lacZ/+}$ activity in the aortic adventitia was highest from postnatal day 1 (P1) to P10, and diminished thereafter, thus correlating with rapid growth of all layers of the aorta including the adventitia during the perinatal period[25]. *Gli1*$^{LacZ/+}$ expression however, does not diminish with age and reporter activity remains high throughout the adult life of the animal.

The mechanism for why Gli1 continues to be robustly expressed in adulthood is still unclear. One study reported similar results that Ptc1 and Gli1 were differentially regulated. In a mouse model of pancreatic cancer, the researchers reported that during tumor progression, expression of Shh and Ptc1 decreased while Gli1 expression remained unchanged. Gli1 levels are still high, even with the ablation of Smo in the pancreatic epithelium, suggesting an uncoupling of Gli1 from canonical signaling. They went on to show that Gli1 was regulated by TGF-β and KRAS in this tumor model[204]. Further evidence shows that Gli1 may differ from other Hh reporter genes due to regulation by Hh-independent mechanisms. Other studies have implicated regulation of Gli1 by TGF-β. In the developing lung, knockout of TGF-β-receptor II in the mesoderm led to upregulation of Ptc1 and Gli1, with no discernible change in levels of Shh[205]. Dennler et al. reported that TGF-beta induces the expression of Gli1 and Gli2 in various human cell types, including normal fibroblasts and keratinocytes, as well as various cancer cell lines. According to their results, Gli2 induction by TGF-β is rapid, independent from Hh receptor signaling, and requires a functional Smad pathway. Gli1 expression is subsequently activated in a Gli2-dependent manner[195]. Other studies have reported negative regulation of Gli1 by Notch[194, 206]. Notch target Hes1, a negative regulator, binds Gli1 at the first intron, and may prevent transcription. In a model of glioblastoma, inhibition of Notch with γ-secretase inhibitors increased levels of Gli1 and activated Hh signaling[206]. Non-canonical signaling of Hedgehog pathway elements is a potential mechanism to account for our results. To test this, it would be informative to treat *Gli1*$^{LacZ/+}$ adult mice with TGF-β receptor inhibitors and examine levels of *Gli1*$^{LacZ/+}$ expression.

Spatially, the distribution of *Ptc1*$^{LacZ/+}$, *Ptc2*$^{LacZ/+}$, and *Gli1*$^{LacZ/+}$ expression patterns are not discernibly different. The adventitias of all large arteries are strongly positive, veins stain weakly, and lymphatic vessels are devoid of expression. Arteries as small as 18μm in diameter show expression of *Gli1*$^{LacZ/+}$ in the brain, mesenterics, uterus, hindlimb, and heart (Supplemental Figure 5). The hedgehog response reporter that differs in its expression pattern from these three is *Gli2*$^{LacZ/+}$. *Gli2*$^{LacZ/+}$ is expressed in both the adventitia and the media, while all other reporters of Hh activity never show staining in the media.

Gli2 has been reported to act as both a repressor and activator[207, 208]. In the absence of Hh ligand, Gli2 and Gli3 are processed to a truncated repressor form. When Hh signaling is activated, Gli1-3 are processed to the active form. A recent study has found that the proteasome processes Gli3 in the absence of Hedgehog pathway stimulation while Gli1 is completely degraded[115]. Gli1 is not processed because two of the three components of the processing signal are ineffective[115]. While this study did not focus on Gli2, it has been shown that Gli2 is a composite of positive and negative regulatory domains and functions as both an activator and repressor, similar to Gli3. In cultured cells, truncation of the activation domain in the C-terminal half results in a protein with repressor activity, while removal of the repression domain at the N-terminus converts Gli2 into a strong activator[116]. It is possible that the SMCs in the media are transcribing Gli2 in the absence of Hh signal as a constitutive repressor, while the adventitia is using Gli2 as an activator. The *Gli2*$^{LacZ/+}$ reporter mice will only be able to determine whether or not the gene

was transcribed. β-galactosidase will not be processed like Gli2 protein will, so we cannot use it as a readout of protein translation or protein modification.

. It is likely that Gli2 is being transcribed in response to a pathway other than Shh. It has been recently reported that Gli2 and Gli3 are transcribed in response to Notch1 signaling in neural progenitors[194]. It has also been shown that Gli2 is a transcriptional target of TGF-β signaling[195, 196]. Both of these pathways have been shown to be active in vascular SMCs to promote differentiation[50, 197, 198]. Further study will be required to determine the mechanism for Gli2 transcription in the medial smooth muscle cells.

There are a few possible experimental approaches to address this question. For one, it would be informative to do a western blot for Gli2 comparing the media to the adventitia. The repressive form of Gli2 is truncated, and will therefore produce a band that is a smaller size than Gli2-activator form. Additionally, ChIP-chip or ChIP-seq analysis would show binding targets for Gli2. These data would then be compared to gene expression data with RT-PCR for these targets to show whether Gli2 was acting to turn on or off these targets.

The limitation to these approaches is that they all rely entirely on a good antibody for Gli2. An antibody free approach to this question would be to make a mouse with a tagged Gli2. Epitope tagging Gli2 on the activator end would allow for biochemical approaches listed above to be performed with antibodies against the epitope tag. Additionally, a construct with different fluorescent tags (i.e. GFP and DS-Red) on both ends of Gli2 would allow visualization of which form of Gli2 was present in the cell type. Truncation of the activation domain in the C-terminal half

results in a protein with repressor activity, while removal of the repression domain at the N-terminus converts Gli2 into a strong activator[116]. If DS-Red were on the N terminus while GFP were on the C-terminus, Gli2 would be green when in its activated form, and red when in its repressive form based on cleavage of the protein.

It would also be informative to look at relative levels of Gli3 between the media and adventitia. Gli3 has most often been described as having the strongest repressive activity, and having the weakest activator function[117]. We predict that the tunica media would have higher levels of Gli3 than the adventitia, and that Gli2 and Gli3 are acting as repressors in SMCs to keep Hh signaling off. For the same reason, we would expect levels of Hedgehog Interacting Protein (Hhip) to be higher in the media than adventitia. Hhip is a target gene of Shh signaling, and acts as a negative regulator by competing for binding of Hh ligand with Ptc, but not transmitting a signal[209, 210].

Next Steps

A necessary next experiment is to verify whole mount confocal staining data with other methods like flow cytometry. There are many advantages to using whole mount confocal imaging in studying the adventitia. It shows the native architecture, and cell-cell physical interactions like clustering that are occurring, and individual cells are more clearly identified than in tissue sections. One disadvantage to this technique is that it is easy to misinterpret signals that are coming from 2 cells stacked on top of each other in the Z plane as co-localization of signal. It will be important to verify co-localization of cell-type specific markers with Hh reporters

using single cell dispersion techniques like flow cytometry. We can also adhere freshly isolated adventitial cells from reporter mice in single cell suspension to a coverslip with poly-L-lysine coating for immunofluorescence analysis.

Another important next step for understanding the role for Shh signaling in the adventitia is to identify target genes in each cell type. Shh has a broad range of targets, and it will be important to identify key factors for establishing and maintaining the adventitial niche. Starting with adventitial CD68-positive macrophages, we will isolate these cells by immunomagnetic selection or FACS. We will then isolate RNA for gene expression analysis by RT-PCR and microarray. These data will help identify candidates for potential niche factors. Candidate genes will most likely be secreted factors, as there are minimal cell-cell contacts between AdvSca1 cells and macrophages. These cells do cluster together, but do not appear to be binding to one another. Gene Ontology will help identify common functions of genes expressed in the CD68-positive cells, as well as gene expression patterns that mimic other well characterized cell types. These tools will be useful in identifying the factor or factors produced by the macrophages that support niche function.

In addition, we will culture these isolated macrophages and collect the conditioned culture medium. The conditioned medium will be used to treat isolated and cultured AdvSca1 cells to see if it suppresses differentiation compared to unconditioned medium. We can also subject the conditioned culture medium to ELISA analysis to look for the presence of the soluble factors found in the gene expression study.

Important variables to control in each of these experiments will be the age of the mice that we collect the macrophages and AdvSca1 cells from and the oxygen tension in the culture conditions. Because of the differences in expression of *Ptc1*$^{LacZ/+}$ in newborn versus adult mice, cells from these mice are going to have different gene expression profiles. Because AdvSca1 cells persist into adulthood and are not only around during the time of *Ptc*$^{LacZ/+}$ expression, it will be important to examine both time points to identify genes that are only expressed during development of the wall, and distinguish them from genes that are involved in maintaining homeostasis of the niche throughout the life of the animal.

Oxygen tension in culture conditions of both macrophages and AdvSca1 cells will be important to monitor as well. It has been shown that stem and progenitor cells are sensitive to oxygen levels and many stem cell niches are more hypoxic than surrounding areas[211-213]. While the *vasa vasorum* provides oxygen to the adventitia, oxygen levels in the adventitia are 10 mmHg lower than the endothelium of the normal rabbit aorta, although not the most hypoxic region of the wall[214]. *In vitro* culture conditions will likely be more hyperoxic than the adventitia *in vivo*, and will undoubtedly affect results. To remove oxygen tension as a variable from our culture conditions, it would be most effective to measure the partial pressure of oxygen in the mouse adventitia, and use that oxygen level in our culture conditions. If this is not possible, we will need to test both hyperoxic and hypoxic conditions to determine the effect of oxygen on our *in vitro* results.

Additional future directions are discussed in the following chapters.

CHAPTER 3

SONIC HEDGEHOG IN THE DEVELOPMENT OF THE ARTERIAL ADVENTITIA

Introduction

Much progress has been made in discerning the genetic and molecular pathways that govern vascular development. However, the majority of the work has been made towards understanding the early steps in vasculogenesis, arterial-venous specification, and patterning of the vasculature[20, 21, 215, 216], while studies toward elucidating the origins and development of the adventitia have been lacking. As a broad outline, vasculogenesis begins when vascular progenitor cells, called angioblasts, are specified by bFGF and BMP4 signaling in the primitive streak. These cells form blood islands that self-assemble into a primitive vascular plexus[217, 218]. A complex remodeling process ensues, and a functional embryonic circulation is formed from the newly fledged network of small arteries, veins, and lymphatic vessels [20, 218-220]. Closely following the remodeling of the endothelial plexus, smooth muscle cells and pericytes begin to invest in the wall (Supplemental Figure 2). Interactions between the endothelial cells and smooth muscle cells through the Notch signaling pathway and biomechanical stimulation from blood flow promote maturation of both cell types[20, 221, 222]. However, there is a gap in information

between these early steps in the development of the media and the mature multilayered vessel wall complete with adventitia.

Since the adventitia is only recently coming to light as an important niche environment, it is not surprising that little attention has been paid to its development. The goal in undertaking this work was to lay a foundation for future studies on origins of the adventitia and discerning the properties of the cells that live there. Knowing the context for how the cells came to reside in the adventitia, we can gain some insight into why they stay, and the factors involved in the maintenance of this critical niche. Additionally, understanding how an organ functions and develops normally can point to potential mechanisms for disease when these processes are misregulated or reactivated inappropriately.

Hypothesis

Shh plays a role in the development of the arterial adventitia and that response to hedgehog is a non-exclusive marker for cells predefined to be adventitia. Tracking these cells, we can observe the developmental timing and morphogenetic movements of the adventitia as it develops.

Materials and Methods

All antibody and reagent product numbers and sources are listed in Appendix B.

Mice

All animal protocols were approved for use by the Institutional Animal Care and Use Committee of the University of North Carolina and Seattle Children's

Research Institute. All mouse strains used were obtained from the Jackson Laboratories. Noon on the day of vaginal plug was designated as day 0.5. The day of birth was designated at P0. We used *Gli1*$^{LacZ/+}$ (*Gli1*^{tm2Alj}/J, Jackson stock number: 008211)[188], and *Shh*$^{EGFP/+}$(*B6.Cg-Shh*$^{tm1(EGFP/cre)Cjt}$/J, Jackson stock number: 005622)[91] mice for this analysis.

See Appendix C for strain specific genotyping information (Page 94). See Appendix D for mouse strain information (Page 96).

Whole Mount Immunostaining

Wild type females crossed to either *Shh*$^{EGFP/+}$ or *Gli1*$^{LacZ/+}$ male mice were sacrificed when embryos were at the stage designated. Embryos were removed from the uterus; their chest cavities were opened, then fixed in 4% PFA for 30 minutes at room temperature, and rinsed with PBS for 15 minutes. Thoracic aortas and carotid arties were then dissected out, as arterial tissues are less fragile when dissected after fixation.

Antigen retrieval methods vary based on the primary antibody used. See appendix B for list of antibodies and dilutions and notes on antigen retrieval conditions.

Samples were blocked at room temperature with 2% normal donkey serum (NDS) + 2% normal goat serum (NGS) + 0.1% bovine serum albumin (BSA) in PBS for one hour. Primary antibody was diluted in 2% normal donkey serum (NDS) + 2% NGS + 0.1% BSA in PBS and incubated overnight at 4^0C. Samples were rinsed in PBS for 15 minutes, and blocked again at room temperature for 1 hour in recycled

blocking solution. Secondary antibody diluted in 0.1% BSA in PBS was applied to the tissue for 2 hours at room temperature. Samples were then rinsed in PBS for 15 minutes. Hoechst dye (Molecular Probes) was diluted to 1µg/ml in H_2O, and applied for 1 minute. Tissue samples were washed briefly in H_2O, followed by a brief wash in PBS before storage in a 1:1 glycerol: PBS solution at 4^0C. Samples can be stored at these conditions protected from light for several months to a year with little to no loss of signal. Samples were imaged on a Leica SP5 Confocal Laser Scanning Microscope.

Xgal Staining

Embryos were fixed using 0.2% gluteraldehyde solution in a 0.1M sodium phosphate buffer pH 7.3 containing 2mM $MgCl_2$ and 5mM EGTA for 1 hour at room temperature. Samples were then washed three times for 30 minutes at room temperature with rinse buffer pH 7.3 containing 0.1M sodium phosphate, 2mM $MgCl_2$, and 0.1% Triton X100. Finally, tissue samples were stained overnight in a shaking incubator at 50 rpm and 37°C using X-gal staining buffer pH7.3, composed of 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 0.02M Tris-Cl, and 1mg/ml Xgal (Bioline) or Rose-β-D-gal (Biotium Inc.) diluted in the rinse buffer described above. Tissue samples were post-fixed in 4% PFA for 30 minutes at room temperature and stored in PBS.

Sectioning

Frozen sections were obtained by first fixing tissues in 4% PFA for 30 minutes at room temperature. Samples were then taken through a sucrose gradient in PBS at 4°C, with 10%, 15%, 20%, and 30% sucrose washes 2 hours each, followed by a 2 hour 1:1 30% sucrose in PBS:OCT wash. Samples were then oriented and embedded in OCT and frozen on dry ice and stored and cut at -20°C. Blocks can be stored at -20°C for 2-3 weeks. After this time, the OCT begins to dry out and sections are more brittle and prone to shredding. If blocks are older than a few weeks, melt, and re-embed in fresh OCT. Sections were cut at 6µm on a Leica Cryostat model CM3050S.

Paraffin sections were obtained by first fixing tissues in 4% PFA for 30 minutes at room temperature. Samples were then dehydrated by taking tissues through an ethanol gradient, with two 15 minute washes each for 30% EtOH + 0.9% NaCl, 50% EtOH + 0.9% NaCl, 70% EtOH, 95% EtOH, 100% EtOH. Samples were cleared by washing twice each with 1:1 100% EtOH: Histoclear (National Diagnostics) and 100% Histoclear for 15 minutes each wash. Tissue samples were next washed in 1:1 Histoclear: paraffin wax at 60°C for an hour, and finally incubated in clean wax at 60°C overnight and oriented and embedded. Sections were cut at 6µm.

Sections were counterstained with Nuclear Fast Red (ENG Scientific Inc.) for approximately 1 minute. Excess stain was washed off briefly in water. Slides were allowed to air dry before coverslipping with Mowiol (Polysciences) dissolved in in glycerol, prepared according to the manufacturer's instructions.

Results

Gli1$^{LacZ/+}$ Expression through Embryonic Development

Starting at e11.5, we examined *Gli1*$^{LacZ/+}$ expression every day through birth. Strikingly, we observe that there is a region of *Gli1*$^{LacZ/+}$-positive cells that lies adjacent to the vessel on the dorsal side from e11.5 through e14.5. Between e13.5 and e14.5, *Gli1*$^{LacZ/+}$ positive cells begin to appear on the ventral side of the wall. This pattern of expression begins in the thoracic descending aorta, followed by the carotid arteries. The aortic arch is void of *Gli1*$^{LacZ/+}$-positive cells until e16.5. The ductus arteriosus, and later the ligamentum arteriosum, never appears to fully be covered in Hh responsive cells (Supplemental Figure 3). The coronary arteries begin expressing *Gli1*$^{LacZ/+}$ between e16.5 and e17.5, and are fully covered by e18.5.

A
B
C
D
D
V
e11.5
E
F
G
H
D
V
e12.5
I
J
K
L
D
V
e13.5
M
N
O
P
D
V
e14.5
Q
R
S
T
D
V
e15.5
U
V
W
X
D
V
e16.5
Y
Z
A'
B'
D
V
e17.5
C'
D'
E'
F'
e18.5
G'
H'
I'
J'
P0

Figure 13: *Hedgehog signaling in adventitial development*. A time course of *Gli1*$^{LacZ/+}$ expression was performed through arterial development from e11.5-birth. *Gli1*$^{LacZ/+}$ expression does not appear on the ventral side of the aorta until e14.5 (P, black arrow). *Gli1*$^{LacZ/+}$ expression does not extend up into the ventral aortic arch until e16.5 (V, white arrow). Coronary arteries express *Gli1*$^{LacZ/+}$ starting between e16.5 and e17.5 (U, Y, white double arrow). Panel Z for e17.5 was stained with Rose-β-D-gal instead of X-gal, showing a magenta pigment instead of blue where β-gal is active.

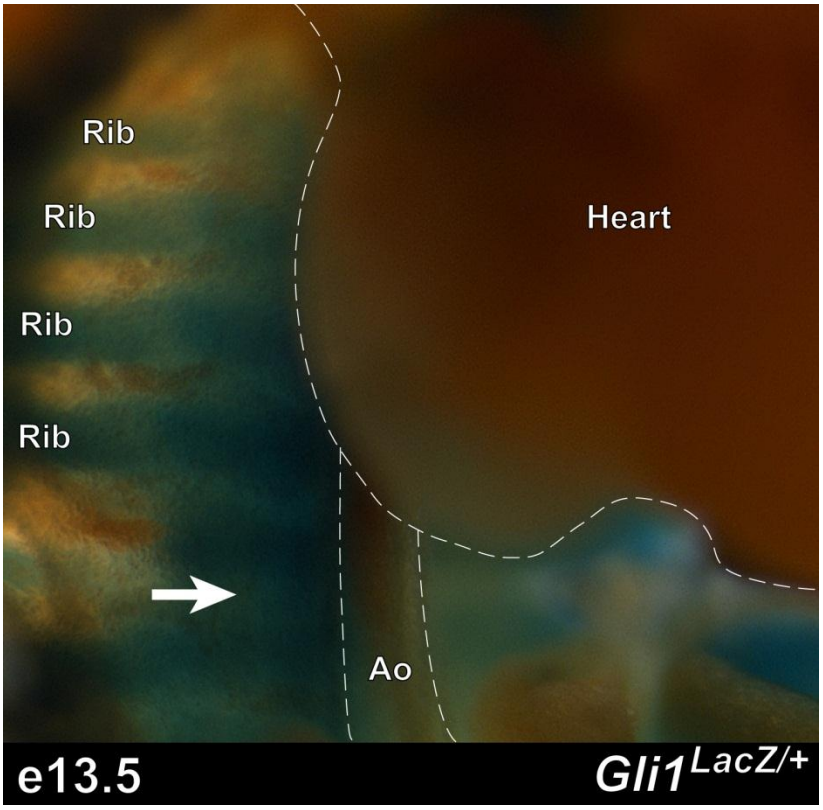


Figure 14: Gli1$^{LacZ/+}$ *positive mesenchyme lies dorsal to the aorta in development*. At embryonic day 13.5, ribs and the perichondrial

mesenchyme between them (arrow) express *Gli1*$^{LacZ/+}$. It is possible that this mesenchyme participates in making adventitia.

Shh Expression in Development

Similar to results with *Gli1*$^{LacZ/+}$, we observe that Shh expressing cells do not invest in the vasculature until around e14.5. At e12.5 and e13.5, nearly no *Shh*$^{EGFP/+}$-positive cells can be found in the aorta and carotids (Figure 15 A, B). By e14.5 however, *Shh*$^{EGFP/+}$-positive cells have invested into the outside of the wall of the descending thoracic aorta (Figure 15 D-F), but not the aortic arch (Figure 15C). At e15.5 and e16.5, the aortic arch becomes invested with Shh expressing cells (Figure 15 G-I). This is consistent with data from *Gli1*$^{LacZ/+}$ reporter mice (Figure 13).

Further analysis is required to verify the cell types that are *Gli1*$^{LacZ/+}$-positive and *Shh*$^{EGFP/+}$ positive in embryonic development. CD68-positive macrophages express Shh-N protein at e14.5 in the thoracic descending aorta (Figure 15F, arrows). AdvSca1 cells do not appear to express Shh protein in mid-late gestation (e15.5, Figure 17), despite expressing *Shh*$^{EGFP/+}$ in the perinatal time period. When AdvSca1 cells begin to express Shh is not yet known, but this analysis is in progress. It is possible that fibroblasts and/or other leukocytes are expressing Shh during development in addition to the CD68-positive macrophages. Adipocytes do not appear until late in gestation, so it is unlikely that these are a primary source of Shh in the development of the adventitia.

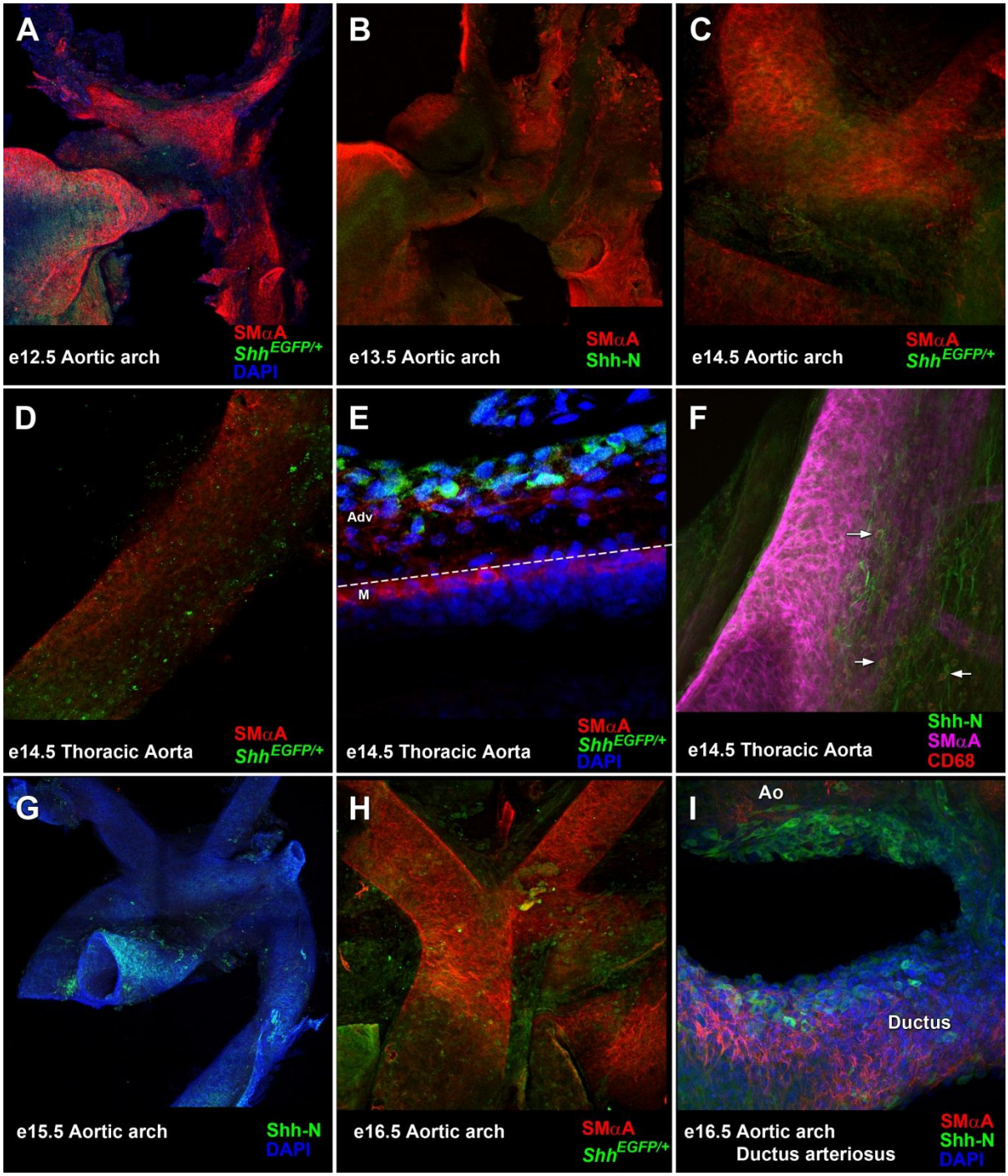


Figure 15: *Shh expression in arterial development.* Few Shh expressing cells are in the aorta at e12.5 and e13.5 (A, B). By e14.5, many Shh expressing cells have invested in the outer layers of the thoracic aorta wall (D, E, F), but not up in the aortic arch (C). The aortic

arch becomes invested in Shh-positive cells at e15.5 and e16.5 (G,H,I), with a concentration of Shh-expressing cells in the aortic root at e15.5 (G) and lesser-curvature of the aorta at e16.5 (I). See Figure 17 for more images of Shh protein distribution at e15.5.

AdvSca1 Cells in Development

Passman et al. reported that AdvSca1 cells were not found until around e16.5 [25]. This analysis was conducted using frozen tissue sections through the aortic root. Using whole mount confocal microscopy, we observe that AdvSca1 cells can be found in the anterior end of the descending thoracic aorta at least as early as e14.5 in a cluster on the dorsal side (Figure 16). Whole mount confocal microscopy is a useful tool for studying the adventitia to better appreciate the three dimensional architecture of this complex layer of the artery wall. This is especially true in development, as the adventitia's spatiotemporal development is not uniform across the whole vasculature.

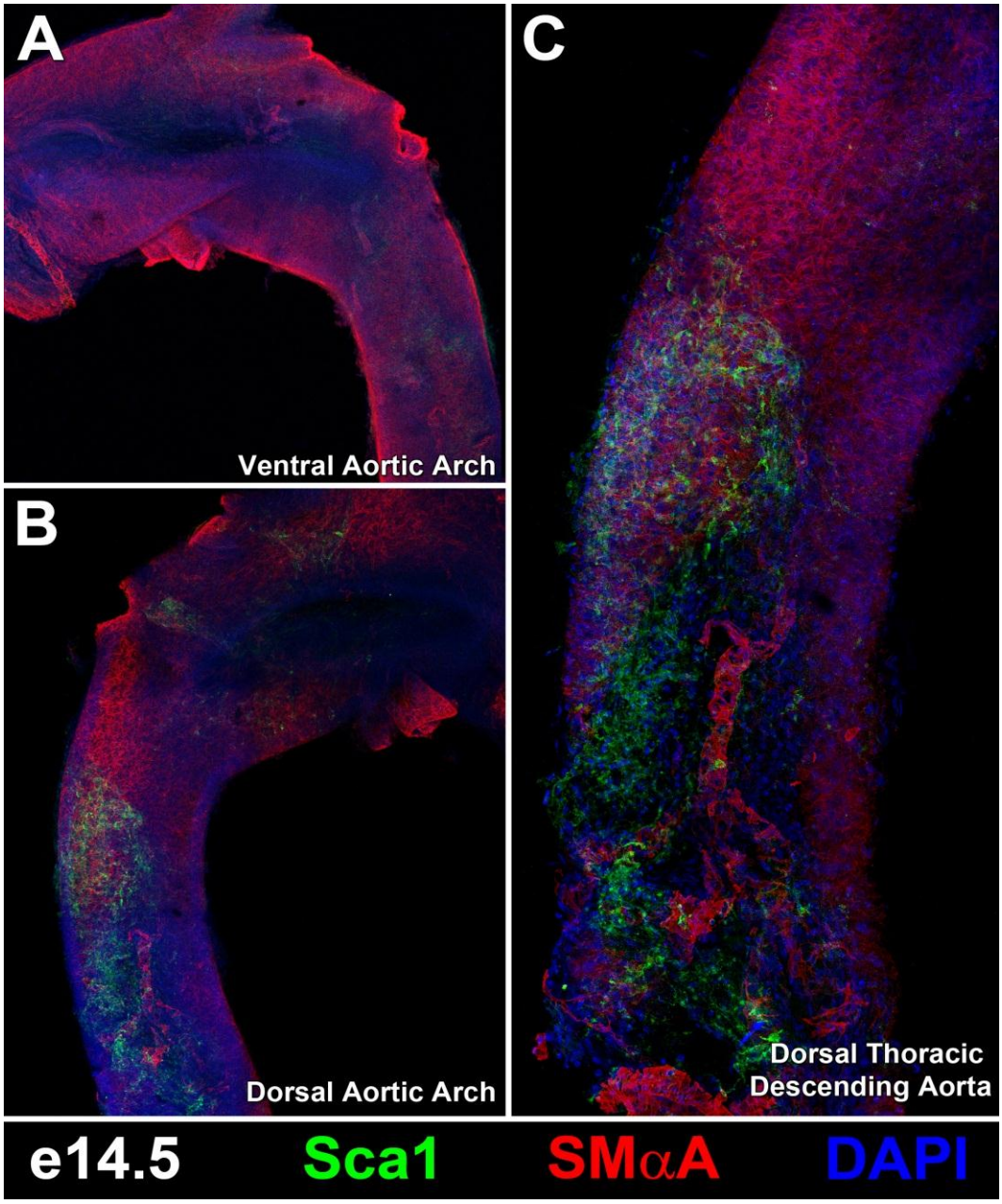


Figure 16: *AdvSca1 in development: e14.5.* AdvSca1 progenitors are located in a large cluster on the dorsal side of the thoracic aorta, with few detected cells on the ventral side, and some smaller clusters on the dorsal aortic arch.

At e15.5, we detect AdvSca1 cells distributed in a similar pattern to e14.5. We observe that they cluster together along the dorsal side of the thoracic aorta. These AdvSca1 cells are found within a region expressing Shh-N, although themselves are not Shh-N-positive at this time (Figure 17D). There are no AdvSca1 cells detected in the carotid arteries, aortic arch, or abdominal aorta at e15.5 (Figure 17).

At e16.5, AdvSca1 cells exist in scattered patches in the vasculature, with most being proximal to the heart, and enrichment for patches on the dorsal side of the vessels (Figure 18). There are many more clusters of cells at e16.5 than previously. No AdvSca1 cells were found posterior to the thoracic aorta. Unlike at e15.5, there are AdvSca1 cells located in the carotids, aortic arch, and several cells can be detected on the ventral side of the vasculature.

We also note that patches of AdvSca1 cells tend to be found in regions of branch points, although not in every occasion. One potential explanation for this is that regions of disturbed flow, such as branch points or the lesser curvature of the aorta, tend to be more hypoxic[223]. It has been shown that stem cells are sensitive to oxygen tension, and many niches tend to be more hypoxic[211-213]. AdvSca1 cells may also be sensitive to oxygen levels, and colonize more hypoxic regions preferentially. Further study will be required to test AdvSca1 cells sensitivity to oxygen.

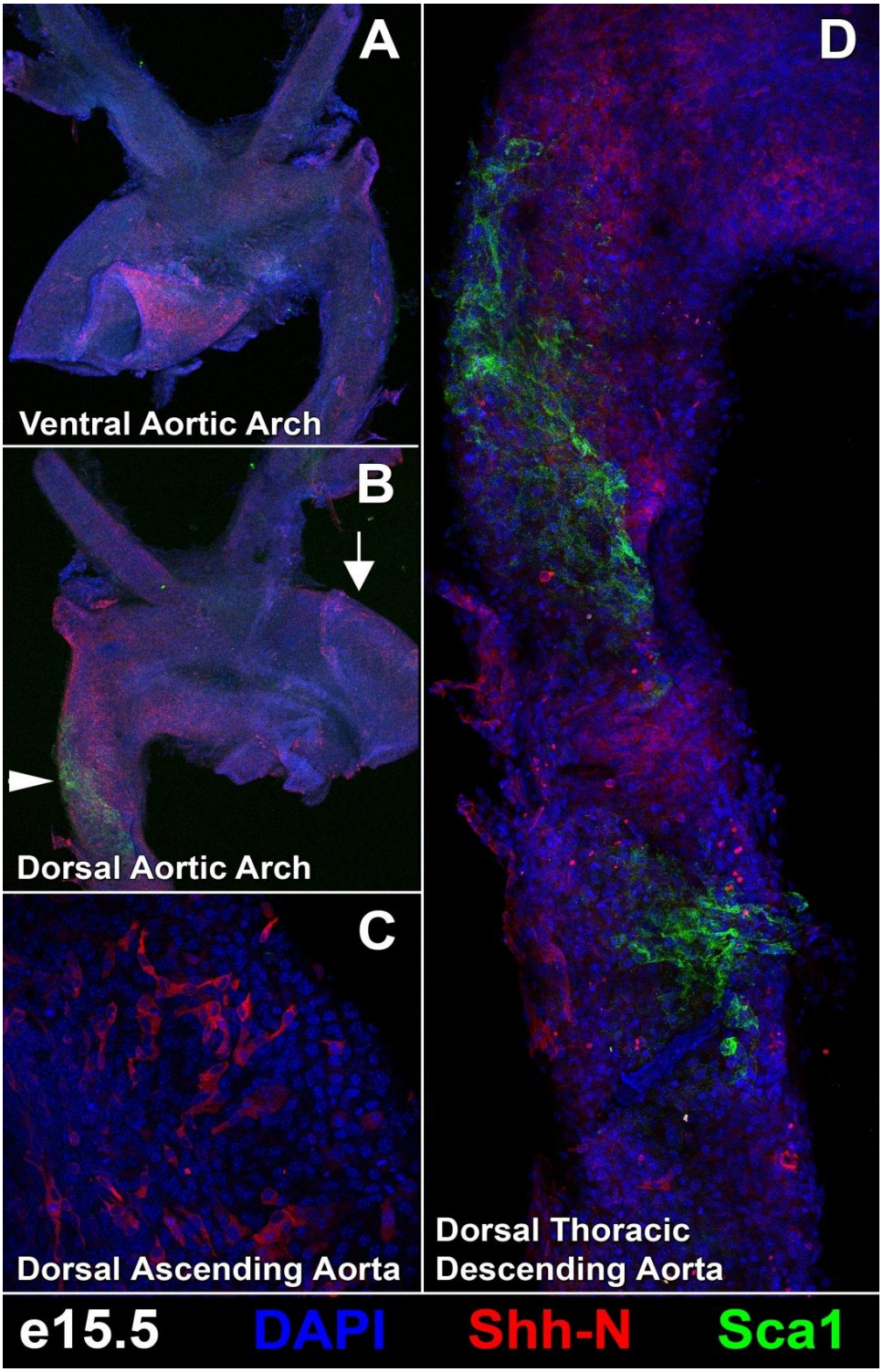


Figure 17: *AdvSca1 cells in development: e15.5*. AdvSca1 cells are found in a cluster on the dorsal side of the thoracic aorta at e15.5 in a region expressing Shh-N. Not all regions that express Shh-N have

AdvSca1 cells, as there are abundant Shh-N-positive cells in the ascending aorta. Arrowhead: region focused in panel D; Arrow: region focused in panel C.

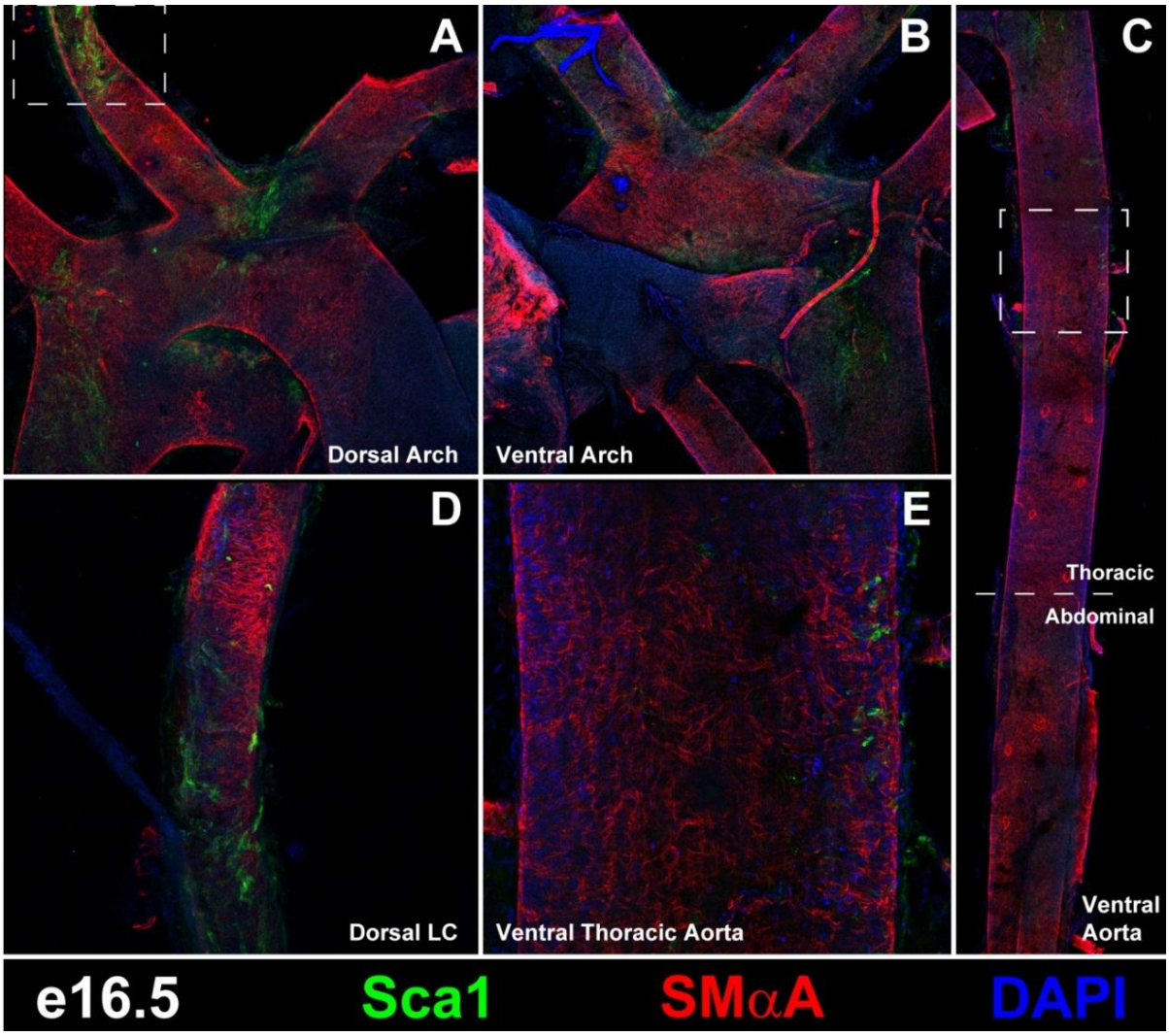


Figure 18: *AdvSca1 cells in development: e16.5.* Sca1-positive progenitors reside in the vasculature at e16.5, but are unevenly distributed. More AdvSca1 cells reside on the dorsal side of the aortic arch and carotids. Few AdvSca1 cells can be found on the ventral side of the thoracic aorta, and no detectable AdvSca1 cells reside in the

abdominal aorta at this time. Dashed square in A is shown in D. Dashed square in C is shown in E. Dashed line in E shows division between the thoracic and abdominal aorta.

Discussion

Vascular Wall Development

The molecular and genetic pathways that govern early vasculogenesis, arterial-venous specification, and vascular patterning have been well studied[147, 217, 221, 224]. Mechanisms that control the later stages of vascular development, and especially adventitial development, are still unclear. Understanding the cessation of the smooth muscle media formation program will be informative to the initiation of adventitia formation, as these two events occur at approximately the same time: around e14.5-e15.5 in the mouse[225].

Work by Wolinsky and Glagov showed that the number of layers of smooth muscle in the media of mature arteries is characteristic of species and vessel type, correlating to the normalization of circumferential wall stress[226]. As an example, the aorta of a mouse has 6-8 layers of smooth muscle and elastin, the rat has 8-10 layers, and human aortas have 40-50 layers[20]. This number of layers is not entirely contingent on body size, and does not change to accommodate changes in mass. This is best shown in a mouse model of gigantism where rat growth hormone under the metallothionein promoter (MtGH) was injected into the pronuclei of fertilized mouse eggs. These mice have an increase in body mass 1.8 fold over its wild type littermates, with proportional increases in the mass of internal organs[227]. To

accommodate increase in demand for blood flow, the aorta exhibited several structural changes from wild type including a 1.22 fold increase in lumen diameter, a 1.24 fold increase in wall thickness, and 30% more SMCs[228]. Despite the increase in the number of SMCs and the thickness of the wall, the number of layers of smooth muscle is unchanged in these mice[221, 228].

Insight into the mechanism to control the number of layers comes from mice hypomorphic for elastin. Knockout of elastin ($Eln^{-/-}$) is perinatal lethal, with the mice developing supravalvular aortic stenosis from over-proliferation and disorganization of SMCs to the point that they occlude the aorta[225]. Mice that are hemizygous for elastin ($Eln^{+/-}$) are viable, and have a 35% increase in the number of layers in the tunica media[229]. The extra layers begin to appear at approximately embryonic day 18.5 (E18.5) and are completed by birth[230, 231]. These ectopic layers are added from the adventitial side of the wall, preserving lumen diameter. Because these ectopic layers are added to the adventitial side of the media, it has been proposed that the ectopic SMCs come from differentiating AdvSca1 cells[184].

$Eln^{+/-}$ mice may represent a small window of time between e18.5 and birth that the artery wall has the potential to adjust the number of smooth muscle layers to normalize wall strain[221]. It is possible that this potential may still be retained by adult vessels to respond to injury, or is pathologically reactivated in situations like pulmonary hypertension[20, 184]. In pulmonary hypertension, hypoxia induces dramatic remodeling of the pulmonary artery. Pulmonary arteries of chronically (2 wk) hypoxic neonatal calves demonstrate marked adventitial thickening, excessive deposition of extracellular matrix proteins such as type I collagen, fibronectin, and tenascin-C,

augmented cell proliferation, myofibroblast accumulation/differentiation, cytokine production such as the active isoform of TGF-β1, and recruitment of circulating leukocytes and fibrocytes[184]. Further study is necessary to pinpoint the factors responsible for maintaining or reactivating plasticity of the artery wall to respond in this fashion. Identification of these pathways will be useful therapeutic targets to inhibit in obstructive arterial disease, or reactivate in medial dissection. These pathways will also likely be informative of the regulative mechanisms controlling the proliferation and differentiation of AdvSca1 cells.

Lineage of AdvSca1 Cells

The developmental origin and lineage of the adventitia, and specifically AdvSca1 cells, is still unclear. Circulating bone marrow derived progenitor cells have been suggested to contribute to neointimal lesion formation. While recent work debates whether this is accurate[232, 233], bone marrow is a potential source of AdvSca1 cells. Hu et al. examined this possibility by reconstituting bone marrow from lethally irradiated wild type mice with genetically labeled *Rosa26*$^{LacZ/+}$ marrow. Six months after bone marrow reconstitution, they examined the cells in the adventitia, and did not find any β-gal positive AdvSca1 cells. Further work from our lab showed that AdvSca1 cells were not labeled by Tie2$^{cre/+}$ mice[40]. Tie2 labels cells of hematopoietic or endothelial origin[234].

Work from our lab further investigated potential lineages for AdvSca1 cells[40]. There are several known origins of vascular smooth muscle cells[235]. Using these

known lineages, our lab used lineage tracing cre mice to determine if AdvSca1 cells were also derived from one or more of these lineages.

Cardiac neural crest cells contribute to the aortic arch and common carotid artery smooth muscle, as well as the neurons of cardiac innervation[224, 235]. They also mediate the complex remodeling of the symmetrical pharyngeal arch arteries into the aortic arch, carotid, and subclavian arteries, as well as septation of the outflow tract into pulmonary and systemic circulation[224]. Additionally, neural crest derived melanocytes (chromatophores) can be found in the frog adventitia (Supplemental figure 4), unlike in mammals. It has been shown that Indian Hedgehog is required for proper migration of *Xenopus* neural crest cells[236]. Neural crest derived cells can be labeled and traced in the mouse using Wnt1^{cre} crossed to R26R. Cross sectional analysis of these mice reveals that the adventitia and AdvSca1 cells do not derive from neural crest.

Somite derived paraxial mesoderm contributes to the smooth muscle of the thoracic aorta. Paraxial mesoderm contributes to several lineages sclerotome (cartilage), myotome (skeletal muscle), syndotome (tendon), dermatome (dermis), and endothelial cells[237]. Work from our lab tested *Nkx3.2*$^{cre/+}$ to label somite derived cells, and did not detect any in the adventitia[40]. Other somite derived paraxial mesoderm cre mouse lines include *Tbx6*$^{cre/+}$ [238] and *Meox1*$^{cre/+}$ [239]. These could be informative of adventitial origins.

Wilm's Tumor 1 cre (*WT1*$^{cre/+}$), which is a marker of cells derived from the mesothelium was also tested, and the adventitia was not labeled.

Other possibilities for lineages for AdvSca1 cells are anterior heart field, splanchnic mesoderm, mesoangioblasts, yolk sac, and foregut. Anterior heart field (*mef2c*$^{cre/+}$), splanchnic mesoderm, and mesoangioblasts have all been shown to contribute to vascular smooth muscle diversity[235], and splanchnic mesoderm in particular also contributes to the myocardium and endocardium of the heart[237, 240]. Yolk sac derived macrophages are involved in angiogenesis in the brain during embryogenesis[170], and contribute to hematopoiesis[241]. It is possible that the yolk sac may provide other cells as well. A recent study by Samokhvalov and colleagues using lineage tracing of Runx1-positive cells (yolk sac derived) found labeled cells that surrounded the dorsal aorta at e11.5 [241]. The study did not go later in development than e11.5, but these cells could possibly contribute to adventitia. Foregut contributes to lung and esophagus: both Hh responsive structures in close contact to the dorsal aortic arch. Further studies will be required to determine the developmental origin of the AdvSca1 cells.

Next Steps

We observe that *Gli1*$^{LacZ/+}$ begins to be expressed on the outer ventral part of the aorta wall between e13.5 and e14.5. We hypothesize that the Hh responsive cells on the dorsal side of the aorta migrate around ventrally at this time. The alternative explanation is that mesenchymal cells already in the ventral part of the wall activate Gli1 de-novo during this time window.

To address this, we will use *Gli1*$^{creERT2/+}$, a tamoxifen inducible cre recombinase[192]. Using Rosa 26 cre reporter strains (R26R), that include a reporter

gene like LacZ behind a stop codon flanked by two LoxP sites, we can irreversibly activate the reporter in Gli1 expressing cells at a specific time in development, and observe where those cells and their progeny end up later in development. We will treat pregnant R26R females crossed to male *Gli1*$^{creERT2/+}$ mice with tamoxifen by oral gavage at time points between e10.5 and e13.5 to label the mesenchyme dorsal to the aorta. Then we will examine newborn pups for expression of the reporter.

It will also be informative to do lineage tracing of Shh producing cells with *Shh*$^{creERT2/+}$, another tamoxifen inducible cre recombinase[91] as some of the cell types that are positive for *Gli1*$^{LacZ/+}$ also appear to be positive for *Shh*$^{EGFP/+}$. We will treat pregnant R26R females crossed to male *Shh*$^{creERT2/+}$ mice with tamoxifen by oral gavage or IP injection at time points between e10.5 and e13.5 to label Shh expressing cells. Then we will examine newborn pups for expression of the reporter. These results will be compared to *Gli1*$^{creERT2/+}$*/R26R*$^{LacZ/+}$ labeled with tamoxifen at the same time points.

An alternative approach to determining if Hh responsive cells migrate around ventrally from the dorsal side of the aorta would be to use transgenic zebrafish for *Shh*$^{GFP/+}$ and *Gli1*$^{GFP/+}$ and time lapse microscopy[242, 243]. This way we can view movements of *Gli1*$^{GFP/+}$-positive or *Shh*$^{GFP/+}$-positive cells as they happen. Time-lapse microscopy with live zebrafish embryos is a common technique and a useful attribute of zebrafish as a model system[244].

The zebrafish circulatory system has been well studied, and many transgenic models are available to visualize blood vessels in live embryos, such as the *Fli1*$^{GFP/+}$ and *Flk1*$^{mCherry/+}$ fish that labels endothelial cells[245]. Vasculogenesis begins early in

the zebrafish, with angioblasts beginning to form around 14 hours post fertilization. At 17 hours, the angioblasts are already expressing markers of arterial/venous specification. The longitudinally aligned major axial vessels of the trunk, the dorsal aorta, and posterior cardinal vein, become patent and blood conducting about 30 hours post fertilization. The axial vessels are linked by dorsoventrally aligned intersegmental arteries and intersegmental veins, nearly all of which are perfused by 3 days post fertilization[245]. Zebrafish embryos lacking Hh signaling display defects of the primary intersegmental vessel sprouting[242]. Shh appears to promote the maturation of blood vessels by regulating the levels of angiopoietin-1 and -2 and VEGF[242]. It is in this second day post fertilization between the formation of the major axial vessels and perfusion of the intersegmental arteries around day 3 as the dorsal aorta is maturing that we would monitor with time lapse imaging for *Shh*$^{GFP/+}$ and *Gli1*$^{GFP/+}$ expression.

An obvious first step to this experiment however, is to determine if zebrafish express markers of Hh signaling in the adventitia of the dorsal aorta. Zebrafish have 2 homologs of the Shh gene, Sonic Hedgehog (Shh A) and Tiggy-Winkle Hedgehog (twhh) (now named Shh B)[246]. Zebrafish also express 2 different Indian Hedgehog genes (Ihha and Ihhb), as well as Desert Hedgehog[247]. An analysis of all 5 Hh genes and the other Hh pathway elements (Ptc, Smo, Gli1-3) will be important to verify before proceeding with time lapse video capture.

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

Summary of Results

1. The adventitia is a remarkably dynamic and diverse niche community of cell types that interact through a number of different signaling pathways, with one major player being Shh.
2. We have identified that CD68-positive tissue resident macrophages, Perilipin A-positive adipocytes, and Sca1-positive adventitial progenitor cells all participate in making and responding to Shh signal, as shown using transgenic reporter mice.
3. Hedgehog responsive cells appear on the ventral side of the developing artery wall between e13.5 and e14.5 in the mouse, and are present on the dorsal side at least as early as e11.5, although these cells may be a part of the perichondrium, and are not integrated into the wall.
4. AdvSca1 cells are present in the adventitia at least as early as e14.5, and are found in clusters that are enriched on the dorsal side of the aorta.

5. There is a rapidly growing microvascular network in the adventitia and periadventitial adipose tissue that closely associates with Hh responsive cells, and resides in a Shh rich matrix.

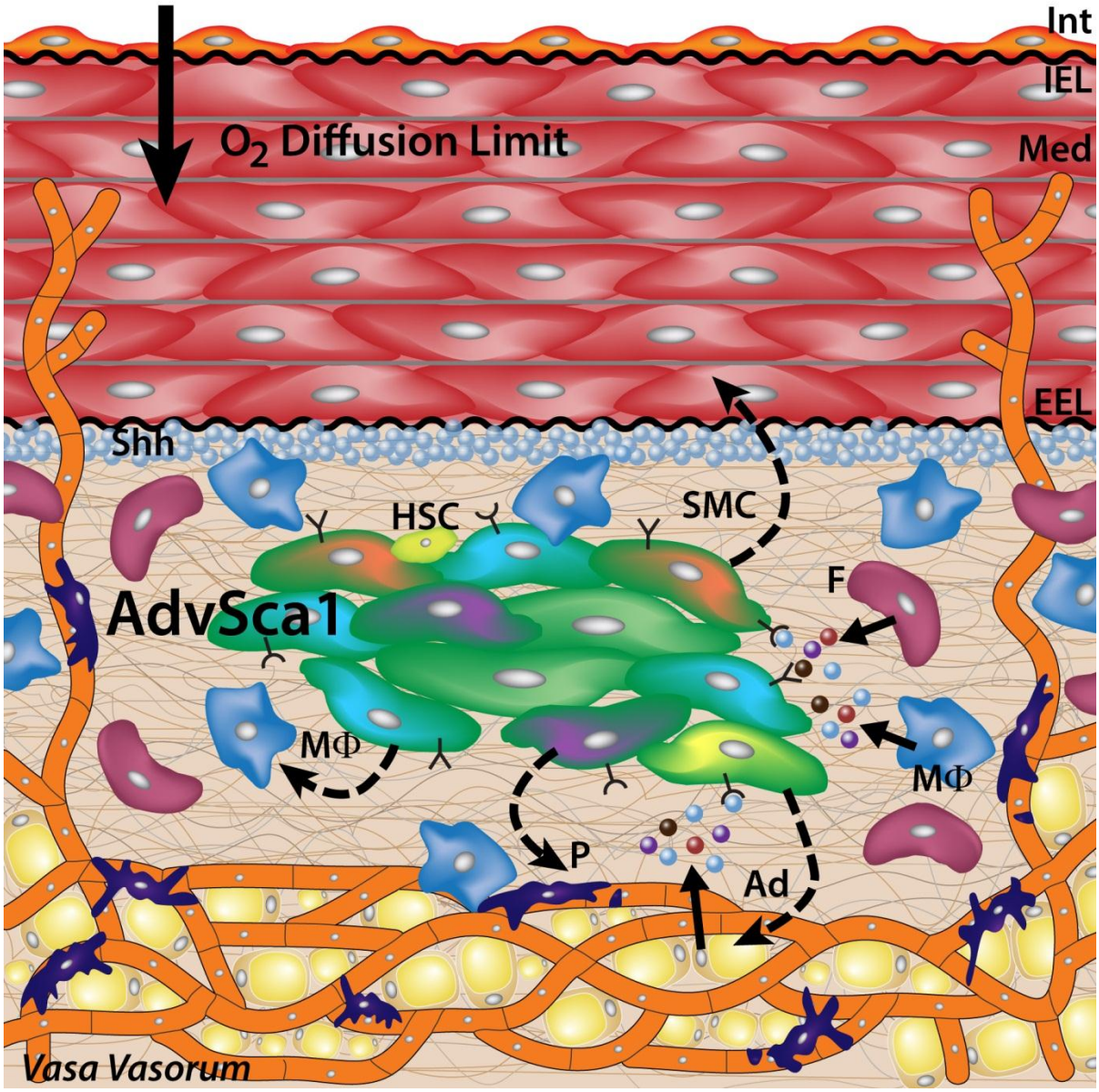


Figure 19: *Model of the adventitial niche*. Adventitial Sca1 positive progenitor cells are a heterogeneous population (Green cells with different tinted centers) with the ability to differentiate into many different cell types (dashed arrows). Some are poised to become

smooth muscle cells (red) to repair and replace aging SMCs in the tunica media (SMC, green cells with red tinted center). Others are poised to become pericytes (dark purple) that support the *vasa vasorum* (P, green cells with purple tinted center). Some are poised to become monocyte/macrophages (MΦ, green cells with turquoise tinted center)[27]. Still others are poised to become adipocytes (Ad, green cell with yellow tinted center). Additionally, the adventitia provides growth factors (Solid arrows, blue, red, purple, brown spheres) and ECM components (grey and brown lines) from fibroblasts (F, light purple cells) tissue resident macrophages (MΦ, blue cells) and adipocytes (Ad, light yellow cells) that build and maintain the AdvSca1 niche. In addition to AdvSca1 cells, the adventitia supports a small population of hematopoietic stem cells (HSC, yellow-green cells)[26-28]. Another important feature and function of the adventitia is the *vasa vasorum* (orange) that provides oxygen and nutrients to the outer layers of the tunica media that cannot receive it by diffusion from the lumen of the vessel.

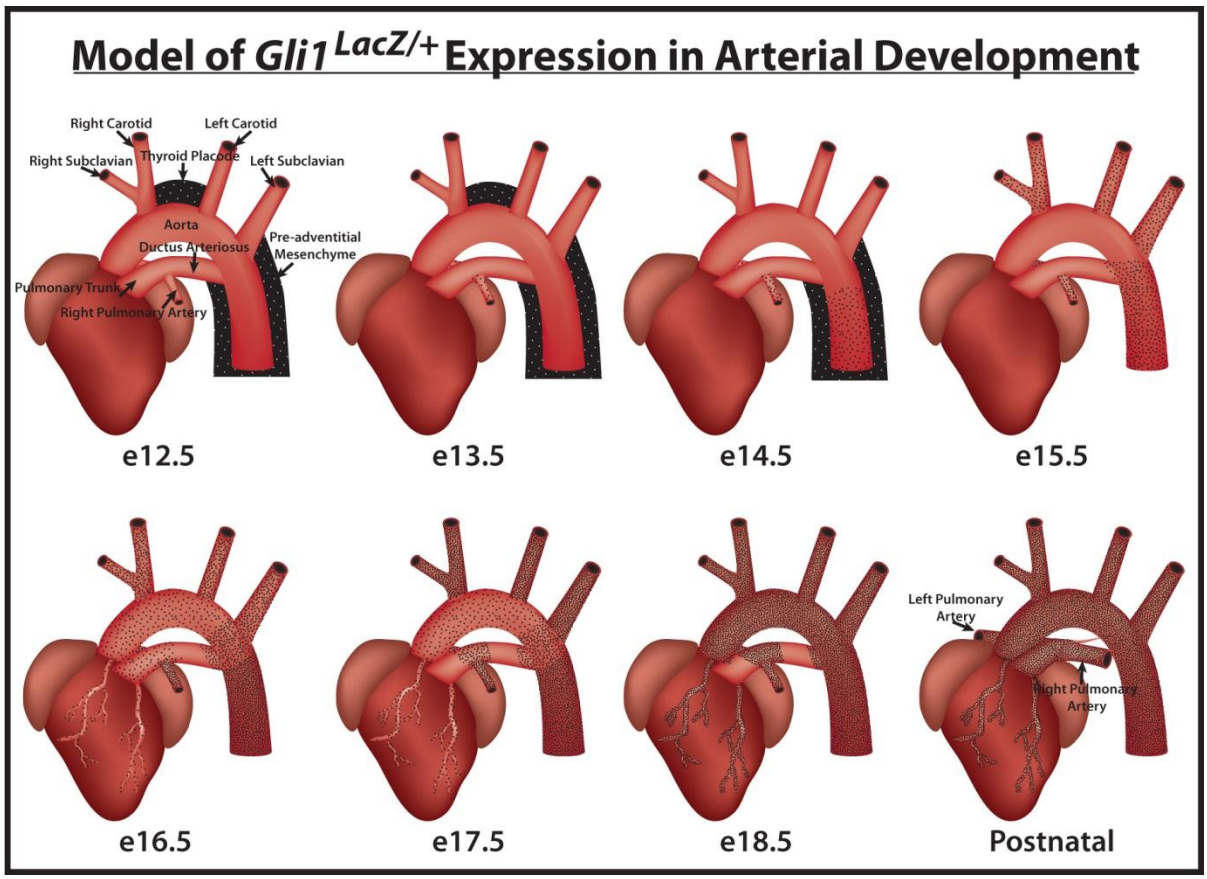


Figure 20: *Model of* Gli1$^{LacZ/+}$ *expression in arterial development*. We hypothesize that *Gli1*$^{LacZ/+}$ expression patterns reflect the development of the adventitia. Hh responsive “pre-adventitia” from the mesenchyme adjacent to the dorsal side of the developing vasculature begins to migrate ventrally sometime between e13.5 and e14.5 to cover the entire vasculature by birth, with the exception of the ductus arteriosus/ligamentum arteriosum.

Future Directions

Determine the Heterogeneity of the Advsca1 Population and Classify Subpopulations

It is likely that the marker Sca1 labels a heterogeneous population of cells. *In vitro* studies of isolated AdvSca1 cells show that roughly ¼ of the cells retain their Sca1 phenotype, ½ differentiate into SMCs, and the remaining ¼ lose Sca1 expression but do not detectably express smooth muscle markers in serum containing medium without supplement of additional growth factors[20, 25, 40]. Growth factor stimulation of AdvSca1 isolated cells only produced a small percentage of osteogenic or adipogenic cells[20, 25]. Psaltis et al. found that aortic adventitial cells generated a broad spectrum of multipotent and lineage-specific hematopoietic colony-forming units, with a preponderance of macrophage colony-forming units[27]. We hypothesize that Sca1 represents a heterogeneous population of progenitors with limited potentials and not a homogeneous population of multipotent cells.

To test this hypothesis, we would use single-cell fluorescence activated cell sorting (FACS) to grow up clones of single AdvSca1 cells. Once sufficient quantities of clones have been grown up, we will test the potential of each clone to produce the lineages we know the AdvSca1 population as a whole is capable of becoming by treatment of cells from each clone with growth factors: PDGF-BB for SMCs[23], insulin + dexamethasone + isobutylmethylxanthine for adipocytes[248], BMP-2 + heparin sulfate for osteocytes[249], VEGF + bFGF + endothelial cell growth supplement for endothelial cells[250], and CSF-1 for macrophages[251]. Multipotent cells should be able to respond to each treatment and become each cell type tested. Data from

Campagnolo et al. show that AdvSca1 cells from the human saphenous vein can be cloned with a success rate of about ~2% of plated cells proliferating sufficiently to make clones. They find that clones are plastic and can differentiate into various mesenchymal cell types, and additionally β-tubulin III-positive nerves[39].

Culture conditions will be important to test in this analysis. Howson et al. found that AdvSca1 cells can form suspended spheroids in serum-free medium supplemented with bFGF, but when serum is added cells stick down to the culture dish and differentiate into SMCs[41]. Passman et al. confirmed that about half of AdvSca1 cells cultured in serum become SMCs, but ¼ of the cells retain Sca1[25]. It will be important to ensure that this subpopulation of cells are not selected for when growing clones as they will not be representative of the whole population of AdvSca1 cells.

Additionally, these experiments will be repeated under hypoxic conditions to see if hypoxia shifts the potential of individual AdvSca1 cells. It has been shown that Sca1-positive cells become more adipogenic when pre-exposed to hypoxic conditions (2% oxygen) before switching to normoxic conditions (21% oxygen)[213]. Hypoxia also enhanced the ability of Sca1-positive adipose tissue derived mesenchymal stem cells to proliferate in culture[213].

Clones will also be tested for gene expression profile differences. If a particular clone is predisposed to make a specific cell type, we hypothesize that it would also have differences in gene expression and epigenetic marks that other clones will not. Microarray analysis will be useful for this study to analyze gene expression as well as to find markers that are specific for sub-populations of

AdvSca1 cells. Additional cell-type specific cell-surface antigens are needed to verify purity of AdvSca1 isolations, select a specific population for study, better understand niche interactions, and profile the relative ratios of each subpopulation. Ratios of subpopulations may be different in disease situations, and may serve as useful biomarkers for disease risk or indicators of disease severity. Psaltis et al. found that in lesion free APOE-knockout mice, there were a greater percentage of macrophage colony forming AdvSca1 cells than wild type mice[27].

Alternatively, if AdvSca1 cells are not amenable to growing in culture, we can sort single cells into 96 well plates, and test each plate for the percentage of single cells that can differentiate into the cell type of choice when subjected to growth medium. While this method is not ideal, the proliferative capacity of AdvSca1 cells may be limiting to clonal analysis.

As an alternative approach to studying gene expression in AdvSca1 cells if clonal analysis does not work to isolate sufficient quantities of RNA by standard methods, we would use single cell RNA isolation and gene expression analysis on a cell by cell basis. This technique is being optimized by several groups[192, 193], and kits are becoming available from a few companies to perform this technique at the bench (Life Technologies, Signosis).

Determine a Role for Shh Signaling in Macrophages in the Development of the *Vasa Vasorum*

The *vasa vasorum* is an important part of maintaining the adventitial niche. Removal of the adventitia from rabbit carotid arteries results in formation of a

hyperplastic intimal lesion[79]. Similarly, removal of rat abdominal aortic adventitia shows an initial severe trauma of the endothelium and intimal hyperplasia as early as one month after injury[80]. The researchers in both of these studies suggested that this response was due to the removal of adventitial microvessels and the ensuing hypoxia in the artery wall. Hypoxia has been shown to enhance adipogenesis in Sca1 cells[213], implicating the *vasa vasorum* as an important component of the adventitial niche.

CD68-positive macrophages are very strongly positive for all of our hedgehog reporter mice, including $Shh^{EGFP/+}$, $Ptc1^{LacZ/+}$, and $Gli1^{LacZ/+}$, indicating that they both make and respond to Shh. These cells closely associate with microvessels, and other recent studies have shown that macrophages play a key role in anastomosis of tip cells in angiogenic vessel plexuses[170]. We hypothesize that Shh signaling, particularly in these resident macrophages, induces the release of growth and angiogenic factors that promote increases in mass of the adventitia and formation of *vasa vasorum* microvessels in the perinatal period *in vivo*.

To test this hypothesis, we would first disrupt Shh signaling and monitor effects on overall growth of the adventitia in the perinatal period, and on development of the *vasa vasorum* within this layer of artery wall. Shh signaling would be disrupted using two approaches *in vivo*. First, we would genetically disrupt signaling using the cre-lox system to target hedgehog signal transduction in macrophages specifically. Mice deficient in the Shh signaling receptor smoothened (Smo) are unable to respond to any of the vertebrate Hh ligands. We would use $Smo^{flox/flox}$ mice[252] crossed with macrophage-specific cre mice ($LysM^{cre/cre}$)[253] to

inhibit macrophages from being able to respond to Hh signal. As an alternative approach, we would disrupt smoothened signaling pharmacologically with the well-studied inhibitor of smoothened signaling, cyclopamine and determine effects on perinatal growth of the adventitia and formation of adventitial microvessels of the *vasa vasorum.*

Conversely, we would look at how constitutive activation of smoothened in these cells affects the growth of the adventitia. We hypothesize that activity of Shh drops to a minimal baseline level of activity after the perinatal period of about 10-14 days after birth, based on data from $Ptc^{LacZ/+}$ mice. Using the mouse line that puts an activated form of smoothened behind a floxed stop codon under the control of the ubiquitous Rosa26 promoter[254], we can constitutively activate smoothened in macrophages when we cross them to the $LysM^{cre/cre}$ mice. Additionally, we can apply recombinant Shh protein to the adventitia in adult mice, as an alternative approach, although this is less specific to macrophages.

Preliminary data

Initial characterization of the $LysM^{cre/+}/R26R^{YFP/+}$ mice reveal no recombination in AdvSca1 cells, adipocytes, or medial SMCs (Figure 21 D, E, F). Minimal recombination occurs in activated macrophages that are Mac2-positive, but this population contributes to a minimal percentage of overall adventitial cells (Figure 21 C). We also observe about 70% recombination in CD68-positive adventitial resident macrophages (Figure 21 A,B). This mouse line will serve as a very useful tool for studying this population of cells.

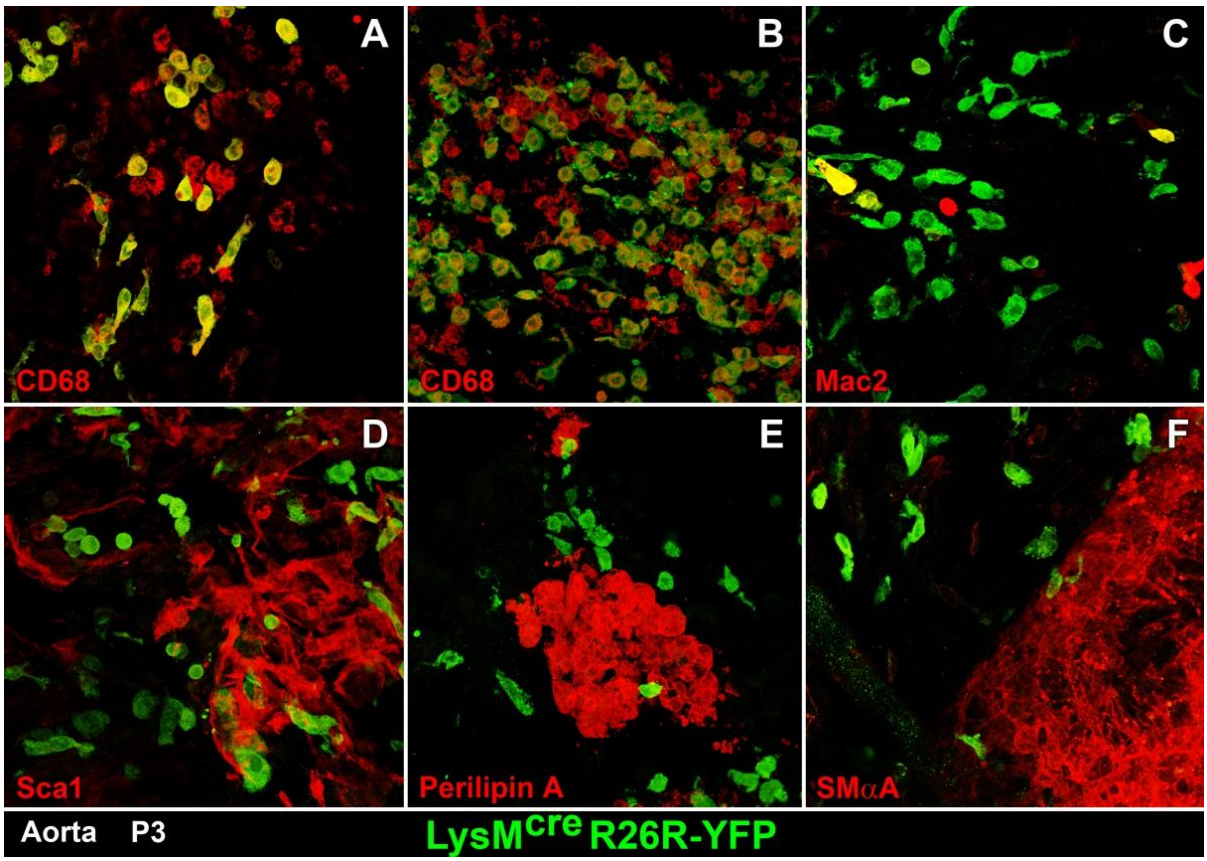


Figure 21: *Characterization of* LysM$^{cre/+}$/R26R$^{YFP/+}$ *mice*. Cre expression and recombination occurs in about 70% of CD68-positive macrophages (A, B), and a few Mac2-positive activated macrophages (C). No recombination is detected in AdvSca1 cells, adipocytes, or smooth muscle (D, E, F).

In a first round of breeding of *LysM*$^{cre/+}$*/Smo*$^{flox/flox}$ mice, we observe that these mice are viable, and are born at expected Mendelian ratios. There are no obvious gross morphological differences between wild type and mutant mice. It is possible that the differential phenotype of these mice will only be seen when aggravated with

an injury or disease stimulus. It is also possible that the wild type macrophages that escape recombination are able to compensate for the lack of normal hedgehog signaling response of the mutant cells. Further characterization is required to determine the effect of this mutation.

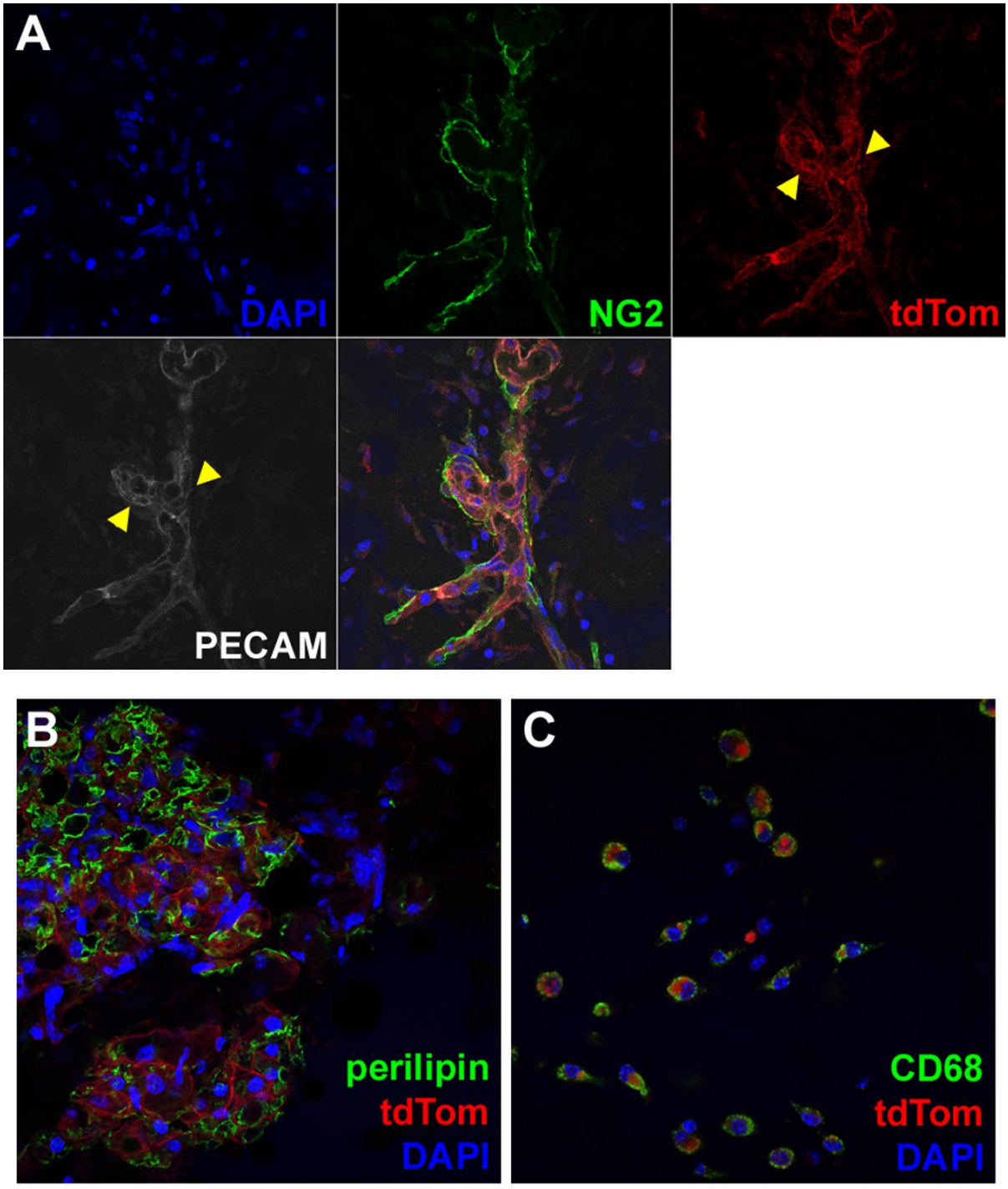
A recent study by Schumacher and colleagues made *LysM*$^{cre/+}$/*Smo*$^{flox/flox}$ mice to examine inflammation in response to gastric bacterial infection[176]. While they were not interested in angiogenesis in these mice, they reported that macrophages use Shh secreted by the stomach as a chemoattractant to respond to infection. *LysM*$^{cre/+}$/*Smo*$^{flox/flox}$ mice had reduced levels of inflammation in response to infection by *H pylori* bacteria[176]. These data suggest that response to infection and injury in these mice will be impaired. It is possible that inflammation in response to vascular injury such as a hindlimb ischemia model, balloon injury, or carotid ligation would be reduced in *LysM*$^{cre/+}$/*Smo*$^{flox/flox}$ compared to controls.

Data from Fantin et al. hint at possible mechanisms for why the phenotypes of this mouse model are so subtle. In studying the role of macrophages on angiogenesis in the brain, they crossed *LysM*$^{cre/cre}$ mice to R26R-diptheria toxin mice, thus specifically killing LysM-positive cells. They found that angiogenesis in the brain was unaffected, reporting that LysM-negative macrophages derived from the yolk sac were responsible for brain angiogenesis[170]. The authors pointed to a study that used macrophage deficient mice in studying footplate remodeling, as it has been shown that macrophages clear the synchronized apoptotic cells between digits[255]. Neighboring mesenchymal cells were able to clear the debris of the apoptotic cells in the absence of tissue macrophages, albeit less efficiently[255]. Fantin

et al. speculate that the angiogenic effects of macrophages might be compensated for by other cell types in mice that lack macrophages[170].

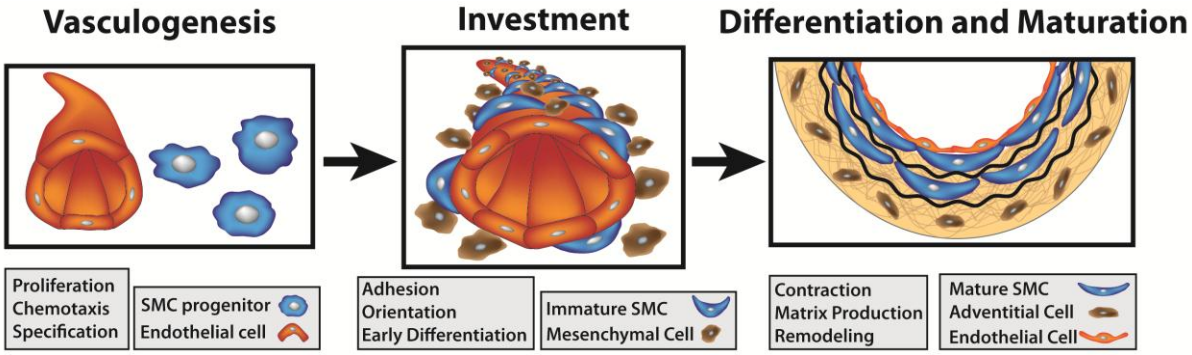
APPENDICES

Appendix A: Supplemental Figures

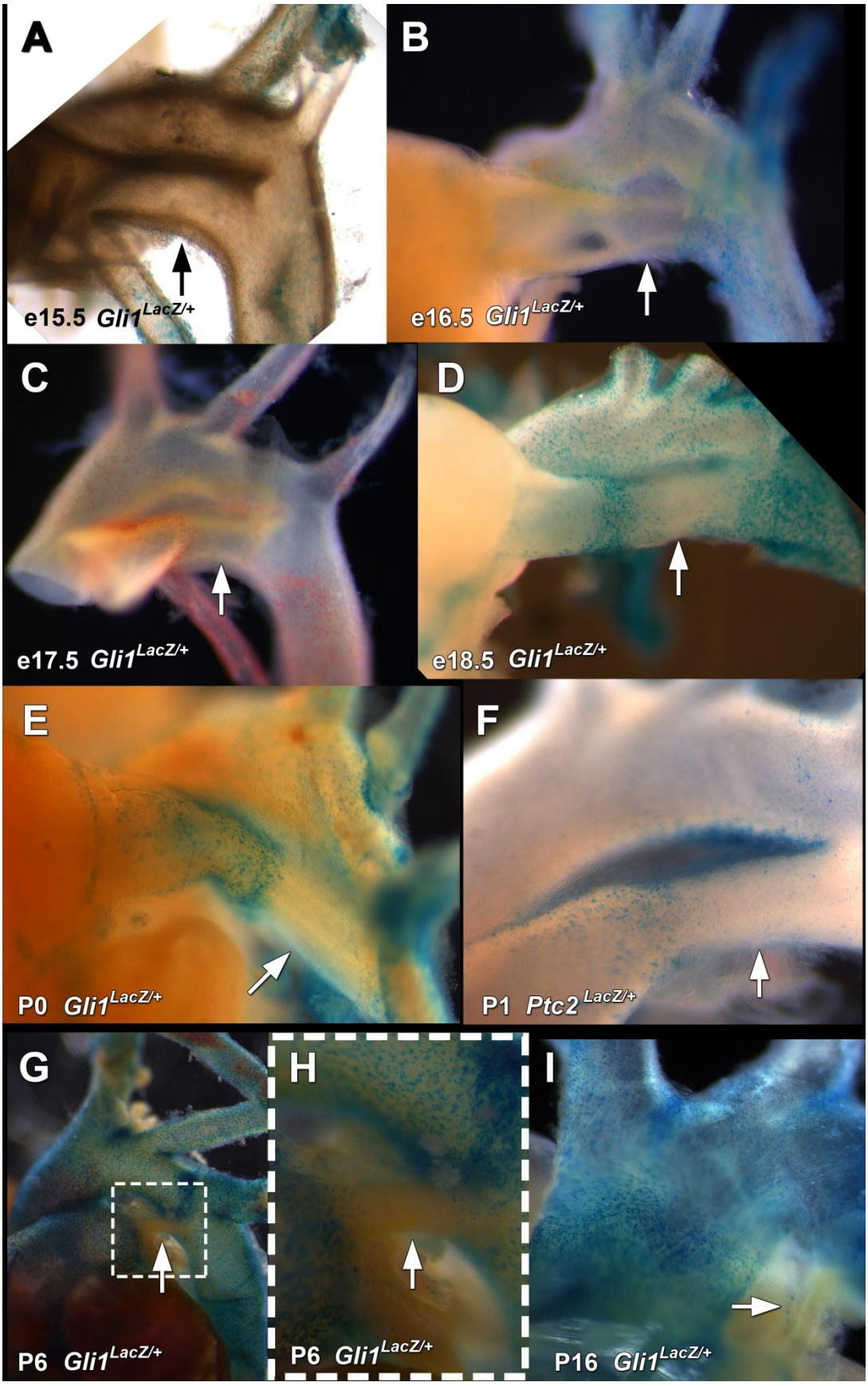


Supplemental Figure 1: *AdvSca1 cell differentiation potential in vivo.* AdvSca1 cells isolated from Rosa26 mice with membrane localized

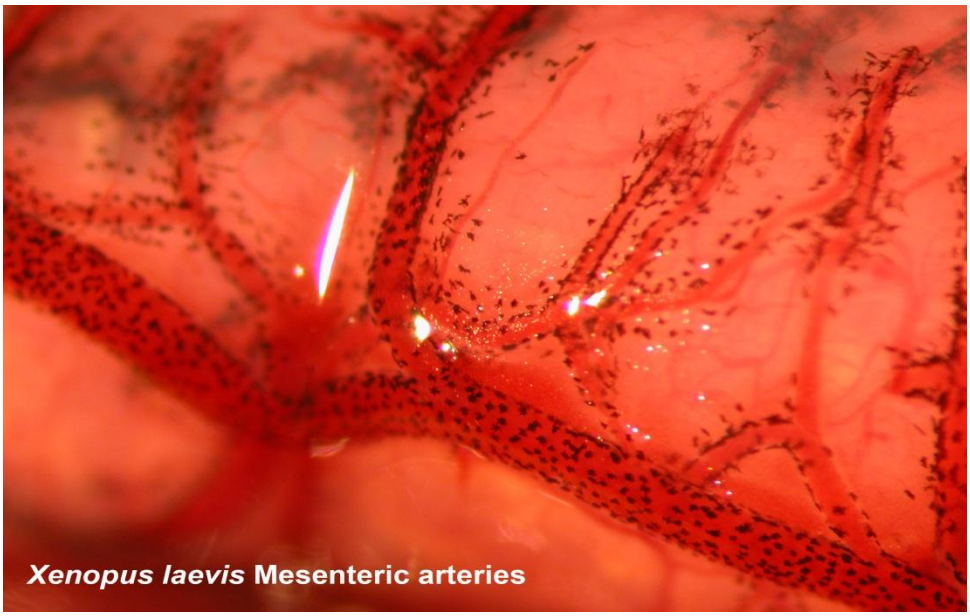
tdTomato (mTmG, see appendix D for mouse strain information) were incubated in Matrigel plugs treated with 0.5 µg/mL human FGF2 and 60 U/mL heparin for 10 days injected into wild type mice. Single confocal Z-sections indicate tdTomato co-localization with endothelial cell marker PECAM (arrowheads, A), Perilipin A, a marker of adipocytes (B), and macrophage marker CD68 (C). Images courtesy of Dr. Jenna Regan [40].



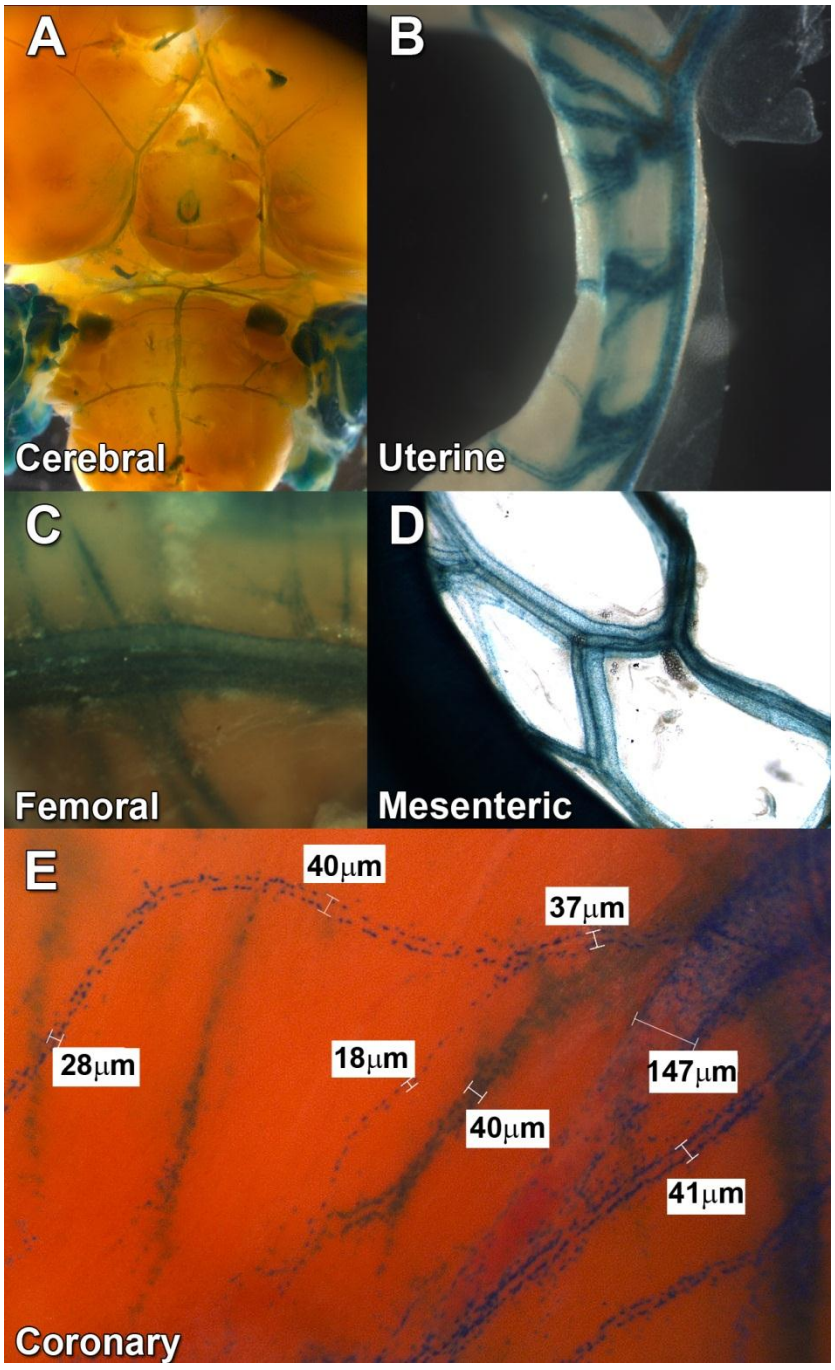
Supplemental Figure 2: *Schematic of early arteriogenesis*. Smooth muscle progenitors are recruited to the endothelial vascular plexus. They begin to differentiate and invest in the wall, and then through Notch signaling, and processes still unclear, the wall undergoes maturation into a multilayered structure complete with adventitia.[256]


A
e15.5 Gli1LacZ/+
B
e16.5 Gli1LacZ/+
C
e17.5 Gli1LacZ/+
D
e18.5 Gli1LacZ/+
E
P0 Gli1LacZ/+
F
P1 Ptc2LacZ/+
G
P6 Gli1LacZ/+
H
P6 Gli1LacZ/+
I
P16 Gli1LacZ/+

Supplemental Figure 3: *The ductus arteriosus and ligamentum arteriosum are largely void of* Gli1$^{LacZ/+}$*-positive cells throughout development.*

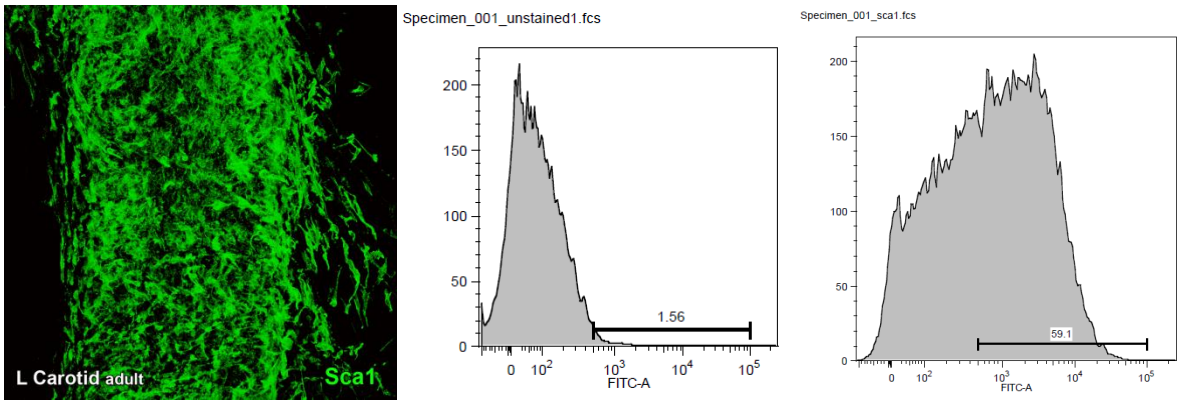


Supplemental Figure 4: *Neural crest derived melanocytes closely associate with the vasculature of the African clawed frog (*Xenopus laevis*).* Image of unstained adult male frog small intestine vasculature. Frog tissue courtesy of Dr. Frank Conlon.



Supplemental Figure 5: Gli1$^{LacZ/+}$ *expression in smaller caliber vessels.* *Gli1*$^{LacZ/+}$ is expressed in the brain, the mesentery, the uterine arteries, the femoral arteries, and fine caliber coronary arteries during neonatal development. Coronaries were measured with Leica LAS software

using the “distance line” analysis tool. All samples are newborn mice P0-P6.



Supplemental Figure 6: *Flow analysis of adventitial cells for AdvSca1.* A) The adult mouse adventitia contains abundant AdvSca1 cells. B) Total adventitial cells from 3 wild type adult (6-8mo) C57Bl/6 mouse thoracic aortas, unstained control, gated with a false positive rate of 1.56% of cells. C) Total adventitial cells from 3 wild type adult (6-8mo) C57Bl/6 mouse thoracic aortas were stained with anti-Sca1-FITC antibody. Using the same gate as A, 59.1% of the cells in the population stained positive for Sca1-FITC.

Appendix B: List of Antibodies and Reagents

Antibody	Host	Source	Cat #	Dilution	Staining Notes
Acetylated α tubulin	Mouse IgG2b	Sigma	M7451	1:1000	Triton
BOC	Mouse IgG2a	R&D	MAB20361	1:100	No antigen retrieval
CD68	Rat IgG2a	AbD Serotec	MCA1957	1:200	No antigen retrieval, but can use methanol
Chicken IgY-Alexa 488	Goat	Molecular Probes	A11039	1:500	Secondary antibody
Chicken IgY-Alexa 594	Goat	Molecular Probes	A11042	1:500	Secondary antibody
GFP	Rabbit	Invitrogen	A11122	1:200	Methanol
GFP	Chicken	Abcam	Ab13970	1:1000	Methanol
Ki67	Mouse IgG1	Pharmingen	550609	1:200	Methanol
KLF4	Rabbit	Santa Cruz	sc20691	1:100	Triton
Mouse IgG1-Alexa 594	Goat	Molecular Probes	A21125	1:500	Secondary antibody
Mouse IgG2a-Alexa 488	Goat	Molecular Probes	A21131	1:500	Secondary antibody
Mouse IgG2a-Alexa 594	Goat	Molecular Probes	A21135	1:500	Secondary antibody
Mouse IgG2a-Alexa 633	Goat	Molecular Probes	A21136	1:500	Secondary antibody
Mouse IgG2b-Alexa 594	Goat	Molecular Probes	A21145	1:500	Secondary antibody
Mouse IgG2b-Alexa 488	Goat	Molecular Probes	A21141	1:500	Secondary antibody
Pecam	Rat IgG2a	Pharmingen	550274	1:200	No antigen retrieval needed, can use methanol

Perilipin A	Rabbit IgG	Sigma	P1998	1:200	No antigen retrieval. Incompatible with Triton
Phospho-Histone H3	Rabbit	Upstate	06-570	1:100	Best with Triton, also works with methanol
Rabbit IgG-Alexa 488	Goat	Molecular Probes	A11008	1:500	Secondary antibody
Rabbit IgG-Alexa 594	Goat	Molecular Probes	A11037	1:500	Secondary antibody
Rat IgG- Alexa 488	Donkey	Molecular Probes	A11034	1:500	Secondary antibody
Rat IgG- Alexa 594	Donkey	Molecular Probes	A21209	1:500	Secondary antibody
Rat IgG- Alexa 633	Goat	Molecular Probes	A21094	1:500	Secondary antibody
Sca1	Rat IgG2a	Pharmingen (BD)	553333	1:200	No antigen retrieval. Incompatible with Triton
Sca1-FITC	Rat IgG2a	Miltenyi	130-092-529	1:20	No antigen retrieval. Incompatible with Triton
Shh-C	Goat	R&D	AF445	1:100	Best with no antigen retrieval or methanol
Shh-N (5E1)	Mouse IgG1	DSHB	5E1	1:50	Incompatible with Triton
SM-MHC	Rabbit IgG	Biomedical Technologies	BT-562	1:100	Methanol or Triton
SMαA	Mouse IgG2a	Sigma	A2547	1:500	Methanol or Triton
β-gal	Mouse IgG2b	Roche	1083104	1:200	Methanol or Triton
β-tubulin III	Mouse IgG2a	Covance	MMS-435P	1:1000	Triton

Reagent	Source	Cat#	Dilution/Notes
Click-It® EdU-Alexa 647	Invitrogen	C10340	Use manufacturer's instructions for staining. 50mg/kg injected/mouse
FITC-Dextran	Sigma	FD2000S	High molecular weight (2000kDa)
Heparin	Sigma	H3393	60 U/ml
Histoclear	National Diagnostics	HS-200	-
Hoechst Dye	Molecular Probes	33258	1µg/ml in H_2O
Matrigel	BD Biosciences	356231	Growth factor reduced
Mowiol	Polysciences	17951-500	Use manufacturer's instructions
Nuclear fast red	ENG Scientific Inc.	9040	Counterstain sections for ~ 1 minute, wash off excess with H_2O
OCT	Tissue-tek	4583	-
Recombinant human FGF basic	Peprotech	100-18B	0.5µg/ml
Rose-β-D-gal	Biotium Inc.	10013	1 mg/ml working concentration
Sca1 isolation kit	Miltenyi Biotec	130-092-529	Sort over 2 columns, follow manufacturer's instructions
X-gal	Bioline	BIO 37035	1 mg/ml working concentration

Appendix C: Genotyping Primers

Strain	Forward Primer	Reverse Primer	Product Length
Gli1LacZ	GGGATCTGTGCCTGAAACTG	AGGCGATTAAGTTGGGTAAC	286bp
Gli1 WT	GGGATCTGTGCCTGAAACTG	AGGTGAGACGACTGCCAAGT	261bp
Gli2LacZ	CCTGGGGTCAGAAGACTGAG	AGGCGATTAAGTTGGGTAAC	350bp
Gli2 WT	CCTGGGGTCAGAAGACTGAG	CTGCTGTCCTCCAAGAGACC	230bp
Ptc1LacZ (not gene specific)	TTGGAGTGACGGCAGTTATCTGGA	TCAACCACCGCACGATAGAGATTC	348bp
Ptc1 WT	CTGCGGCAAGTTTTTGGTTG	AGGGCTTCTCGTTGGCTACAAG	200bp
Ptc2LacZ	GGGTGGGATTAGTATAATGCCTGCTCT	TCAAACATCTGGAGGGACTGTGTAG	476bp
Ptc2 WT	CCTAGAAATCCCTGTCTGAGATCTC	TCAAACATCTGGAGGGACTGTGTAG	222bp
ShhEGFP	GGGACAGCTCACAAGTCCTC	GGTGCGCTCCTGGACGTA	333bp
Shh WT	GGGACAGCTCACAAGTCCTG	CTCGGCTACGTTGGGAATAA	200bp
LysMcre	CTTGGGCTGCCAGAATTTCTC	CCCAGAAATGCCAGATTACG	701bp
LysM WT	CTTGGGCTGCCAGAATTTCTC	TTACAGTCGGCCAGGCTGAC	346bp
Smo flox	CCACTGCGAGCCTTTGCGCTAC	GGCGCTACCGGTGGATGTGG	400bp
Smo WT	CCACTGCGAGCCTTTGCGCTAC	CCCATCACCTCCGCGTCGCA	170bp
R26R YFP	AAAGTCGCTCTGAGTTGTTAT	AAGACCGCGAAGAGTTTGTC	320bp
R26 WT	AAAGTCGCTCTGAGTTGTTAT	GGAGCGGGAGAAATGGATATG	600bp

Appendix D: Mouse Strain Information

Gli1$^{LacZ/+}$ mice were made by Alexandra Joyner. (*Gli1*^{tm2Alj}*/J*, Jackson stock number: 008211)[188]. To make the *Gli1*$^{LacZ/+}$ mice, a targeting vector was designed to insert a nuclear localized β-galactosidase (*LacZ*) cDNA and *loxP*-flanked, reverse-oriented neo cassette into the first coding exon (exon 2) and delete the coding sequences in exons two to seven of the targeted gene. This construct was electroporated into 129S6/SvEvTac-derived W4 embryonic stem (ES) cells. Correctly targeted ES cells were injected into recipient blastocysts and chimeric mice were bred with 129SvEv (Taconic) mice. Next, mutant mice were bred to TK-*Cre* transgenic mice (129SvEv genetic background) to remove the floxed neo cassette. The resulting *Gli1*$^{LacZ/+}$ offspring were selectively bred to remove the TK-Cre transgene and then subsequently maintained by breeding to Swiss Webster mice and also interbreeding for many generations prior to arrival at The Jackson Laboratory.[188]

Gli2$^{LacZ/+}$ mice were made by Alexandra Joyner (*Gli2*$^{tm2.1Alj}$*/J*, Jackson stock number: 007922)[189]. To make the *Gli2*$^{LacZ/+}$ mice, a targeting vector was designed with a nuclear localized β-galactosidase cDNA (with 1.7 kb of 3' UTR and three tandem SV40 poly A sequence repeats downstream) and followed by a reverse-oriented loxP-flanked neo cassette. This construct was used to replace a portion of the first coding exon (exon 2) of the targeted gene (including the ATG start codon) via electroporation into 129S6/SvEvTac-derived W4 embryonic stem (ES) cells. Correctly targeted ES cells were injected into recipient blastocysts and chimeric mice were bred with Black Swiss Webster outbred mice. The resulting *Gli2*^{nlzki}

heterozygotes were then bred to cre expressing mice (on a Swiss Webster genetic background) to remove the neo cassette, generating *Gli2*$^{LacZ/+}$ offspring (with a single loxP site remaining after the SV40 polyA). These *Gli2*$^{LacZ/+}$ mutant mice were bred to Swiss Webster mice for many generations prior to arrival at The Jackson Laboratory, and are maintained by crossing heterozygotes to wild type littermates.[189]

Ptc1$^{LacZ/+}$ mice were made by Matthew Scott (*Ptch1*^{tm1Mps}*/J*, Jackson stock number: 003081)[186]. To make *Ptc1*$^{LacZ/+}$ mice, a targeting vector, KO1, containing sequence encoding nuclear localized beta-galactosidase (LacZ), neomycin resistance gene, mouse protamine 1 (mP1) intron, and a polyadenylation site was electroporated into 129-derived R1 embryonic stem (ES) cells. Correctly targeted ES cells were injected into C57BL/6 blastocysts. The resulting chimeric male mice were then mated to B6D2F1 females and backcrossed onto C57BL/6. This strain is also segregating the coat color allele dilute (Myo5ad).

Ptc2$^{LacZ/+}$ mice were made by Deltagen Inc. (*Ptch2*^{tm1Dgen}*/J*, Jackson stock number: 005827)[187]. The construct insert is reported to be "Lac0-SA-IRES-LacZ-Neo555G/Kan." These mice are maintained on C57BL/6 background.

Shh$^{EGFP/+}$ mice were made by Clifford Tabin. (*B6.Cg-Shh*$^{tm1(EGFP/cre)Cjt}$*/J,* Jackson stock number: 005622)[91] To make the *Shh*$^{EGFP/+}$ mice, a targeting vector containing a fusion product involving Enhanced Green Fluorescent Protein (EGFP) and Cre recombinase was inserted at the ATG of Shh and disrupted the sequence encoding the first 12 amino acids. The construct was electroporated into 129S6/SvEv-derived TC-1 embryonic stem (ES) cells. Correctly targeted ES cells

were injected into recipient blastocysts. The resulting chimeric animals were crossed to C57BL/6 mice and are maintained on this background.[91]

LysM$^{cre/cre}$ mice were made by Irmgard Foerster. (*B6.129P2-Lyz2*$^{tm1(cre)Ifo}$*/J,* Jackson stock number: 004781)[253]. To make the *LysM*$^{cre/cre}$ mice, a targeting vector containing a FRT-flanked neomycin resistance gene, herpes simplex virus thymidine kinase genes and NLS (nuclear localization signal) -Cre cDNA, was inserted into the endogenous ATG-start site within exon 1 of the *Lyzs* gene. The construct was electroporated into 129P2/OlaHsd-derived E14.1 embryonic stem (ES) cells. Correctly targeted ES cells were transiently transfected with a *Flp* expression vector for the purpose of removing the selectable marker cassette. ES cells that had successfully undergone Flp recombination were injected in blastocysts. These mice are maintained on a C57Bl6/J background.

Smo$^{flox/flox}$ mice were made by Andrew McMahon. (*Smo*^{tm2AMC}*/J*, Jackson stock number: 004526)[252]. To make these mice, a loxP site flanked targeting vector containing a 400bp fragment of exon 1 and an FRT-flanked neomycin resistance gene was utilized in the construction of this mutant. This construct was electroporated into 129X1/SvJ derived AV3 embryonic stem (ES) cells. Correctly targeted ES cells were injected into C57BL/6J blastocysts. The resulting male chimeric animals were crossed to Swiss Webster females. These mice are maintained by intercrossing homozygous flox littermates.

R26R$^{YFP/YFP}$ mice were made by Frank Costantini. (*B6.129X1-Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$*/J*, Jackson stock number:00). To make these mice, a targeting vector was designed to contain the Enhanced Yellow Fluorescent Protein

gene (from the pEYFP-N1 plasmid, Clonetech) downstream of a loxP-flanked stop sequence (neomycin resistance gene and a trimer of the SV40 polyadenylation sequence). This entire construct was inserted into the Gt(ROSA)26Sor locus via electroporation of 129X1/SvJ-derived JM-1 embryonic stem (ES) cells. Correctly targeted ES cells were microinjected in C57BL/6J blastocysts. Chimeric male Y8-8 was bred to a C57BL/6J female mouse. Mutant male progeny were backcrossed to C57BL/6J for 6 generations and then made homozygous. These mice are maintained by intercrossing homozygous R26R littermates.

Appendix E: Curriculum Vitae

Virginia Jean Hoglund

Education

University of North Carolina, Chapel Hill, NC — August 2007-May 2012
M.S. Genetics and Molecular Biology
Thesis: *Characterization of the Arterial Adventitia as a Sonic Hedgehog Responsive Niche*

University of Florida, Gainesville, FL — August 2003-May 2007
B.S. Zoology, Cum Laude

Fellowship Support

Hoglund VJ. American Heart Association Predoctoral Fellowship-Mid Atlantic Affiliate, 09PRE2060165. "Paracrine hedgehog signaling pathways in the arterial adventitia." 2009. This application received (1.37, 5.77%)

Hoglund VJ. Integrative Vascular Biology Training Program, National Institutes of Health 2T32-HL069768. "Paracrine hedgehog signaling pathways in the arterial adventitia." 2009. This fellowship was awarded at the same time as the AHA Predoctoral Fellowship (above). The AHA award was accepted. An appointment to the IVB training grant was accepted without stipend support to avoid overlap with the AHA award.

Publications

Hoglund VJ & Majesky MW (2012) Patterning the artery wall by lateral induction of notch signaling. *Circulation* 125(2):212-215.

Majesky MW, Dong XR, Hoglund V, Daum G, & Mahoney WM, Jr. (2012) The adventitia: a progenitor cell niche for the vessel wall. *Cells Tissues Organs* 195(1-2):73-81.

Majesky MW, Dong XR, Hoglund V, Mahoney WM, Jr., & Daum G (2011) The adventitia: a dynamic interface containing resident progenitor cells. *Arterioscler Thromb Vasc Biol* 31(7):1530-1539.

Majesky MW, Dong XR, Regan JN, & Hoglund VJ (2011) Vascular smooth muscle progenitor cells: building and repairing blood vessels. *Circ Res* 108(3):365-377.

Majesky MW, Dong XR, & Hoglund VJ (2011) Parsing aortic aneurysms: more surprises. *Circ Res* 108(5):528-530.

Hoglund VJ, Dong XR, & Majesky MW (2010) Neointima formation: a local affair. *Arterioscler Thromb Vasc Biol* 30(10):1877-1879.

Abstracts

Hoglund V, Regan J, Maguire C, Enderlein C, Levenson M, Dong XR, Majesky M (2011). Building an Artery Wall: Characterizing the Development of a Sonic Hedgehog Responsive Adventitia. *Seattle Children's Research Institute Annual Symposium*, Seattle Children's Research Institute, Seattle, WA

Hoglund V, Regan J, Maguire C, Enderlein C, Levenson M, Dong XR, Majesky M (2011). Building an Artery Wall: Characterizing the Development of a Sonic Hedgehog Responsive Adventitia. *University of North Carolina Department of Genetics Retreat*, Myrtle Beach, SC

Hoglund VJ, Regan JN, Maguire CT, Dong XR, Majesky MW (2010). Sonic Hedgehog Signaling in Tissue Resident Macrophages in the Arterial Adventitia. *University of North Carolina Department of Genetics Retreat*, Myrtle Beach, SC

Hoglund VJ, Regan JN, Maguire CT, Dong XR, Majesky MW (2010). Characterization of a Novel Sonic Hedgehog Signaling Domain and Stem Cell Niche in the Arterial Adventitia. *Seattle Children's Research Institute Symposium*, Seattle, WA

Hoglund VJ, Regan JN, Maguire CT, Dong XR, Majesky MW (2010). Hedgehog Signaling in the Arterial Adventitia. *Curriculum in Genetics Student Seminars*, University of North Carolina, Chapel Hill, NC

Hoglund VJ, Regan JN, Maguire CT, Dong XR, Majesky MW (2010). Hedgehog Signaling in the Arterial Adventitia. *Integrative Vascular Biology Symposium*, University of North Carolina, Chapel Hill, NC

Majesky MW, Dong XR, Regan J, Hoglund V (2010). Vascular Smooth Muscle Progenitor Cells. *Federation European Physiological Societies*, Copenhagen, Denmark. Acta Physiol Scand, Volume 198, Supplement 677 :S-SAT-1-1

Hoglund VJ, Regan JN, Maguire CT, Dong XR, Majesky MW (2009). Hedgehog Signaling in a Novel Progenitor Cell Niche Within the Arterial Adventitia. *University of North Carolina Department of Genetics Retreat*, Asheville, NC

Passman J, Dong XR, Hoglund VJ, Maguire CT, Chen Z, Tzima E, Majesky MW. (2009) Hedgehog signaling and KLF4 contribute to maintenance of smooth muscle progenitor cells in the arterial adventitia. *Experimental Biology Meetings*, New Orleans, LA. FASEBJ 23:300.1

Majesky MW, Dong XR, Passman J, Hoglund V, Maguire C. (2009). Developmental origins of coronary smooth muscle. *Keystone Symposium: Dissecting the Vasculature*, Vancouver, BC, Canada

Hoglund VJ, Passman JN, Maguire CT, Dong XR, Majesky MW (2009). Hedgehog signaling and angiogenesis in the arterial adventitia. *Vasculata*, Cleveland, OH.

Hoglund VJ, Passman JN, Maguire CT, Majesky MW (2009) Hedgehog signaling and angiogenesis in the arterial adventitia. *Integrative Vascular Biology Symposium*, University of North Carolina, Chapel Hill, NC

Hoglund VJ, Passman JN, Maguire CT, Majesky MW (2008) Characterization of a novel progenitor cell niche in the adventitia of the artery wall. *University of North Carolina Department of Genetics Retreat*, Atlantic Beach, NC

Organizations

North American Vascular Biology Organization

Phi Beta Kappa

Golden Key Honor Society

National Society of Collegiate Scholars

Society for Developmental Biology

Awards

Certificate of recognition for outstanding abstract and poster presentation, title: Hedgehog signaling and angiogenesis in the arterial adventitia. (2009). *Vasculata*, Cleveland, OH

REFERENCES

1. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature*. 2001;414:98-104

2. Hartman TR, Zinshteyn D, Schofield HK, Nicolas E, Okada A, O'Reilly AM. Drosophila boi limits hedgehog levels to suppress follicle stem cell proliferation. *J Cell Biol*. 2010;191:943-952

3. O'Reilly AM, Lee HH, Simon MA. Integrins control the positioning and proliferation of follicle stem cells in the drosophila ovary. *J Cell Biol*. 2008;182:801-815

4. Morrison SJ, Spradling AC. Stem cells and niches: Mechanisms that promote stem cell maintenance throughout life. *Cell*. 2008;132:598-611

5. Xie T, Spradling AC. A niche maintaining germ line stem cells in the drosophila ovary. *Science*. 2000;290:328-330

6. Losick VP, Morris LX, Fox DT, Spradling A. Drosophila stem cell niches: A decade of discovery suggests a unified view of stem cell regulation. *Dev Cell*. 2011;21:159-171

7. Song X, Xie T. DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the drosophila ovary. *Proc Natl Acad Sci U S A*. 2002;99:14813-14818

8. Forbes AJ, Lin H, Ingham PW, Spradling AC. Hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in drosophila. *Development*. 1996;122:1125-1135

9. Forbes AJ, Spradling AC, Ingham PW, Lin H. The role of segment polarity genes during early oogenesis in drosophila. *Development*. 1996;122:3283-3294

10. Zhang Y, Kalderon D. Hedgehog acts as a somatic stem cell factor in the drosophila ovary. *Nature*. 2001;410:599-604

11. Licht T, Goshen I, Avital A, Kreisel T, Zubedat S, Eavri R, Segal M, Yirmiya R, Keshet E. Reversible modulations of neuronal plasticity by vegf. *Proc Natl Acad Sci U S A*. 2011;108:5081-5086

12. Kokovay E, Goderie S, Wang Y, Lotz S, Lin G, Sun Y, Roysam B, Shen Q, Temple S. Adult SVZ lineage cells home to and leave the vascular niche via differential responses to sdf1/cxcr4 signaling. *Cell Stem Cell*. 2010;7:163-173

13. Rafii S, Shapiro F, Pettengell R, Ferris B, Nachman RL, Moore MA, Asch AS. Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. *Blood*. 1995;86:3353-3363

14. Rafii S, Shapiro F, Rimarachin J, Nachman RL, Ferris B, Weksler B, Moore MA, Asch AS. Isolation and characterization of human bone marrow microvascular endothelial cells: Hematopoietic progenitor cell adhesion. *Blood*. 1994;84:10-19

15. Davis TA, Robinson DH, Lee KP, Kessler SW. Porcine brain microvascular endothelial cells support the in vitro expansion of human primitive hematopoietic bone marrow progenitor cells with a high replating potential: Requirement for cell-to-cell interactions and colony-stimulating factors. *Blood*. 1995;85:1751-1761

16. Chute JP, Muramoto GG, Fung J, Oxford C. Soluble factors elaborated by human brain endothelial cells induce the concomitant expansion of purified human BM CD34+CD38- cells and scid-repopulating cells. *Blood*. 2005;105:576-583

17. Chute JP, Saini AA, Chute DJ, Wells MR, Clark WB, Harlan DM, Park J, Stull MK, Civin C, Davis TA. Ex vivo culture with human brain endothelial cells increases the SCID-repopulating capacity of adult human bone marrow. *Blood*. 2002;100:4433-4439

18. Doan PL, Chute JP. The vascular niche: Home for normal and malignant hematopoietic stem cells. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K*. 2012;26:54-62

19. Yoshida S, Sukeno M, Nabeshima Y. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science*. 2007;317:1722-1726

20. Majesky MW, Dong XR, Hoglund V, Mahoney WM, Jr., Daum G. The adventitia: A dynamic interface containing resident progenitor cells. *Arterioscler Thromb Vasc Biol*. 2011;31:1530-1539

21. Majesky MW, Dong XR, Hoglund V, Daum G, Mahoney WM, Jr. The adventitia: A progenitor cell niche for the vessel wall. *Cells Tissues Organs*. 2012;195:73-81

22. Corselli M, Chen CW, Sun B, Yap S, Rubin JP, Peault B. The tunica adventitia of human arteries and veins as a source of mesenchymal stem cells. *Stem Cells Dev*. 2011

23. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in apoe-deficient mice. *J Clin Invest*. 2004;113:1258-1265

24. Klein D, Weisshardt P, Kleff V, Jastrow H, Jakob HG, Ergun S. Vascular wall-resident CD44+ multipotent stem cells give rise to pericytes and smooth muscle cells and contribute to new vessel maturation. *PLoS One*. 2011;6:e20540

25. Passman JN, Dong XR, Wu SP, Maguire CT, Hogan KA, Bautch VL, Majesky MW. A sonic hedgehog signaling domain in the arterial adventitia supports resident sca1+ smooth muscle progenitor cells. *Proc Natl Acad Sci U S A*. 2008;105:9349-9354

26. Psaltis PJ, Harbuzariu A, Delacroix S, Holroyd EW, Simari RD. Resident vascular progenitor cells--diverse origins, phenotype, and function. *J Cardiovasc Transl Res*. 2011;4:161-176

27. Psaltis PJ, Harbuzariu A, Delacroix S, Witt TA, Holroyd EW, Spoon DB, Hoffman SJ, Pan S, Kleppe LS, Mueske CS, Gulati R, Sandhu GS, Simari RD. Identification of a monocyte-predisposed hierarchy of hematopoietic progenitor cells in the adventitia of postnatal murine aorta. *Circulation*. 2012;125:592-603

28. Yoder MC. Aortic tissue as a niche for hematopoiesis. *Circulation*. 2012;125:565-567

29. Zengin E, Chalajour F, Gehling UM, Ito WD, Treede H, Lauke H, Weil J, Reichenspurner H, Kilic N, Ergun S. Vascular wall resident progenitor cells: A source for postnatal vasculogenesis. *Development*. 2006;133:1543-1551

30. Ergun S, Tilki D, Klein D. Vascular wall as a reservoir for different types of stem and progenitor cells. *Antioxid Redox Signal*. 2011;15:981-995

31. Hoshino A, Chiba H, Nagai K, Ishii G, Ochiai A. Human vascular adventitial fibroblasts contain mesenchymal stem/progenitor cells. *Biochem Biophys Res Commun*. 2008;368:305-310

32. Sartore S, Chiavegato A, Faggin E, Franch R, Puato M, Ausoni S, Pauletto P. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: From innocent bystander to active participant. *Circ Res*. 2001;89:1111-1121

33. Ryan ST, Koteliansky VE, Gotwals PJ, Lindner V. Transforming growth factor-beta-dependent events in vascular remodeling following arterial injury. *J Vasc Res*. 2003;40:37-46

34. Chen H, Liu Y, Slipchenko MN, Zhao X, Cheng JX, Kassab GS. The layered structure of coronary adventitia under mechanical load. *Biophysical journal*. 2011;101:2555-2562

35. Heistad DD, Marcus ML, Larsen GE, Armstrong ML. Role of vasa vasorum in nourishment of the aortic wall. *Am J Physiol*. 1981;240:H781-787

36. Moulton KS, Vakili K, Zurakowski D, Soliman M, Butterfield C, Sylvin E, Lo KM, Gillies S, Javaherian K, Folkman J. Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. *Proc Natl Acad Sci U S A*. 2003;100:4736-4741

37. Drinane M, Mollmark J, Zagorchev L, Moodie K, Sun B, Hall A, Shipman S, Morganelli P, Simons M, Mulligan-Kehoe MJ. The antiangiogenic activity of rpai-1(23) inhibits vasa vasorum and growth of atherosclerotic plaque. *Circ Res*. 2009;104:337-345

38. Haas KS, Phillips SJ, Comerota AJ, White JV. The architecture of adventitial elastin in the canine infrarenal aorta. *The Anatomical record*. 1991;230:86-96

39. Campagnolo P, Cesselli D, Al Haj Zen A, Beltrami AP, Krankel N, Katare R, Angelini G, Emanueli C, Madeddu P. Human adult vena saphena contains perivascular progenitor cells endowed with clonogenic and proangiogenic potential. *Circulation*. 2010;121:1735-1745

40. Regan JN. Regulation of adventitia-resident progenitor cells. *Curriculum in Genetics and Molecular Biology*. 2010;Doctor of Philosophy:140

41. Howson KM, Aplin AC, Gelati M, Alessandri G, Parati EA, Nicosia RF. The postnatal rat aorta contains pericyte progenitor cells that form spheroidal colonies in suspension culture. *American journal of physiology. Cell physiology*. 2005;289:C1396-1407

42. Xiao Q, Wang G, Luo Z, Xu Q. The mechanism of stem cell differentiation into smooth muscle cells. *Thrombosis and haemostasis*. 2010;104:440-448

43. Shore P, Sharrocks AD. The MADS-box family of transcription factors. *European journal of biochemistry / FEBS*. 1995;229:1-13

44. McDonald OG, Wamhoff BR, Hoofnagle MH, Owens GK. Control of srf binding to CARG box chromatin regulates smooth muscle gene expression in vivo. *J Clin Invest*. 2006;116:36-48

45. Hautmann MB, Thompson MM, Swartz EA, Olson EN, Owens GK. Angiotensin II-induced stimulation of smooth muscle alpha-actin expression by serum response factor and the homeodomain transcription factor mhox. *Circ Res*. 1997;81:600-610

46. Liu Y, Sinha S, McDonald OG, Shang Y, Hoofnagle MH, Owens GK. Kruppel-like factor 4 abrogates myocardin-induced activation of smooth muscle gene expression. *J Biol Chem*. 2005;280:9719-9727

47. Hayashi K, Nakamura S, Nishida W, Sobue K. Bone morphogenetic protein-induced msx1 and msx2 inhibit myocardin-dependent smooth muscle gene transcription. *Mol Cell Biol*. 2006;26:9456-9470

48. Zhou J, Hu G, Herring BP. Smooth muscle-specific genes are differentially sensitive to inhibition by elk-1. *Mol Cell Biol*. 2005;25:9874-9885

49. Miralles F, Posern G, Zaromytidou AI, Treisman R. Actin dynamics control srf activity by regulation of its coactivator mal. *Cell*. 2003;113:329-342

50. Tang Y, Yang X, Friesel RE, Vary CP, Liaw L. Mechanisms of tgf-beta-induced differentiation in human vascular smooth muscle cells. *J Vasc Res*. 2011;48:485-494

51. Goss AM, Tian Y, Cheng L, Yang J, Zhou D, Cohen ED, Morrisey EE. Wnt2 signaling is necessary and sufficient to activate the airway smooth muscle program in the lung by regulating myocardin/mrtf-b and fgf10 expression. *Dev Biol*. 2011;356:541-552

52. Liu ZP, Wang Z, Yanagisawa H, Olson EN. Phenotypic modulation of smooth muscle cells through interaction of foxo4 and myocardin. *Dev Cell*. 2005;9:261-270

53. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663-676

54. Tsai SY, Clavel C, Kim S, Ang YS, Grisanti L, Lee DF, Kelley K, Rendl M. Oct4 and klf4 reprogram dermal papilla cells into induced pluripotent stem cells. *Stem Cells*. 2010;28:221-228

55. Kim SI, Bresnick EH, Bultman SJ. Brg1 directly regulates nucleosome structure and chromatin looping of the alpha globin locus to activate transcription. *Nucleic acids research*. 2009;37:6019-6027

56. Spin JM, Maegdefessel L, Tsao PS. Vascular smooth muscle cell phenotypic plasticity: Focus on chromatin remodelling. *Cardiovascular research*. 2012

57. Conboy IM, Conboy MJ, Smythe GM, Rando TA. Notch-mediated restoration of regenerative potential to aged muscle. *Science*. 2003;302:1575-1577

58. Iakova P, Awad SS, Timchenko NA. Aging reduces proliferative capacities of liver by switching pathways of c/ebpalpha growth arrest. *Cell*. 2003;113:495-506

59. Conboy IM, Rando TA. Aging, stem cells and tissue regeneration: Lessons from muscle. *Cell Cycle*. 2005;4:407-410

60. Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16ink4a. *Nature*. 2006;443:421-426

61. Olive M, Harten I, Mitchell R, Beers JK, Djabali K, Cao K, Erdos MR, Blair C, Funke B, Smoot L, Gerhard-Herman M, Machan JT, Kutys R, Virmani R, Collins FS, Wight TN, Nabel EG, Gordon LB. Cardiovascular pathology in Hutchinson-Gilford progeria: Correlation with the vascular pathology of aging. *Arterioscler Thromb Vasc Biol*. 2010;30:2301-2309

62. Wagner W, Horn P, Bork S, Ho AD. Aging of hematopoietic stem cells is regulated by the stem cell niche. *Experimental gerontology*. 2008;43:974-980

63. Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA. Increased wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science*. 2007;317:807-810

64. Fox RG, Magness ST, Kujoth GC, Prolla TA, Maeda N. Mitochondrial DNA polymerase editing mutation, polgd257a, disturbs stem-progenitor cell cycling in the small intestine and restricts excess fat absorption. *Am J Physiol Gastrointest Liver Physiol*. 2012; Epub ahead of print. DOI:10.1152/ajpgi.00402.2011

65. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature*. 2005;433:760-764

66. Mahoney WM, Jr., Fleming JN, Schwartz SM. A unifying hypothesis for scleroderma: Identifying a target cell for scleroderma. *Current rheumatology reports*. 2011;13:28-36

67. Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially l-selectin dependent. *J Exp Med*. 2006;203:1273-1282

68. Mayranpaa MI, Trosien JA, Fontaine V, Folkesson M, Kazi M, Eriksson P, Swedenborg J, Hedin U. Mast cells associate with neovessels in the media and adventitia of abdominal aortic aneurysms. *J Vasc Surg*. 2009;50:388-395; discussion 395-386

69. Moos MP, John N, Grabner R, Nossmann S, Gunther B, Vollandt R, Funk CD, Kaiser B, Habenicht AJ. The lamina adventitia is the major site of immune cell accumulation in standard chow-fed apolipoprotein e-deficient mice. *Arterioscler Thromb Vasc Biol*. 2005;25:2386-2391

70. Swedenborg J, Mayranpaa MI, Kovanen PT. Mast cells: Important players in the orchestrated pathogenesis of abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2011;31:734-740

71. Tang PC, Qin L, Zielonka J, Zhou J, Matte-Martone C, Bergaya S, van Rooijen N, Shlomchik WD, Min W, Sessa WC, Pober JS, Tellides G. Myd88-dependent, superoxide-initiated inflammation is necessary for flow-mediated inward remodeling of conduit arteries. *J Exp Med*. 2008;205:3159-3171

72. Zhou J, Tang PC, Qin L, Gayed PM, Li W, Skokos EA, Kyriakides TR, Pober JS, Tellides G. Cxcr3-dependent accumulation and activation of perivascular macrophages is necessary for homeostatic arterial remodeling to hemodynamic stresses. *J Exp Med*. 2010;207:1951-1966

73. Erami C, Zhang H, Tanoue A, Tsujimoto G, Thomas SA, Faber JE. Adrenergic catecholamine trophic activity contributes to flow-mediated arterial remodeling. *American journal of physiology. Heart and circulatory physiology*. 2005;289:H744-753

74. Haubold M, Gilsbach R, Hein L. {alpha}2b-adrenoceptor deficiency leads to postnatal respiratory failure in mice. *J Biol Chem*. 2010;285:34213-34219

75. Tieu BC, Lee C, Sun H, Lejeune W, Recinos A, 3rd, Ju X, Spratt H, Guo DC, Milewicz D, Tilton RG, Brasier AR. An adventitial il-6/mcp1 amplification loop accelerates macrophage-mediated vascular inflammation leading to aortic dissection in mice. *J Clin Invest*. 2009;119:3637-3651

76. Havelka GE, Kibbe MR. The vascular adventitia: Its role in the arterial injury response. *Vasc Endovascular Surg*. 2011;45:381-390

77. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, Goronzy J, Weyand C, Harrison DG. Role of the t cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J Exp Med*. 2007;204:2449-2460

78. Tsuruda T, Kato J, Matsui E, Hatakeyama K, Masuyama H, Imamura T, Kitamura K, Asada Y, Eto T. Adrenomedullin alleviates not only neointimal formation but also perivascular hyperplasia following arterial injury in rats. *European journal of pharmacology*. 2005;508:201-204

79. Barker SG, Tilling LC, Miller GC, Beesley JE, Fleetwood G, Stavri GT, Baskerville PA, Martin JF. The adventitia and atherogenesis: Removal initiates intimal proliferation in the rabbit which regresses on generation of a 'neoadventitia'. *Atherosclerosis*. 1994;105:131-144

80. Chignier E, Eloy R. Adventitial resection of small artery provokes endothelial loss and intimal hyperplasia. *Surgery, gynecology & obstetrics*. 1986;163:327-334

81. Nusslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in drosophila. *Nature*. 1980;287:795-801

82. Yu J, Carroll TJ, McMahon AP. Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development*. 2002;129:5301-5312

83. Bhardwaj G, Murdoch B, Wu D, Baker DP, Williams KP, Chadwick K, Ling LE, Karanu FN, Bhatia M. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via bmp regulation. *Nat Immunol*. 2001;2:172-180

84. Corcoran RB, Scott MP. Oxysterols stimulate sonic hedgehog signal transduction and proliferation of medulloblastoma cells. *Proc Natl Acad Sci U S A*. 2006;103:8408-8413

85. Rowitch DH, B SJ, Lee SM, Flax JD, Snyder EY, McMahon AP. Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. *J Neurosci*. 1999;19:8954-8965

86. Kusano KF, Allendoerfer KL, Munger W, Pola R, Bosch-Marce M, Kirchmair R, Yoon YS, Curry C, Silver M, Kearney M, Asahara T, Losordo DW. Sonic hedgehog induces arteriogenesis in diabetic vasa nervorum and restores function in diabetic neuropathy. *Arterioscler Thromb Vasc Biol*. 2004;24:2102-2107

87. Ahmed RP, Haider KH, Shujia J, Afzal MR, Ashraf M. Sonic hedgehog gene delivery to the rodent heart promotes angiogenesis via inos/netrin-1/pkc pathway. *PLoS One*. 2010;5:e8576

88. Lee SW, Moskowitz MA, Sims JR. Sonic hedgehog inversely regulates the expression of angiopoietin-1 and angiopoietin-2 in fibroblasts. *Int J Mol Med*. 2007;19:445-451

89. Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake Pepinsky R, Shapiro R, Taylor FR, Baker DP, Asahara T, Isner JM. The morphogen sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med*. 2001;7:706-711

90. Kolesova H, Roelink H, Grim M. Sonic hedgehog is required for the assembly and remodeling of branchial arch blood vessels. *Dev Dyn*. 2008;237:1923-1934

91. Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. Evidence for an expansion-based temporal shh gradient in specifying vertebrate digit identities. *Cell*. 2004;118:517-528

92. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev*. 1999;13:2072-2086

93. Haraguchi R, Mo R, Hui C, Motoyama J, Makino S, Shiroishi T, Gaffield W, Yamada G. Unique functions of sonic hedgehog signaling during external genitalia development. *Development*. 2001;128:4241-4250

94. Ingham PW, McMahon AP. Hedgehog signaling in animal development: Paradigms and principles. *Genes Dev*. 2001;15:3059-3087

95. Barakat MT, Humke EW, Scott MP. Learning from jekyll to control hyde: Hedgehog signaling in development and cancer. *Trends Mol Med*. 2010;16:337-348

96. Yan M, Wang L, Zuo H, Zhang Z, Chen W, Mao L, Zhang P. Hh/gli signalling as a new therapeutic target for patients with oral squamous cell carcinoma. *Oral oncology*. 2011;47:504-509

97. Lauth M, Rohnalter V, Bergstrom A, Kooshesh M, Svenningsson P, Toftgard R. Antipsychotic drugs regulate hedgehog signaling by modulation of 7-dehydrocholesterol reductase levels. *Mol Pharmacol*. 2010;78:486-496

98. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function. *Nature*. 1996;383:407-413

99. Bitgood MJ, Shen L, McMahon AP. Sertoli cell signaling by desert hedgehog regulates the male germline. *Curr Biol*. 1996;6:298-304

100. Porter JA, Young KE, Beachy PA. Cholesterol modification of hedgehog signaling proteins in animal development. *Science*. 1996;274:255-259

101. Pepinsky RB, Zeng C, Wen D, Rayhorn P, Baker DP, Williams KP, Bixler SA, Ambrose CM, Garber EA, Miatkowski K, Taylor FR, Wang EA, Galdes A. Identification of a palmitic acid-modified form of human sonic hedgehog. *J Biol Chem*. 1998;273:14037-14045

102. Chamoun Z, Mann RK, Nellen D, von Kessler DP, Bellotto M, Beachy PA, Basler K. Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. *Science*. 2001;293:2080-2084

103. Lee JD, Kraus P, Gaiano N, Nery S, Kohtz J, Fishell G, Loomis CA, Treisman JE. An acylatable residue of hedgehog is differentially required in drosophila and mouse limb development. *Dev Biol*. 2001;233:122-136

104. Farzan SF, Singh S, Schilling NS, Robbins DJ. The adventures of sonic hedgehog in development and repair. III. Hedgehog processing and biological activity. *Am J Physiol Gastrointest Liver Physiol*. 2008;294:G844-849

105. Tian H, Jeong J, Harfe BD, Tabin CJ, McMahon AP. Mouse disp1 is required in sonic hedgehog-expressing cells for paracrine activity of the cholesterol-modified ligand. *Development*. 2005;132:133-142

106. Tenzen T, Allen BL, Cole F, Kang JS, Krauss RS, McMahon AP. The cell surface membrane proteins cdo and boc are components and targets of the hedgehog signaling pathway and feedback network in mice. *Dev Cell*. 2006;10:647-656

107. Allen BL, Song JY, Izzi L, Althaus IW, Kang JS, Charron F, Krauss RS, McMahon AP. Overlapping roles and collective requirement for the coreceptors gas1, cdo, and boc in shh pathway function. *Dev Cell*. 2011;20:775-787

108. Allen BL, Tenzen T, McMahon AP. The hedgehog-binding proteins gas1 and cdo cooperate to positively regulate shh signaling during mouse development. *Genes Dev*. 2007;21:1244-1257

109. Chen MH, Wilson CW, Li YJ, Law KK, Lu CS, Gacayan R, Zhang X, Hui CC, Chuang PT. Cilium-independent regulation of gli protein function by sufu in hedgehog signaling is evolutionarily conserved. *Genes Dev*. 2009;23:1910-1928

110. Hammerschmidt M, Bitgood MJ, McMahon AP. Protein kinase a is a common negative regulator of hedgehog signaling in the vertebrate embryo. *Genes Dev*. 1996;10:647-658

111. Tuson M, He M, Anderson KV. Protein kinase a acts at the basal body of the primary cilium to prevent gli2 activation and ventralization of the mouse neural tube. *Development*. 2011;138:4921-4930

112. Tukachinsky H, Lopez LV, Salic A. A mechanism for vertebrate hedgehog signaling: Recruitment to cilia and dissociation of sufu-gli protein complexes. *J Cell Biol*. 2010;191:415-428

113. Humke EW, Dorn KV, Milenkovic L, Scott MP, Rohatgi R. The output of hedgehog signaling is controlled by the dynamic association between suppressor of fused and the gli proteins. *Genes Dev*. 2010;24:670-682

114. Wen X, Lai CK, Evangelista M, Hongo JA, de Sauvage FJ, Scales SJ. Kinetics of hedgehog-dependent full-length gli3 accumulation in primary cilia and subsequent degradation. *Mol Cell Biol*. 2010;30:1910-1922

115. Schrader EK, Harstad KG, Holmgren RA, Matouschek A. A three-part signal governs differential processing of gli1 and gli3 proteins by the proteasome. *J Biol Chem*. 2011;286:39051-39058

116. Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H. Regulation of gli2 and gli3 activities by an amino-terminal repression domain: Implication of gli2 and gli3 as primary mediators of shh signaling. *Development*. 1999;126:3915-3924

117. Ruiz i Altaba A, Mas C, Stecca B. The gli code: An information nexus regulating cell fate, stemness and cancer. *Trends in cell biology*. 2007;17:438-447

118. Devine CA, Sbrogna JL, Guner B, Osgood M, Shen MC, Karlstrom RO. A dynamic gli code interprets hh signals to regulate induction, patterning, and endocrine cell specification in the zebrafish pituitary. *Dev Biol*. 2009;326:143-154

119. Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV. Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature*. 2003;426:83-87

120. Rohatgi R, Scott MP. Patching the gaps in hedgehog signalling. *Nat Cell Biol*. 2007;9:1005-1009

121. Maloverjan A, Piirsoo M, Kasak L, Peil L, Osterlund T, Kogerman P. Dual function of unc-51-like kinase 3 (ulk3) in the sonic hedgehog signaling pathway. *J Biol Chem*. 2010;285:30079-30090

122. Maloverjan A, Piirsoo M, Michelson P, Kogerman P, Osterlund T. Identification of a novel serine/threonine kinase ulk3 as a positive regulator of hedgehog pathway. *Exp Cell Res*. 2010;316:627-637

123. Katoh Y, Katoh M. Hedgehog signaling pathway and gastrointestinal stem cell signaling network (review). *Int J Mol Med*. 2006;18:1019-1023

124. Zhang Q, Shi Q, Chen Y, Yue T, Li S, Wang B, Jiang J. Multiple ser/thr-rich degrons mediate the degradation of ci/gli by the cul3-hib/spop e3 ubiquitin ligase. *Proc Natl Acad Sci U S A*. 2009;106:21191-21196

125. Palma V, Carrasco H, Reinchisi G, Olivares G, Faunes F, Larrain J. Shh activity and localization is regulated by perlecan. *Biol Res*. 2011;44:63-67

126. Eggenschwiler JT, Anderson KV. Cilia and developmental signaling. *Annu Rev Cell Dev Biol*. 2007;23:345-373

127. Lancaster MA, Schroth J, Gleeson JG. Subcellular spatial regulation of canonical wnt signalling at the primary cilium. *Nat Cell Biol*. 2011;13:700-707

128. Schneider L, Cammer M, Lehman J, Nielsen SK, Guerra CF, Veland IR, Stock C, Hoffmann EK, Yoder BK, Schwab A, Satir P, Christensen ST. Directional cell migration and chemotaxis in wound healing response to PDGF-AA are coordinated by the primary cilium in fibroblasts. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2010;25:279-292

129. Zaghloul NA, Brugmann SA. The emerging face of primary cilia. *Genesis*. 2011;49:231-246

130. Goetz SC, Anderson KV. The primary cilium: A signalling centre during vertebrate development. *Nat Rev Genet*. 2010;11:331-344

131. Tobin JL, Beales PL. The nonmotile ciliopathies. *Genetics in medicine : official journal of the American College of Medical Genetics*. 2009;11:386-402

132. Roy S. Cilia and hedgehog: When and how was their marriage solemnized? *Differentiation*. 2012;83:S43-48

133. Satir P, Christensen ST. Overview of structure and function of mammalian cilia. *Annual review of physiology*. 2007;69:377-400

134. Satir P, Pedersen LB, Christensen ST. The primary cilium at a glance. *J Cell Sci*. 2010;123:499-503

135. Cortellino S, Wang C, Wang B, Bassi MR, Caretti E, Champeval D, Calmont A, Jarnik M, Burch J, Zaret KS, Larue L, Bellacosa A. Defective ciliogenesis, embryonic lethality and severe impairment of the sonic hedgehog pathway caused by inactivation of the mouse complex a intraflagellar transport gene ift122/wdr10, partially overlapping with the DNA repair gene med1/mbd4. *Dev Biol*. 2009;325:225-237

136. Gorivodsky M, Mukhopadhyay M, Wilsch-Braeuninger M, Phillips M, Teufel A, Kim C, Malik N, Huttner W, Westphal H. Intraflagellar transport protein 172 is essential for primary cilia formation and plays a vital role in patterning the mammalian brain. *Dev Biol*. 2009;325:24-32

137. Ocbina PJ, Anderson KV. Intraflagellar transport, cilia, and mammalian hedgehog signaling: Analysis in mouse embryonic fibroblasts. *Dev Dyn*. 2008;237:2030-2038

138. Chang CF, Ramaswamy G, Serra R. Depletion of primary cilia in articular chondrocytes results in reduced gli3 repressor to activator ratio, increased hedgehog signaling, and symptoms of early osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2012;20:152-161

139. Qin J, Lin Y, Norman RX, Ko HW, Eggenschwiler JT. Intraflagellar transport protein 122 antagonizes sonic hedgehog signaling and controls ciliary localization of pathway components. *Proc Natl Acad Sci U S A*. 2011;108:1456-1461

140. Cheung HO, Zhang X, Ribeiro A, Mo R, Makino S, Puviindran V, Law KK, Briscoe J, Hui CC. The kinesin protein kif7 is a critical regulator of gli transcription factors in mammalian hedgehog signaling. *Science signaling*. 2009;2:ra29

141. Endoh-Yamagami S, Evangelista M, Wilson D, Wen X, Theunissen JW, Phamluong K, Davis M, Scales SJ, Solloway MJ, de Sauvage FJ, Peterson AS. The mammalian cos2 homolog kif7 plays an essential role in modulating hh signal transduction during development. *Curr Biol*. 2009;19:1320-1326

142. Liem KF, Jr., He M, Ocbina PJ, Anderson KV. Mouse kif7/costal2 is a cilia-associated protein that regulates sonic hedgehog signaling. *Proc Natl Acad Sci U S A*. 2009;106:13377-13382

143. Farzan SF, Ascano M, Jr., Ogden SK, Sanial M, Brigui A, Plessis A, Robbins DJ. Costal2 functions as a kinesin-like protein in the hedgehog signal transduction pathway. *Curr Biol*. 2008;18:1215-1220

144. Rink JC, Gurley KA, Elliott SA, Sanchez Alvarado A. Planarian Hh signaling regulates regeneration polarity and links Hh pathway evolution to cilia. *Science*. 2009;326:1406-1410

145. Glazer AM, Wilkinson AW, Backer CB, Lapan SW, Gutzman JH, Cheeseman IM, Reddien PW. The zn finger protein iguana impacts hedgehog signaling by promoting ciliogenesis. *Dev Biol*. 2010;337:148-156

146. Lawson ND, Vogel AM, Weinstein BM. Sonic hedgehog and vascular endothelial growth factor act upstream of the notch pathway during arterial endothelial differentiation. *Dev Cell*. 2002;3:127-136

147. Williams C, Kim SH, Ni TT, Mitchell L, Ro H, Penn JS, Baldwin SH, Solnica-Krezel L, Zhong TP. Hedgehog signaling induces arterial endothelial cell formation by repressing venous cell fate. *Dev Biol*. 2010;341:196-204

148. Byrd N, Becker S, Maye P, Narasimhaiah R, St-Jacques B, Zhang X, McMahon J, McMahon A, Grabel L. Hedgehog is required for murine yolk sac angiogenesis. *Development*. 2002;129:361-372

149. Straface G, Aprahamian T, Flex A, Gaetani E, Biscetti F, Smith RC, Pecorini G, Pola E, Angelini F, Stigliano E, Castellot JJ, Jr., Losordo DW, Pola R. Sonic hedgehog regulates angiogenesis and myogenesis during post-natal skeletal muscle regeneration. *J Cell Mol Med*. 2009;13:2424-2435

150. Nagase T, Nagase M, Yoshimura K, Fujita T, Koshima I. Angiogenesis within the developing mouse neural tube is dependent on sonic hedgehog signaling: Possible roles of motor neurons. *Genes Cells*. 2005;10:595-604

151. Surace EM, Balaggan KS, Tessitore A, Mussolino C, Cotugno G, Bonetti C, Vitale A, Ali RR, Auricchio A. Inhibition of ocular neovascularization by hedgehog blockade. *Mol Ther*. 2006;13:573-579

152. Lamont RE, Vu W, Carter AD, Serluca FC, MacRae CA, Childs SJ. Hedgehog signaling via angiopoietin1 is required for developmental vascular stability. *Mech Dev*. 2010;127:159-168

153. Renault MA, Roncalli J, Tongers J, Misener S, Thorne T, Jujo K, Ito A, Clarke T, Fung C, Millay M, Kamide C, Scarpelli A, Klyachko E, Losordo DW. The hedgehog transcription factor gli3 modulates angiogenesis. *Circ Res*. 2009;105:818-826

154. Kanda S, Mochizuki Y, Suematsu T, Miyata Y, Nomata K, Kanetake H. Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. *J Biol Chem*. 2003;278:8244-8249

155. Renault MA, Roncalli J, Tongers J, Thorne T, Klyachko E, Misener S, Volpert OV, Mehta S, Burg A, Luedemann C, Qin G, Kishore R, Losordo DW. Sonic hedgehog induces angiogenesis via rho kinase-dependent signaling in endothelial cells. *Journal of molecular and cellular cardiology*. 2010;49:490-498

156. Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. *Neuron*. 2003;39:937-950

157. Dahmane N, Ruiz i Altaba A. Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development*. 1999;126:3089-3100

158. Wallace VA. Purkinje-cell-derived sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum. *Curr Biol*. 1999;9:445-448

159. Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by sonic hedgehog. *Neuron*. 1999;22:103-114

160. Ahn S, Joyner AL. In vivo analysis of quiescent adult neural stem cells responding to sonic hedgehog. *Nature*. 2005;437:894-897

161. Sicklick JK, Li YX, Melhem A, Schmelzer E, Zdanowicz M, Huang J, Caballero M, Fair JH, Ludlow JW, McClelland RE, Reid LM, Diehl AM. Hedgehog signaling maintains resident hepatic progenitors throughout life. *Am J Physiol Gastrointest Liver Physiol*. 2006;290:G859-870

162. Brownell I, Guevara E, Bai CB, Loomis CA, Joyner AL. Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. *Cell Stem Cell*. 2011;8:552-565

163. Adolphe C, Narang M, Ellis T, Wicking C, Kaur P, Wainwright B. An in vivo comparative study of sonic, desert and indian hedgehog reveals that hedgehog pathway activity regulates epidermal stem cell homeostasis. *Development*. 2004;131:5009-5019

164. Leek RD, Talks KL, Pezzella F, Turley H, Campo L, Brown NS, Bicknell R, Taylor M, Gatter KC, Harris AL. Relation of hypoxia-inducible factor-2 alpha (hif-2 alpha) expression in tumor-

infiltrative macrophages to tumor angiogenesis and the oxidative thymidine phosphorylase pathway in human breast cancer. *Cancer Res*. 2002;62:1326-1329

165. Leek RD, Harris AL. Tumor-associated macrophages in breast cancer. *J Mammary Gland Biol Neoplasia*. 2002;7:177-189

166. Schmid MC, Varner JA. Chapter 15. Methods to study myeloid cell roles in angiogenesis. *Methods Enzymol*. 2008;445:343-371

167. Lewis C, Murdoch C. Macrophage responses to hypoxia: Implications for tumor progression and anti-cancer therapies. *Am J Pathol*. 2005;167:627-635

168. Gelati M, Aplin AC, Fogel E, Smith KD, Nicosia RF. The angiogenic response of the aorta to injury and inflammatory cytokines requires macrophages. *J Immunol*. 2008;181:5711-5719

169. Rymo SF, Gerhardt H, Wolfhagen Sand F, Lang R, Uv A, Betsholtz C. A two-way communication between microglial cells and angiogenic sprouts regulates angiogenesis in aortic ring cultures. *PLoS One*. 2011;6:e15846

170. Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozhij S, Peri F, Wilson SW, Ruhrberg C. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of vegf-mediated endothelial tip cell induction. *Blood*. 2010;116:829-840

171. Jongstra-Bilen J, Haidari M, Zhu SN, Chen M, Guha D, Cybulsky MI. Low-grade chronic inflammation in regions of the normal mouse arterial intima predisposed to atherosclerosis. *J Exp Med*. 2006;203:2073-2083

172. Crompton T, Outram SV, Hager-Theodorides AL. Sonic hedgehog signalling in T-cell development and activation. *Nat Rev Immunol*. 2007;7:726-735

173. Rowbotham NJ, Hager-Theodorides AL, Cebecauer M, Shah DK, Drakopoulou E, Dyson J, Outram SV, Crompton T. Activation of the hedgehog signaling pathway in T-lineage cells inhibits tcr repertoire selection in the thymus and peripheral T-cell activation. *Blood*. 2007;109:3757-3766

174. Rowbotham NJ, Hager-Theodorides AL, Furmanski AL, Crompton T. A novel role for hedgehog in t-cell receptor signaling: Implications for development and immunity. *Cell Cycle*. 2007;6:2138-2142

175. Wakelin SJ, Forsythe JL, Garden OJ, Howie SE. Commercially available recombinant sonic hedgehog up-regulates ptc and modulates the cytokine and chemokine expression of human macrophages: An effect mediated by endotoxin contamination? *Immunobiology*. 2008;213:25-38

176. Schumacher MA, Donnelly JM, Engevik AC, Xiao C, Yang L, Kenny S, Varro A, Hollande F, Samuelson LC, Zavros Y. Gastric sonic hedgehog acts as a macrophage chemoattractant during the immune response to helicobacter pylori. *Gastroenterology*. 2012; Epub ahead of print. DOI: 10.1053/j.gastro.2012.01.029

177. Cao Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest*. 2007;117:2362-2368

178. Cao Y. Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. *Nat Rev Drug Discov*. 2010;9:107-115

179. Tang W, Zeve D, Suh JM, Bosnakovski D, Kyba M, Hammer RE, Tallquist MD, Graff JM. White fat progenitor cells reside in the adipose vasculature. *Science*. 2008;322:583-586

180. Cao R, Brakenhielm E, Wahlestedt C, Thyberg J, Cao Y. Leptin induces vascular permeability and synergistically stimulates angiogenesis with fgf-2 and vegf. *Proc Natl Acad Sci U S A*. 2001;98:6390-6395

181. Pospisilik JA, Schramek D, Schnidar H, Cronin SJ, Nehme NT, Zhang X, Knauf C, Cani PD, Aumayr K, Todoric J, Bayer M, Haschemi A, Puviindran V, Tar K, Orthofer M, Neely GG, Dietzl G, Manoukian A, Funovics M, Prager G, Wagner O, Ferrandon D, Aberger F, Hui CC, Esterbauer H, Penninger JM. Drosophila genome-wide obesity screen reveals hedgehog as a determinant of brown versus white adipose cell fate. *Cell*. 2010;140:148-160

182. Police SB, Thatcher SE, Charnigo R, Daugherty A, Cassis LA. Obesity promotes inflammation in periaortic adipose tissue and angiotensin ii-induced abdominal aortic aneurysm formation. *Arterioscler Thromb Vasc Biol*. 2009;29:1458-1464

183. Korshunov VA, Berk BC. Flow-induced vascular remodeling in the mouse: A model for carotid intima-media thickening. *Arterioscler Thromb Vasc Biol*. 2003;23:2185-2191

184. Stenmark KR, Davie N, Frid M, Gerasimovskaya E, Das M. Role of the adventitia in pulmonary vascular remodeling. *Physiology*. 2006;21:134-145

185. Varjosalo M, Taipale J. Hedgehog: Functions and mechanisms. *Genes Dev*. 2008;22:2454-2472

186. Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science*. 1997;277:1109-1113

187. Deltagen-Inc. NIH initiative supporting placement of deltagen, inc. Mice into public repositories. Mgi ref id j:101679 2005

188. Bai CB, Auerbach W, Lee JS, Stephen D, Joyner AL. Gli2, but not gli1, is required for initial shh signaling and ectopic activation of the shh pathway. *Development*. 2002;129:4753-4761

189. Bai CB, Joyner AL. Gli1 can rescue the in vivo function of gli2. *Development*. 2001;128:5161-5172

190. Shen Q, Wang Y, Kokovay E, Lin G, Chuang SM, Goderie SK, Roysam B, Temple S. Adult SVZ stem cells lie in a vascular niche: A quantitative analysis of niche cell-cell interactions. *Cell Stem Cell*. 2008;3:289-300

191. Song X, Zhu CH, Doan C, Xie T. Germline stem cells anchored by adherens junctions in the drosophila ovary niches. *Science*. 2002;296:1855-1857

192. Ahn S, Joyner AL. Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell*. 2004;118:505-516

193. Garcia AD, Petrova R, Eng L, Joyner AL. Sonic hedgehog regulates discrete populations of astrocytes in the adult mouse forebrain. *J Neurosci*. 2010;30:13597-13608

194. Li Y, Hibbs MA, Gard AL, Shylo NA, Yun K. Genome-wide analysis of n1icd/rbpj targets in vivo reveals direct transcriptional regulation of wnt, shh, and hippo pathway effectors by notch1. *Stem Cells*. 2012;Epub ahead of print:DOI: 10.1002/stem.1030

195. Dennler S, Andre J, Alexaki I, Li A, Magnaldo T, ten Dijke P, Wang XJ, Verrecchia F, Mauviel A. Induction of sonic hedgehog mediators by transforming growth factor-beta: Smad3-dependent activation of gli2 and gli1 expression in vitro and in vivo. *Cancer Res*. 2007;67:6981-6986

196. Dennler S, Andre J, Verrecchia F, Mauviel A. Cloning of the human gli2 promoter: Transcriptional activation by transforming growth factor-beta via smad3/beta-catenin cooperation. *J Biol Chem*. 2009;284:31523-31531

197. Boucher JM, Peterson SM, Urs S, Zhang C, Liaw L. The mir-143/145 cluster is a novel transcriptional target of jagged-1/notch signaling in vascular smooth muscle cells. *J Biol Chem*. 2011;286:28312-28321

198. Tang Y, Urs S, Boucher J, Bernaiche T, Venkatesh D, Spicer DB, Vary CP, Liaw L. Notch and transforming growth factor-beta (tgfbeta) signaling pathways cooperatively regulate vascular smooth muscle cell differentiation. *J Biol Chem*. 2010;285:17556-17563

199. Sanchez-Camacho C, Bovolenta P. Autonomous and non-autonomous shh signalling mediate the in vivo growth and guidance of mouse retinal ganglion cell axons. *Development*. 2008;135:3531-3541

200. Liu Z, Owen T, Zhang L, Zuo J. Dynamic expression pattern of sonic hedgehog in developing cochlear spiral ganglion neurons. *Dev Dyn*. 2010;239:1674-1683

201. Calloni GW, Glavieux-Pardanaud C, Le Douarin NM, Dupin E. Sonic hedgehog promotes the development of multipotent neural crest progenitors endowed with both mesenchymal and neural potentials. *Proc Natl Acad Sci U S A*. 2007;104:19879-19884

202. Olivetti G, Anversa P, Melissari M, Loud AV. Morphometric study of early postnatal development of the thoracic aorta in the rat. *Circ Res*. 1980;47:417-424

203. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84:767-801

204. Nolan-Stevaux O, Lau J, Truitt ML, Chu GC, Hebrok M, Fernandez-Zapico ME, Hanahan D. Gli1 is regulated through smoothened-independent mechanisms in neoplastic pancreatic ducts and mediates pdac cell survival and transformation. *Genes Dev*. 2009;23:24-36

205. Li M, Li C, Liu YH, Xing Y, Hu L, Borok Z, Kwong KY, Minoo P. Mesodermal deletion of transforming growth factor-beta receptor II disrupts lung epithelial morphogenesis: Cross-talk between tgf-beta and sonic hedgehog pathways. *J Biol Chem*. 2008;283:36257-36264

206. Schreck KC, Taylor P, Marchionni L, Gopalakrishnan V, Bar EE, Gaiano N, Eberhart CG. The notch target hes1 directly modulates gli1 expression and hedgehog signaling: A potential mechanism of therapeutic resistance. *Clin Cancer Res*. 2010;16:6060-6070

207. Bhatia N, Thiyagarajan S, Elcheva I, Saleem M, Dlugosz A, Mukhtar H, Spiegelman VS. Gli2 is targeted for ubiquitination and degradation by beta-trcp ubiquitin ligase. *J Biol Chem*. 2006;281:19320-19326

208. Kesper DA, Didt-Koziel L, Vortkamp A. Gli2 activator function in preosteoblasts is sufficient to mediate ihh-dependent osteoblast differentiation, whereas the repressor function of gli2 is dispensable for endochondral ossification. *Dev Dyn*. 2010;239:1818-1826

209. Bishop B, Aricescu AR, Harlos K, O'Callaghan CA, Jones EY, Siebold C. Structural insights into hedgehog ligand sequestration by the human hedgehog-interacting protein hhip. *Nature structural & molecular biology*. 2009;16:698-703

210. Chuang PT, Kawcak T, McMahon AP. Feedback control of mammalian hedgehog signaling by the hedgehog-binding protein, hip1, modulates fgf signaling during branching morphogenesis of the lung. *Genes Dev*. 2003;17:342-347

211. Bar EE, Lin A, Mahairaki V, Matsui W, Eberhart CG. Hypoxia increases the expression of stem-cell markers and promotes clonogenicity in glioblastoma neurospheres. *Am J Pathol*. 2010;177:1491-1502

212. Roy S, Tripathy M, Mathur N, Jain A, Mukhopadhyay A. Hypoxia improves expansion potential of human cord blood-derived hematopoietic stem cells and marrow repopulation efficiency. *European journal of haematology*. 2012; Epub ahead of print. DOI: 10.1111/j.1600-0609.2012.01759.x

213. Valorani MG, Germani A, Otto WR, Harper L, Biddle A, Khoo CP, Lin WR, Hawa MI, Tropel P, Patrizi MP, Pozzilli P, Alison MR. Hypoxia increases sca-1/CD44 co-expression in murine mesenchymal stem cells and enhances their adipogenic differentiation potential. *Cell Tissue Res*. 2010;341:111-120

214. Niinikoski J, Heughan C, Hunt TK. Oxygen tensions in the aortic wall of normal rabbits. *Atherosclerosis*. 1973;17:353-359

215. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nature reviews. Molecular cell biology*. 2007;8:464-478

216. Swift MR, Weinstein BM. Arterial-venous specification during development. *Circ Res*. 2009;104:576-588

217. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and vegf in vascular development. *Nature*. 2005;438:937-945

218. Ellertsdottir E, Lenard A, Blum Y, Krudewig A, Herwig L, Affolter M, Belting HG. Vascular morphogenesis in the zebrafish embryo. *Dev Biol*. 2010;341:56-65

219. Morrow D, Guha S, Sweeney C, Birney Y, Walshe T, O'Brien C, Walls D, Redmond EM, Cahill PA. Notch and vascular smooth muscle cell phenotype. *Circ Res*. 2008;103:1370-1382

220. Kameda Y. Hoxa3 and signaling molecules involved in aortic arch patterning and remodeling. *Cell Tissue Res*. 2009;336:165-178

221. Hoglund VJ, Majesky MW. Patterning the artery wall by lateral induction of notch signaling. *Circulation*. 2012;125:212-215

222. Manderfield LJ, High FA, Engleka KA, Liu F, Li L, Rentschler S, Epstein JA. Notch activation of jagged1 contributes to the assembly of the arterial wall. *Circulation*. 2012;125:314-323

223. Santilli SM, Stevens RB, Anderson JG, Payne WD, Caldwell MD. Transarterial wall oxygen gradients at the dog carotid bifurcation. *Am J Physiol*. 1995;268:H155-161

224. Brown CB, Baldwin HS. Neural crest contribution to the cardiovascular system. *Advances in experimental medicine and biology*. 2006;589:134-154

225. Li DY, Brooke B, Davis EC, Mecham RP, Sorensen LK, Boak BB, Eichwald E, Keating MT. Elastin is an essential determinant of arterial morphogenesis. *Nature*. 1998;393:276-280

226. Wolinsky H, Glagov S. A lamellar unit of aortic medial structure and function in mammals. *Circ Res*. 1967;20:99-111

227. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature*. 1982;300:611-615

228. Dilley RJ, Schwartz SM. Vascular remodeling in the growth hormone transgenic mouse. *Circ Res*. 1989;65:1233-1240

229. Li DY, Faury G, Taylor DG, Davis EC, Boyle WA, Mecham RP, Stenzel P, Boak B, Keating MT. Novel arterial pathology in mice and humans hemizygous for elastin. *J Clin Invest*. 1998;102:1783-1787

230. Faury G, Pezet M, Knutsen RH, Boyle WA, Heximer SP, McLean SE, Minkes RK, Blumer KJ, Kovacs A, Kelly DP, Li DY, Starcher B, Mecham RP. Developmental adaptation of the mouse cardiovascular system to elastin haploinsufficiency. *J Clin Invest*. 2003;112:1419-1428

231. Wagenseil JE, Mecham RP. Vascular extracellular matrix and arterial mechanics. *Physiol Rev*. 2009;89:957-989

232. Daniel JM, Bielenberg W, Stieger P, Weinert S, Tillmanns H, Sedding DG. Time-course analysis on the differentiation of bone marrow-derived progenitor cells into smooth muscle cells during neointima formation. *Arterioscler Thromb Vasc Biol*. 2010;30:1890-1896

233. Iwata H, Manabe I, Fujiu K, Yamamoto T, Takeda N, Eguchi K, Furuya A, Kuro-o M, Sata M, Nagai R. Bone marrow-derived cells contribute to vascular inflammation but do not differentiate into smooth muscle cell lineages. *Circulation*. 2010;122:2048-2057

234. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-cre transgenic mice: A new model for endothelial cell-lineage analysis in vivo. *Dev Biol*. 2001;230:230-242

235. Majesky MW. Developmental basis of vascular smooth muscle diversity. *Arterioscler Thromb Vasc Biol*. 2007;27:1248-1258

236. Aguero TH, Fernandez JP, Vega Lopez GA, Tribulo C, Aybar MJ. Indian hedgehog signaling is required for proper formation, maintenance and migration of xenopus neural crest. *Dev Biol*. 2012; Epub ahead of print. DOI: 10.1016/j.ydbio.2012.01.020

237. Gilbert SF. *Developmental biology, eighth edition*. Sunderland, MA: Sinauer Associates, Inc.; 2006.

238. Peter Lopez T, Fan CM. A transgenic tbx6;creER(t2) line for inducible gene manipulation in the presomitic mesoderm. *Genesis*. 2011

239. Jukkola T, Trokovic R, Maj P, Lamberg A, Mankoo B, Pachnis V, Savilahti H, Partanen J. Meox1cre: A mouse line expressing cre recombinase in somitic mesoderm. *Genesis*. 2005;43:148-153

240. Watanabe Y, Buckingham M. The formation of the embryonic mouse heart: Heart fields and myocardial cell lineages. *Ann N Y Acad Sci*. 2010;1188:15-24

241. Samokhvalov IM, Samokhvalova NI, Nishikawa S. Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. *Nature*. 2007;446:1056-1061

242. Yang H, Xiang J, Wang N, Zhao Y, Hyman J, Li S, Jiang J, Chen JK, Yang Z, Lin S. Converse conformational control of smoothened activity by structurally related small molecules. *J Biol Chem*. 2009;284:20876-20884

243. Neumann CJ, Nuesslein-Volhard C. Patterning of the zebrafish retina by a wave of sonic hedgehog activity. *Science*. 2000;289:2137-2139

244. Ariga J, Walker SL, Mumm JS. Multicolor time-lapse imaging of transgenic zebrafish: Visualizing retinal stem cells activated by targeted neuronal cell ablation. *Journal of visualized experiments : JoVE*. 2010

245. Holden BJ, Bratt DG, Chico TJ. Molecular control of vascular development in the zebrafish. *Birth defects research. Part C, Embryo today : reviews*. 2011;93:134-140

246. Haga Y, Dominique VJ, 3rd, Du SJ. Analyzing notochord segmentation and intervertebral disc formation using the twhh:Gfp transgenic zebrafish model. *Transgenic Res*. 2009;18:669-683

247. Avaron F, Hoffman L, Guay D, Akimenko MA. Characterization of two new zebrafish members of the hedgehog family: Atypical expression of a zebrafish indian hedgehog gene in skeletal elements of both endochondral and dermal origins. *Dev Dyn*. 2006;235:478-489

248. Elmendorf JS, Chen D, Pessin JE. Guanosine 5'-o-(3-thiotriphosphate) (gtpgammas) stimulation of glut4 translocation is tyrosine kinase-dependent. *J Biol Chem*. 1998;273:13289-13296

249. Bramono DS, Murali S, Rai B, Ling L, Poh WT, Lim ZX, Stein GS, Nurcombe V, van Wijnen AJ, Cool SM. Bone marrow-derived heparan sulfate potentiates the osteogenic activity of bone morphogenetic protein-2 (bmp-2). *Bone*. 2011

250. Vishnubalaji R, Manikandan M, Al-Nbaheen M, Kadalmani B, Aldahmash A, Alajez NM. In vitro differentiation of human skin-derived multipotent stromal cells into putative endothelial-like cells. *BMC developmental biology*. 2012;12:7

251. Manzanero S. Generation of mouse bone marrow-derived macrophages. *Methods Mol Biol*. 2012;844:177-181

252. Long F, Zhang XM, Karp S, Yang Y, McMahon AP. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development*. 2001;128:5099-5108

253. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using lysmcre mice. *Transgenic Res*. 1999;8:265-277

254. Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev*. 2004;18:937-951

255. Wood W, Turmaine M, Weber R, Camp V, Maki RA, McKercher SR, Martin P. Mesenchymal cells engulf and clear apoptotic footplate cells in macrophageless pu.1 null mouse embryos. *Development*. 2000;127:5245-5252

256. Majesky MW, Dong XR, Regan JN, Hoglund VJ. Vascular smooth muscle progenitor cells: Building and repairing blood vessels. *Circ Res*. 2011;108:365-377