Patterning the Artery Wall by Lateral Induction of Notch Signaling

Running title: Hoglund et al.; Lateral induction of Notch signaling

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Journal Subject Codes: [162] Smooth muscle proliferation and differentiation; [139] Developmental biology; [145] Genetically altered mice

Key words: editorial, vascular development, smooth muscle progenitor cell, notch signaling, neural crest
Blood vessels exhibit a common structure composed of layers of cells encircling a central lumen. Arteries generally have more layers than veins, and different arteries in the same individual can have different numbers of layers. Since all blood vessels are built around a monolayer of endothelial cells, then the variation in wall structure is due to variable numbers of smooth muscle-containing layers in the tunica media. Indeed, Wolinsky and Glagov’s classic paper showed that the relation between lumen diameter and wall thickness across a wide range of mammalian species is a function of the number of layers of smooth muscle and elastic fibers that are present in the arterial media. Yet precisely how layers of smooth muscle are formed during vascular development and what molecular mechanisms operate to produce different numbers of layers in different blood vessels is still poorly understood.

Lessons from Transgenic Mice

For many years the prevailing hypothesis has been that wall thickness is determined by wall tension and the number of layers found in a given blood vessel is that number sufficient to normalize wall tension to values (dynes/cm²) within a narrow physiological range per layer. The advent of transgenic mouse technology generated new animal models that provided for interesting tests of the accepted paradigms for development of vessel wall structure. For example, mice expressing a growth hormone transgene driven by a metallothionein promoter (MtGH) reached a body mass 1.8-fold that of wild type littermate mice. Most of the internal organs in these mice exhibited proportional increases in mass compared to wild type littermates (e.g., kidney = 1.9-fold; heart = 1.8-fold). To accommodate the increases in blood flow required to support these larger bodies, the aortas of transgenic MtGH mice exhibited a number of structural changes. For example, aortic lumen diameter was increased (1.22-fold), the wall was thicker (1.24-fold), and the number of smooth muscle cells (SMCs) in the tunica media was
increased by 30%. Despite these increases in overall wall size, there were no changes in the number of layers in any of the arteries examined in MtGH transgenic mice. Thus the aortas of MtGH transgenic mice were able to compensate for additional demands for cardiac output and consequent increases in wall tension necessary to support a larger animal by increasing the number and size of SMCs found within thicker individual layers, but the number of SMC-containing layers seemed to be fixed. Since the increases in circulating growth hormone in MtGH transgenic mice become apparent after birth, these findings pointed to mechanisms that operate in utero to determine the characteristic number of layers in the tunica media that are found in different arteries in adult animals. More recently, a second transgenic model was described that confirmed and extended this conclusion. Mice that are completely deficient in elastin (Eln<sup>−/−</sup>) die in the neonatal period from obstructive arterial disease. However, Eln<sup>+/−</sup> mice survive and exhibit various structural adaptations in their elastic arteries. The walls of ascending aorta, common carotid artery, and abdominal aorta are thinner and these arteries are smaller in overall diameter than wild type. Yet, Eln<sup>+/−</sup> mice exhibit an increase of ~35% in the number of layers of elastic lamellae in thoracic and abdominal aorta. The extra layers begin to appear around E18.5 and are completed by birth. Since the normal number of smooth muscle-containing layers in the aorta is laid down by E14.5 to E15.5, the Eln<sup>+/−</sup> mice would appear to define a second window of time from E18.5 to birth in which the artery wall can adjust the number of smooth muscle layers to normalize wall strain – but after birth this adaptive mechanism appears to no longer operate.

**The Role of Notch Signaling in Layer Formation in Developing Arteries**

Recently, evidence has been accumulating to suggest that at least one important mechanism that functions to organize and pattern SMCs in artery walls is Notch signaling. The earliest steps in
formation of the tunica media involve establishment of adhesive contacts between endothelial
cells and smooth muscle progenitors via heterotypic cell-cell adhesion mediated by N-cadherin.\textsuperscript{8} This brings endothelial cell surface molecules into contact with partners on adjacent SMC progenitors triggering SMC differentiation in the progenitors and the initiation of tunica media formation. With continued development, an iterative sequence is set into motion that assembles SMC progenitors into the number of layers characteristic for a given artery.\textsuperscript{1} This process appears to involve physical contact between incoming cells and cells already present in the developing vessel wall.\textsuperscript{8,9} Notch signaling is initiated by four Notch receptors (Notch 1-4) interacting with five Notch ligands (Jagged 1, 2 and Delta-like 1, 3 and 4).\textsuperscript{10-12} Both the ligands and receptors are transmembrane proteins whose activation is promoted by cell-cell contact. Evidence that Notch signaling plays an important role in endothelial contact-dependent differentiation of smooth muscle progenitor cells \textit{in vivo} was provided by the results of cell-specific deletion of Notch signaling activity in neural crest-derived progenitors\textsuperscript{13} and in experiments where the Notch ligand Jagged-1 (Jag1) was deleted specifically in endothelial cells.\textsuperscript{14}

\textbf{Positive Feedback Regulation of Jag1 Expression in Smooth Muscle Cells}

As a further test of the hypothesis that notch mediates patterning of the artery wall, Manderfield et al report in this issue of \textit{Circulation} that expression of dominant-negative mastermind-like protein (DN-MAML), an inhibitor of target gene activation by all forms of notch receptor\textsuperscript{15}, produced significant reductions in Jag1 expression in neural crest-derived SMCs surrounding paired aortic arch arteries.\textsuperscript{16} Moreover, when embryonic day 17.5 (E17.5) aortic SMCs were plated on a substrate that contained immobilized Jag1-Fc fragments that are known to activate Notch receptors, an increase in expression of Jag1 was observed suggesting a positive feedback
regulation of Jag1 expression by these cells. Evidence supporting the idea that notch signaling directly regulates Jag1 expression in aortic SMCs was obtained by identification of an evolutionarily conserved region (ECR) in intron 2 of the Jag1 locus that bound both Rbp-j/CSL and Notch ICD and drove expression of a reporter gene in a Notch-responsive fashion in cultured SMCs. This genomic element, termed ECR6, was then examined in transient transgenic mice and found to be sufficient to direct LacZ reporter gene expression in a temporal and spatial pattern that closely matched that of the endogenous Jag1 gene. Interestingly, the ECR6 reporter transgene in stable transgenics exhibited robust activity in SMCs derived from cardiac neural crest, but was not active in descending aortic SMCs that originate from paraxial mesoderm. Finally loss of Jag1 expression in developing neural crest-derived aortic SMCs produced congenital heart defects consisting of abnormal aortic arch artery patterning, reduced expression of the canonical notch target gene Hrt1, and reduced expression of SMC differentiation markers.

Role of Lateral Induction in Assembly of the Artery Wall

Therefore, the current evidence strongly suggests that endothelial cells of developing aortic arch arteries express Jag1 on their cell surface that then engages a Notch receptor on adjacent neural crest-derived SMC progenitor cells to promote differentiation and assembly of the first layer of the tunica media. Results presented by Manderfield et al\textsuperscript{16} as well as by Feng et al\textsuperscript{20} and by Liu et al\textsuperscript{21} argue that the initial signal is then propagated to the next layer of artery wall by lateral induction of Jag1 expression in newly differentiated SMCs (\textbf{Figure 1}). This simple feed-forward pathway is an elegant switch acting sequentially to turn on SMC differentiation and assemble the next layer of the media in a repeating pattern. Yet for a more complete understanding of this
important process of artery wall assembly and patterning, a number of remaining questions will need to be addressed in future studies:

**Remaining Questions**

1. We know that individual layers in the arterial media are composed of more than SMCs alone. Indeed the term *elastic lamellae* is often used to describe these layers, a reference to the abundance of cross-linked elastin, fibrillin, lysyl oxidase, and elastic fiber proteins that also alternate in layers across the artery wall. Does notch signaling by lateral induction also drive expression of genes encoding tropoelastin and the multiple elastic fiber proteins that are required to make a mature layer of artery wall? If so, is this a direct effect of notch signaling on individual matrix protein gene regulatory elements, or is it an indirect effect of triggering SMC differentiation in appropriate progenitor cells?

2. How is lateral induction of notch signaling coordinated with other signals that are important for artery wall formation such as TGF-b, BMP and MRTF-B?

3. The genomic regulatory element identified here, ECR6, mediates lateral induction of Jag1 expression in neural crest-derived progenitors during SMC differentiation. Yet ECR6 does not seem to have this activity for SMCs that differentiate from non-neural crest progenitors. Since the structure of the artery wall with respect to layers of SMCs and elastic fibers is not obviously different in arterial segments of non-neural crest origin, does lateral induction of notch signaling also explain layer formation in these non-neural crest arterial segments? If so, what genomic elements are responsive to lateral induction in the Jag1 locus in non-neural crest-derived SMC progenitor cells? If not, then what signaling pathway(s) mediate smooth muscle layer formation in non-neural crest derived arteries?
4. What molecular mechanisms become active to produce more elastic layers in aortas of Eln\(^{+/−}\) mice?\(^5,6,23\) Is notch signaling by lateral induction reactivated late in artery wall development (~E18.5) to mediate formation of an ectopic series of elastic lamellae in Eln\(^{+/−}\) aorta?\(^5−7\)

5. Given the consistently observed correlation between the physical forces producing wall tension and the structural component of wall thickness in many different arteries, is lateral induction of Notch signaling responsive to changes in wall tension during vascular development? If so, what is the biochemical nature of the coupling mechanism?

6. If all arteries begin wall formation in the same way – endothelial expression of Jag1 activates Notch signaling in SMC progenitors leading to Jag1 expression etc, what mechanisms terminate the iterative layer sequence to produce arteries with different numbers of SMC layers in their tunica media? Do morphogenic pathways associated with development of the tunica adventitia, such as sonic hedgehog signaling,\(^24\) act to terminate smooth muscle layer formation, or does the lateral induction signal decay at some point thus ending the repeating sequence?

7. While mammals produce artery walls with SMCs as essentially the only cell type within elastic lamellae, other species including avians and reptiles produce alternating layers of SMCs and non-SMCs (called interlaminar cells) within the media of large elastic arteries.\(^25,26\) Does lateral induction of Notch signaling also operate to build a multilayered artery wall in these phylogenetically-distinct cases as well?

**Summary**

Lateral induction of Notch signaling is an attractive mechanism to play an important role in building a multilayered artery wall. Such a role is consistent with Jag1 loss of function
phenotypes in mice\textsuperscript{14,20} and in patients with Alagille syndrome where haploinsufficiency for Jag1 leads to congenital heart disease and mispatterning of the great vessels.\textsuperscript{27,28} While many questions remain to be answered about morphogenesis of the tunica media, pursuit of Notch signaling through Jag1 in SMC progenitors promises to be a very fruitful way forward.

**Funding Sources:** The authors were supported by National Institutes of Health Grants HL93594 and HL19242 (to M.W.M.), American Heart Association Grant 09PRE2060165 (to V.J.H.), the Curriculum in Genetics and Molecular Biology at the University of North Carolina at Chapel Hill; and the Seattle Children’s Research Institute.

**Conflict of Interest Disclosures:** None

**References:**


**Figure Legend:**

**Figure 1.** Artery wall formation begins with the interaction of jagged1 (Jag1)-expressing endothelial cells with mural cell progenitors expressing notch receptors (Notch). This interaction both promotes smooth muscle differentiation and upregulates expression of Jag1 in the newly formed smooth muscle cells thus initiating a feed-forward pathway in the nascent medial layer (Layer 1). Jag1 expressed by cells in Layer 1 engages notch receptors in surrounding mural cell progenitors leading to smooth muscle differentiation and upregulation of Jag1 expression in mural cells that will go on to form Layer 2. This positive feedback response of Jag1 induction by Notch signaling in the developing artery wall is called lateral induction. By this sequential lateral induction process a multilayered artery wall is formed.
Endothelial Cells

Jag1

Smooth Muscle
Layer 1

Notch

Jag1

Layer 2

Notch

Lateral Induction

jag1

Multi-layered artery wall
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Circulation. published online December 6, 2011;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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