Notch and Vascular Smooth Muscle Cell Phenotype

David Morrow, Shaunta Guha, Catherine Sweeney, Yvonne Birney, Tony Walsh, Colm O’Brien, Dermot Walls, Eileen M. Redmond, Paul A. Cahill

Abstract—The Notch signaling pathway is critical for cell fate determination during embryonic development, including many aspects of vascular development. An emerging paradigm suggests that the Notch gene regulatory network is often recapitulated in the context of phenotypic modulation of vascular smooth muscle cells (VSMC), vascular remodeling, and repair in adult vascular disease following injury. Notch ligand receptor interactions lead to cleavage of receptor, translocation of the intracellular receptor (Notch IC), activation of transcriptional CBF-1/RBP-J

–dependent and –independent pathways, and transduction of downstream Notch target gene expression. Hereditary mutations of Notch components are associated with congenital defects of the cardiovascular system in humans such as Alagille syndrome and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Recent loss- or gain-of-function studies have provided insight into novel Notch-mediated CBF-1/RBP-J

–dependent and –independent signaling and cross-regulation to other molecules that may play a critical role in VSMC phenotypic switching. Notch receptors are critical for controlling VSMC differentiation and dictating the phenotypic response following vascular injury through interaction with a triad of transcription factors that act synergistically to regulate VSMC differentiation. This review focuses on the role of Notch receptor ligand interactions in dictating VSMC behavior and phenotype and presents recent findings on the molecular interactions between the Notch components and VSMC-specific genes to further understand the function of Notch signaling in vascular tissue and disease. (Circ Res. 2008;103:1370-1382.)

Key Words: Notch ■ vascular phenotype ■ differentiation ■ disease

Over the last couple of years, it has become increasingly clear that the Notch signaling pathway plays a pivotal role in the development and homeostasis of the cardiovascular system.1 As progress is made in the dissection of the gene regulatory networks that govern vascular morphogenesis during development, it is important that these data are evaluated and validated in adult vascular smooth muscle cells (VSMC) to better our understanding of genetic factors and pathways that increase susceptibility to phenotypic modulation and remodeling following vascular injury. The rationale has been driven by the clinical importance and therapeutic potential of modulating vascular phenotype during various cardiovascular disease states.2 An emerging paradigm suggests that developmental gene regulatory networks are often recapitulated in the context of phenotypic modulation, vascular remodeling, and repair in adult vascular disease (Figure 1).3 This concept has emerged from loss- or gain-of-function analysis4–6 and from the discovery that several hereditary cardiovascular disorders7,8 originate from gene mutations that have a direct impact on Notch signaling. An in-depth knowledge of expression patterns of the various signaling components and a comprehensive understanding of

Original received October 17, 2008; resubmission received September 15, 2008; revised resubmission received October 20, 2008; accepted October 22, 2008.

From the Vascular Health Research Centre (D.M., S.G., C.S., Y.B., T.W., P.A.C.), Faculty of Science and Health; and School of Biotechnology (D.W.), National Centre for Sensor Research, Dublin City University, Ireland; Department of Surgery (D.M., E.M.R.), University of Rochester, NY; Schepens Eye Research Institute (T.W.), Harvard Medical School, Boston, Mass; and Mater Misericordiae Hospital (C.O.), Institute of Ophthalmology, The Conway Institute of Biomolecular and Biomedical Research, Dublin, Ireland.

Correspondence to Paul A. Cahill, Vascular Health Research Centre, School of Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland. E-mail paul.cahill@dcu.ie

© 2008 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.108.187534
downstream targets as it relates to vascular morphogenesis and phenotypic switching following injury is therefore warranted.

As VSMC are not terminally differentiated,9–11 the molecular mechanisms regulating phenotypic switching and the maintenance of the contractile phenotype are relevant to understanding the pathogenesis of common vascular proliferative syndromes including atherosclerosis, restenosis and hypertension. Recent studies suggest that differentiation of VSMC is regulated by a sophisticated transcriptional program9,12–15; however, little is presently known about how gene regulatory networks that govern vascular morphogenesis impact directly on the molecular mechanisms underlying the regulation of contractile VSMC genes, in particular, in response to environmental cues and following vascular injury. One such gene regulatory network, the Notch signaling pathway, mediated by basic helix–loop–helix (bHLH) transcriptional repression,16 controls VSMC differentiation17 and modulates the transcription of endogenous contractile genes in VSMC.18–22 This review assesses our present understanding of the transcriptional programs that control VSMC differentiation and begin to define, at a molecular level, the basis for VSMC phenotypic modulation and switching by Notch. It further discusses the controversies regarding recapitulation of this developmental gene regulatory network in controlling phenotypic changes in response to vascular injury.

The Notch Signaling Pathway
Originally described in developmental studies using *Drosophila*, Notch receptor–ligand interactions are a highly conserved mechanism that regulates intercellular communication and directs individual cell fate decisions.16,23 Although a more detailed understanding of how Notch selects between CBF-1/RBP-Jκ–dependent (canonical) and –independent...
(noncanonical) pathways is lacking, Notch exists at the cell surface as a heterodimeric form (cleaved by furin in the trans–Golgi) or as an intact (colinear) protein.24–26 In general, association between Notch ligands and receptors occurs between cells (homotypic or heterotypic) resulting in trans-signaling events.24,27 Recent experimental evidence suggests that not all receptor/ligand interactions result in downstream signaling.28 Moreover, downstream βHlH transcriptional activity can occur independent of Notch signaling.29

The 4 mammalian Notch receptors (Notch1 to -4) and 4 ligands (Jagged1 and -2; Delta-like1, -3, and -4) all contain transmembrane domains such that ligand–receptor signaling occurs between adjacent cells. The engagement of Notch by ligand results in extracellular processing of the Notch receptor by a disintegrin-metalloprotease, thought to be tumor necrosis factor α-converting enzyme (TACE/ADAM17)30,31 that releases the intracellular domain of Notch to the nucleus and facilitates an association with the transcription factor CBF-1 (also known as RBP-Jκ or CSL). The subsequent recruitment of the coactivator, mastermind-like (MAML) protein,32 promotes transcriptional activation of downstream effectors (Figure 1). Established vascular target genes of the Notch cascade are the Hes and Hey (Hey1, Hey2, and Hey L) gene families, the latter also known as Hesr, Herp, Hrt, Chf, or gridlock.33 A number of excellent reviews have recently been published on the specific subject of Notch signaling.34–37 Because, in most cases, Notch function requires ligand-dependent cleavage of the intracellular (IC) domain, enforced expression of Notch IC provides a constitutively active signaling form of the receptor and has been successfully used to examine VSMC differentiation and proliferation and, more recently, apoptotic pathways of several mammalian cell types.38,39

Genetic work in Drosophila and differentiation assays in mammalian cells have provided compelling evidence that Notch can signal through both CBF-1/RBP-Jκ–dependent and –independent pathways with similar or complementary downstream effects.40–42 A series of genetic reconstitution studies in Notch mutants indicates that a variety of Notch-induced changes in Drosophila cannot be rescued by a complementary approach with Su(H)/CBF-1/RBP-Jκ.31 In addition, studies from mammalian differentiation assays have reported that truncated forms of Notch IC (which are unable to activate CBF-1/RBP-Jκ–dependent promoters) demonstrate some activity even in the presence of a dominant negative CBF-1/RBP-Jκ.43,44

**Notch Transcriptional Regulation:**

**CBF-1/RBP-Jκ–Dependent**

The levels of Notch signaling activity are solely dependent on the nuclear concentration of Notch IC that seems to act at very low concentrations within the nucleus.45 The specificity for expression of Notch target genes is CBF-1/RBP-Jκ, which binds to the DNA target gene regions and in the absence of Notch IC recruits corepressors like silencing mediator of retinoid and thyroid receptors (SMRT)/nuclear receptor corepressor, CBF-1–interacting corepressor, hairless and split ends (SPEN) also called SHARP (SMRT/HDAC-1–associated protein)46–49 (Figure 1). The corepressors associate with histone deacetylase (HDAC) complexes keeping the chromatin in a transcriptional silent mode. When Notch signaling is activated, Notch IC displaces the corepressors and associates with CBF-1/RBP-Jκ in what becomes a ternary complex involving Mastermind.46–50 The ternary complex recruits transcription factors such as p300/CBP-associated factor/general control of amino acids synthesis protein 5 (PCAF/GCN5) and CREB-binding protein (CBP)/p300-activating responsive genes.50 Although CBF-1/RBP-Jκ occupancy is significantly and transiently increased following Notch activation, a more dynamic interaction with targets is now suggested in which CBF-1/RBP-Jκ may not be constitutively bound to DNA at all promoters.51

Notch-mediated transcriptional activation is downregulated by the degradation of Notch IC.52 The mechanism that stops the signaling event involves Mastermind and a protein named Ski-interacting protein (SKIP), which can associate both with the CBF-1/RBP-Jκ corepressors and with the CBF-1/RBP-Jκ–Notch IC–Mastermind ternary complex.48 SKIP and Mastermind are able to recruit kinases that specifically phosphorylate Notch IC in the TAD and PEST domains. Fbw7/Sell10 ubiquitination of the phosphorylated sites leads to Notch IC degradation and stops the signaling process in the absence of new Notch IC entering the nucleus.48 Glycogen synthase kinase (GSK)3β is a serine/threonine kinase and a component of the Wnt/wingless signaling cascade that binds and phosphorylates Notch.53 Thus protein degradation is an effective method of signal regulation and one that is clearly present to keep the levels of Notch IC just above functional threshold. However, one corollary is that for continuous signaling, a continuous ligand input is needed.

In the last few years, endocytic trafficking has also emerged as a central process in the regulation of the levels and activity of Notch.54–56 The observations that different defects in vesicular trafficking affect Notch signaling supports the concept of endocytosis as an important component for Notch IC–mediated signaling and, probably, release. However, it is also possible that there exists ligand-independent Notch signaling because some of the activation resulting from alterations in the trafficking machinery does not require ligands.56 This notion of ligand-dependent and ligand-independent modes of Notch regulation has recently been demonstrated by examining Lgd, a trafficking-associated protein that modulates ligand-independent Notch signaling separate from ligand-dependent activation.57

**Notch Transcriptional Regulation:**

**CBF-1/RBP-Jκ–Independent**

Several lines of evidence support an integration of the Notch and nuclear factor (NF)-κB signaling pathways58–62 in differentiation/maturation of a diverse range of cell types (Figure 1). It is notable that Notch and NF-κB signaling pathways share many common features: (1) both are activated by common stimuli such as tumor necrosis factor α and hypoxia; (2) activated Notch (Notch IC) and NF-κB mediate transcription by regulating corepressors such as SMRT/N-COR; and (3) both regulate similar target genes such as Hes-1/Hey and IκBα. Original studies suggested that the N-terminal portion of Notch IC (Notch1) is responsible for the inhibitory effects...
of Notch on NF-κB–directed gene expression and NF-κB DNA binding activity where Notch IC inhibited p50 DNA binding and interacted specifically with p50 subunit, not p65 of NF-κB. More recent studies however suggest that Notch IC may contribute to the DNA binding/transcriptional capacity of NF-κB.60 The discrepancies between studies might be attributable, in part, to the size of the Notch IC constructs used, which are distinctly different from the physiological, in vivo–generated Notch IC.

Most of the direct evidence for a CBF-1/RBP-Jκ–independent activity of Notch is derived from Drosophila, and the alleles of Notch: the Atrubptex (Ax) and the Microchaetae defective (Mcd) classes.40,63 The mutants exhibit gain of function phenotypes that are independent of CBF-1/RBP-Jκ but dependent on shaggy, which encodes the Drosophila homolog of GSK3β and plays a central role in Wnt signaling.64,65 In addition, Wnt3a alone increased Hes1 expression in the absence of Notch ligand suggesting that Notch signaling presumably induced by ligand-expressing cells is affected by Wnt signaling.66

Notch has also recently been shown to activate integrins without affecting integrin expression (Figure 1), and this activation is dependent on Notch IC to both activate R-Ras and inhibit H-Ras but importantly independent of CBF-1/RBP-Jκ-transcription.57,64 This new CBF-1/RBP-Jκ–independent Notch/R-Ras pathway provides a molecular mechanism to explain Notch, integrin, and Ras cross-talk during development and may present implications for Notch control of VSMC phenotype. Indeed, a role for Ras activation has been reported for VSMC differentiation in response to insulin-like growth factor 1.69

**Notch and Early Vascular Development**

The important in vivo role of Notch genes during vascular development and postnatal arteriogenesis has been primarily addressed by gene targeting and mutation studies (Figure 2).4,6,70–72 Targeted mutagenesis and transgenic studies in mice demonstrated a specific role in embryonic vascular development for receptors, Notch170,73 and Notch4,62,72 the ligands Jag16 and Dll474,75; the Notch transcriptional regulator CBF-1/RBP-Jκ; the E3 ubiquitin ligase Mib177,78; components of the secretase complex, such as nicastrin,79 presenilin 1, and presenilin 280; and the Notch pathway downstream effector bHLH proteins Hey1 and -2.81,82 Most of these mutants exhibit a similar phenotype characterized by the absence of angiogenic vascular remodeling in the extraembryonic yolk sac, placenta, and embryo proper. Although the Notch3-null mice are viable and fertile, a detailed

---

**Figure 2.** Two models of Notch function within the vasculature. Cell fate determination during arterial venous differentiation and morphogenesis and phenotypic switching, modulation, and cell maintenance following vascular injury.
analysis revealed that expression of this gene is necessary for the VSMC differentiation and acquisition of arterial identity. Absence of Notch3 results in enlarged arteries with abnormal distribution of elastic laminae, reinforcing the importance of Notch3 in arterial differentiation within vessel development. In contrast, loss of global Notch1 function results in early embryonic lethality, due primarily to a vascular defect in the endothelial cell. Unlike embryonic development, where the Notch ligand Dll4 is a prerequisite, Dll1 has been shown to be an essential regulator of postnatal arteriogenesis. Despite that important difference, and reminiscent with embryonic development, there is perivascular induction of a proangiogenic milieu, including vascular endothelial growth factor (VEGF), fibroblast growth factor-2, and EphB4. Endothelial Dll1 is strongly upregulated to activate Notch signaling and induce EphB4, thereby allowing arteries to grow postnatally.

Similar studies in zebrafish have validated the important role of Notch in vascular morphogenesis and development. Their embryos are transparent and develop externally. In addition, their early development is quite rapid so that by 36 hour post fertilization, a functional circulatory network, complete with beating heart, patent blood vessels, and flowing blood is observed. Original seminal studies by Lawson et al demonstrated the important role of Notch in arterial venous differentiation during development by repressing venous differentiation within developing arteries. More recently, Notch signaling has been shown to restrict angiogenic cell behavior to tip cells in developing segmental arteries in the zebrafish embryo. Proper specification of cell identity, position, and behavior in a developing blood vessel sprout is required for normal angiogenesis and the Notch signaling pathway is implicated in this process (Figure 2). Although the primary factor that regulates the differentiation of arteries and veins was initially considered to be blood flow, it is now established that genetic prepatternning, largely mediated by the Notch pathway, plays a primary role in regulating arteriovenous differentiation. Indeed, recent work in zebrafish has also established that a single hemangioblast, the bipotential precursor of a subset of hematopoietic and endothelial cells, can give rise to endothelial cell progeny that populate both arteries and veins.

The role of the Notch pathway in regulating early embryonic vascular development is intertwined with that of other major regulators of vascular development and physiology, most notably, the morphogen, sonic hedgehog (Shh) and VEGF-A. The roles and interdependence of the Notch, Shh, and VEGF-A pathways in regulating the formation of the large axial blood vessels of the trunk (the dorsal aorta and the posterior cardinal vein) was first studied in zebrafish. Notch-deficient embryos exhibited a poorly formed dorsal aorta and posterior cardinal vein with accompanying arteriovenous malformations (the fusion of arteries and veins without an intervening capillary bed). A similar phenotype was observed in embryos mutant for the bHLH transcriptional repressor Hey2 (also referred to in zebrafish as the gridlock gene). Similar to VEGF-A-deficient embryos, Shh mutant zebrafish embryos also exhibit a loss of arterial differentiation as Shh acts upstream of VEGF-A. Studies in mammalian cell culture have also placed the Notch pathway downstream of the VEGF-A, whereas Notch-1 and Dll4 expression is induced in human arterial endothelial cells by VEGF-A, but not in venous endothelial cells. Targeted mutagenesis studies in mice have also demonstrated that VEGF-A is essential for vascular development. In addition to regulating arterial specification of endothelial cells, Notch signaling also regulates arterial specification of VSMC. The Notch3 gene is predominantly expressed in vascular smooth muscle cells of arteries, but not in those of veins. Marked arterial defects occur in Notch3-deficient mice, including enlarged arteries with a thinner VSMC coat than is found in wild-type arteries. These defects arise postnatally, because arterial vessels fail to mature. Morphologically, arterial VSMC of Notch3-deficient mice resemble those surrounding veins in wild-type mice. However, the expression of smoothelin and regulatory elements of the SM22 promoter are markedly downregulated in arteries of Notch deficient mice suggesting that these arteries have acquired a venous fate. Notably, in arteries of Notch3-deficient mice, normal expression of several endothelial cell arterial markers, including that of ephrin B2 and connexin 40, was evident suggesting that arterial identity of endothelial cells, and of the VSMC surrounding them, is specified independently.

In addition to its contribution to early vascular morphogenesis and arterial-fate specification, the Notch signaling pathway also impacts on vascular homeostasis (Figure 2). This important relationship between Notch3 and the vasculature was first recognized when mutations in members of the pathway were found to be responsible for certain late-onset hereditary vascular anomalies in humans. Human Notch genes are linked to Alagille syndrome, a developmental disorder with vascular defects, and CADASIL, a cerebral arteriopathy where unpaired cysteine residues in the mutated epidermal growth factor repeats of the Notch3 receptor are thought to be responsible and result in abnormal conformation and accumulation of the ectodomain of the receptor at the cell surface. Several studies have shown that these mutations lead to abnormal accumulation of Notch3 protein but have variable effects on Notch3 signaling through CBF-1/RBP-Jk. More recently, transgenic mice that better recapitulate the characteristic vascular lesions observed in CADASIL have been generated and indicate that Notch3 or electron-dense granular osmiophilic material accumulation are unlikely to be the prerequisites for the induction of VSMC degeneration. They further suggest that degeneration of VSMC may be triggered by the disruption of their normal anchorage, based on the important role of adhesion for cell survival. Moreover, the functional significance of an archetypal CADASIL-associated mutation (R90C) in brain arteries demonstrated that the mutant Notch3 protein remains functional and does not exhibit dominant negative interfering activity, even when the extracellular domain of Notch3 accumulates. Collectively, these data suggest a model that invokes novel pathogenic roles for the mutant Notch3 protein rather than compromised function as the primary determinant of the CADASIL arteriopathy. Ultrastructurally, CADASIL deposits of granular osmiophilic material are often located inside indentations in the VSMC membrane that resemble
endocytic vesicles suggesting that damage to the VSMC may be associated with aberrant ubiquitin-dependent endocytosis of the Notch3 ligand in CADASIL, and increased accumulation of ubiquitin on the vessel wall may be a manifestation of this aberration.103

Recent studies also confirm that endothelial Jagged-1 (Jag1) is essential for vascular morphogenesis because endothelial-specific deletion of Jag1 results in embryonic lethality and cardiovascular defects, recapitulating the Jag1 null phenotype.8,104 These embryos show striking deficits in VSMC, whereas endothelial Notch activation and arterial-venous differentiation appear normal. Endothelial Jag1 mutant embryos are phenotypically distinct from embryos in which Notch signaling is inhibited within the endothelium thereby suggesting that the primary role of endothelial Jag1 is to potentiate the development and differentiation of neighboring VSMC.104

**Notch and Vascular Smooth Muscle Phenotype**

Unlike skeletal and cardiac muscle cell lineages, where cellular differentiation is functionally coupled to irreversable exit from the cell cycle, VSMC retain their capacity to proliferate and modulate their phenotype during postnatal development.9–11 VSMC are pleiotropic, expressing unique permutations and combinations of both contractile and synthetic genes that are continuously modulated in response to various stimuli during development,105 and biochemical and environmental cues including transforming growth factor (TGF)-β, extracellular matrix proteins (integrins, adhesion molecules, collagen, and elastin), mechanical forces, neuronal influences, oxygen homeostasis, and cell–cell interactions important following injury.106–109

Differential display and bioinformatic analyses have been performed to compare transcriptional profiles of contractile and synthetic VSMC.110–113 The modulation of quiescent human coronary artery VSMC to the proliferative and migratory phenotype following platelet-derived growth factor (PDGF) activation revealed several discrete transcriptional changes.113 More than 100 genes were differentially expressed by at least 2-fold including multiple genes involved in cell–cell signaling and cell–matrix interaction.113 Further expression profiles derived from the quantitative distribution of cDNA data in mice classified based on their profile similarity to known reference genes demonstrated a large majority (>90%) of known VSMC-specific genes together with novel candidates.113 Moreover, gene array expression analysis identified extensive differences to conclude that plaque and medial VSMC are distinctly different VSMC cell types.114

Given the recent report of a hierarchy for Notch and PDGF within the vasculature,115 an analysis of these differentially expressed VSMC genes should provide insights into how Notch signaling perturbs VSMC differentiation and function through PDGF.

This VSMC plasticity and phenotypic modulation plays a pivotal role in the overall response of the vasculature to injury.107,116–118 As progress is made in the dissection of the gene regulatory networks that govern vascular remodeling, several insights have now emerged of the genetic factors and pathways that increase susceptibility to clinical phenotypes of vascular remodeling. One such paradigm suggests that the Notch gene regulatory network is recapitulated in the context of vascular remodeling and repair in adult vascular disease to control, in part, phenotypic switching.119–121

Transcriptional pathways regulating VSMC genes include GATA-6,122,123 serum response factor (SRF),124 and myocardin.125 There is accumulating evidence that suggests Notch is an additional pathway critical for VSMC differentiation that engages this triad of transcription factors to regulate the transcription of differentiated VSMC-specific genes.18,21,119,126 Mutations of the Notch pathway in Alagille syndrome and CADASIL8,127 further emphasize the critical importance of this developmental gene regulatory pathway in contributing to human vascular malformations. Although Notch has been shown to be involved in phenotypic modulation of VSMC during development,128 some controversy remains regarding both: (1) the expression of Notch and Hey family members postnatally following phenotypic modulation of VSMC caused by vascular injury, most likely resulting from differences in the animal models used and/or temporal assessment of Notch and Hey expression following injury; and (2) the role for Notch signaling in repressing/enhancing VSMC differentiation marker expression.

**Notch, Hey Genes, and VSMC Phenotype**

Interaction with serum response factor (SRF) and its numerous accessory cofactors with CarG box [CC(A/T-rich)6GG] DNA sequences within promoter chromatin of specific VSMC differentiation genes is a nexus for integrating signals that control VSMC differentiation in both development and disease.18,20,84,115–131 SRF activates VSMC contractile genes by physically associating with the recently identified cardiac and VSMC-restricted SAP domain transcription factor, myocardin.125 These transcription factors act synergistically to regulate the transcription of differentiated VSMC-specific genes.132 Interactions between SRF/CarG box, TAAT sequence/homeobox-binding protein, and GATA-6/GATA binding site act together to promote transcription of VSMC proteins specific to the differentiated phenotype.110,133,134 Collectively, a model is now emerging whereby Notch target gene expression converges on and functionally antagonizes myocardin-dependent VSMC differentiation and the contractile VSMC phenotype (Figure 3).18,135

A ligand–receptor pathway would constitute an instructive signal for VSMC differentiation through a CBF-1/RBP-Jκ-dependent mechanism and would augment gene expression mediated by the myocardin-SRF/CarG complex. Indeed, initial early reports suggested that ligand activation of Notch promoted VSMC differentiation.126,130 However, subsequent studies demonstrated that Notch also inhibited VSMC differentiation in vitro through CBF-1/RBP-Jκ-dependent mechanisms in as much as it either positively or negatively regulated the expression of VSMC-restrictive genes (smooth muscle α-actin [SMA], calponin, smooth muscle myosin heavy chain [SM-MHC], and smoothelin).19,20 Further studies confirmed that Notch-induced Hey gene expression represses myocardin-induced VSMC transcription and gene expression.18,21 Furthermore, Hey2 repressed multiple transcrip-
tional regulatory elements controlling the expression of VSMC contractile genes in VSMC. Surprisingly, the repressive function of Hey2 was not mediated by disruption of myocardin-SRF protein complexes bound to DNA; nor does it reflect recruitment of HDAC activity by Hey2. Structure–function studies also revealed that Notch-mediated repression is dependent on the basic DNA binding domain and C terminus of Hey2. Little is presently known about the mechanism of synergistic activation by Notch IC–CBF-1/RBP-J and myocardin-SRF complexes in controlling VSMC differentiation. Although cis elements for both Notch IC–CBF-1/RBP-J and myocardin-SRF proteins are required for the cooperative effect, there is some disagreement about whether a physical interaction with the myocardin-SRF complex at the protein level is operational. Given that multiple transcription factors plus promoter/enhancer regions of DNA often constitute higher order three-dimensional complexes, termed enhanceosomes, a common transcriptional coactivator for Notch IC and myocardin such as p300 may facilitate higher complex formation, which augments transcription.

Despite the initial evidence that Notch and downstream targets modulate adult VSMC differentiation, there still remain discrepancies about whether Notch signaling either directly or indirectly promotes or inhibits differentiation. It is accepted that regulation of myocardin target genes by Notch/Hey genes does occur in VSMC. However, in vitro studies have also demonstrated that Notch signaling induces VSMC differentiation and identified SMA and SM-MHC as Notch IC/CBF-1 targets. These contrasting studies are only reconciled if Notch IC directly activates markers such as SMA and SM-MHC, but the concomitant temporal expression of Hey 1 or Hey2 would be sufficient to turn off these signals. Hence a hypothetical regulatory loop was initially proposed that would allow for temporally restricted Notch signaling and induction of VSMC target gene expression until Hey production rises above a threshold required to antagonize the signal. Notch IC released from the plasma membrane specifically by ligand binding directly activates the transcription of VSMC marker genes, via myocardin-SRF complexes but concurrent induction of Hey expression by the same Notch IC to sufficient levels would inhibit the Notch IC– and myocardin-mediated expression of VSMC differentiation marker genes (Figure 3).

These discrepancies were recently addressed experimentally using cocultured VSMC and endothelial cells in vitro (to provide a physiological stimulus for Notch IC signaling via ligand).

Figure 3. Context-dependent (1) Notch IC activation and (2) Notch target gene dependent inhibition of VSMC differentiation. Notch IC stimulates VSMC differentiation via interactions with myocardin, whereas concurrent Notch target gene expression (Hey2) inhibiting of SRF/myocardin to CArG elements. Injury-induced suppression of VSMC marker genes may involve loss of positive differentiation signaling pathways through environmental changes that alter MAPK activity and downstream effectors.
Hey2 are repressors of SMA expression, a marker for VSMC differentiation, but are also able to antagonize the initial Notch-induced SMA expression in vitro. Hey does not inhibit Notch IC/CBF-1 complex formation, although it decreases Notch IC/CBF-1 binding and activation of the SMA promoter. VSMC responsiveness to Notch signaling, therefore, may be limited temporally and spatially by the production of Hey factors, which feed back to inhibit specific Notch target gene activation.21 Hey is thus proposed as a candidate suppressor of mature VSMC markers, including SMA, which are downregulated following vascular injury in vivo.13 The physiological relevance of this regulatory feedback loop was highlighted by the conserved regulation of SMA in an endothelial/SMC coculture, a condition that activates Notch signaling via ligand dependent activation in VSMC. Whether this repressive effect of Hey on Notch IC activation of SMA holds true for other VSMC-restrictive differentiation markers remains to be seen.

The involvement of other select signaling molecules in promoting VSMC differentiation and phenotypic modulation through Notch has also recently been addressed. These include TGF-β, signaling cascade, which has been assigned multiple roles in these cells. TGF-β1 promoted the expression of VSMC differentiation genes (SM22α) through the inhibition of Notch3 and activation of Hes1.136 The repression of Notch3 was mediated by SMAD activity and p38 mitogen-activated protein (MAP) kinase, whereas analysis of the Hes1 promoter revealed direct activation by Smad2 but not Smad3. Furthermore, the Hes1 repressor protein augmented Smad3 transactivation of the SM22α promoter.

Notch signaling has also been recently linked to PDGF signaling, a key determinant of VSMC biology, where PDGF receptor (PDGFR)-β is a novel immediate Notch target gene.135 In newborn Notch3-deficient mice, PDGFR-β expression was strongly reduced in VSMC that later develop an aberrant morphology. In VSMC from a CADASIL patient carrying a NOTCH3 missense mutation, upregulation of PDGFR-β mRNA and protein in response to ligand-induced Notch activation was significantly also reduced135 underscoring the important hierarchy for these 2 signaling pathways within the vasculature and how dysfunctional Notch3 signaling perturbs VSMC differentiation and function.

**Notch and Cell Transdifferentiation**

Notch has also been identified as a critical player in controlling cell transdifferentiation. Studies in mice confirm, at least during development, that Notch acts as a positive regulator of VSMC differentiation from neural crest cells.128 Whether these findings are likely to be applicable to a broad range of smooth muscle–related disorders,137 including those involving non–neural crest–derived smooth muscle remains unanswered. Patient cohorts with Alagille syndrome are also predisposed to multiple vascular pathologies affecting blood vessels that contain non–neural crest–derived smooth muscle, including stenosis of the peripheral pulmonary vascular tree and intracranial aneurysms.138 Jagged-1–induced Notch signaling promotes VSMC differentiation from mesenchymal cells.126 Overexpression of Notch IC upregulates the expression of multiple VSMC marker genes including VSMC-

**Notch Control of Vascular Cell Differentiation**

Notch and Id Function

Inhibitor of differentiation (Id) family of helix-loop-helix (HLH) transcription factors act as important regulators of growth and phenotypic modulation in VSMC140 and in the vascular response to injury by modulating the formation of active class A–class B bHLH complexes.141 The proteins of the Id family dictate VSMC phenotypes by prompting undifferentiated precursor cells to initiate a program of differentiation in response to Notch signaling.140 Recent studies have also addressed the interaction between the Id family of proteins and Notch. Extramacrochaetae (Emc), the *Drosophila* homolog of Id, interacts genetically with components of the Notch pathway in the fly eye and wing.142 Recent studies examining Notch and Delta mutant zebrafish embryos revealed relatively normal levels of Id2 expression indicating that other Notch receptors/ligands may be involved or alternatively, various Notch and Delta components might be necessary to cooperatively regulate Id2.143

The recently reported CBF-1/RBP-Jκ–independent Notch/R-Ras pathway68 may also be relevant to VSMC phenotypic switching and VSMC differentiation. In differentiated cells, insulin-like growth factor 1 is required for maintaining the differentiated phenotype69 and through an IRS-1/SHP-2 Ras activation that acts as a switch controlling VSMC phenotype–dependent signaling.69

**Vascular Injury, Notch, and Phenotypic Switching**

One of the paradigms of Notch signaling is the observation that the biological response to receptor activation is highly sensitive to dosage, developmental timing, and cellular context.27,45,144 The conflicting data on Notch control of VSMC differentiation has also left unresolved the question of the physiological role of individual Notch receptors and downstream targets on VSMC differentiation in response to vascular injury.

Several studies have characterized the expression of Notch receptors and their downstream target genes following vascular injury. The expression of several Notch pathway components, including Notch1, Notch3, Jagged-1, Jagged-2,
Hey1, and Hey2 are regulated in a temporal manner after experimentally induced vascular injury. Similarly, Jagged-1 and Jagged-2 are expressed in regenerating endothelium as well as VSMC after vascular injury. In subsequent studies, a biphasic response in which Jagged-1, Notch-3, and Hey1 were acutely downregulated in medial VSMC within the first 2 days after vascular injury but became upregulated 7 to 14 days after injury compared with uninjured vessels. In support of a functional role for the modulation of Notch pathway components during the response to vascular injury, intimal hyperplasia after vascular injury was significantly decreased in Hey2−/− mice. Primary VSMC from Hey2−/− mice revealed that these mutant cells proliferate at a reduced rate compared with wild-type cells, whereas the overexpression of Hey 147 in VSMC led to increased VSMC proliferation associated with reduced levels of the cyclin-dependent kinase inhibitors p21waf1/cip1 (Cdkn1a) or p27kip1 (Cdkn1b). In the latter study, Hey2 protein directly interacts with the p27kip1 promoter to repress transcription.

Although recent in vitro studies have suggested that both Notch1 and -3 may be important in regulating SMC proliferation and differentiation, the pathophysiological correlate of these findings in vivo, especially in the postnatal period, has not been demonstrated. This is attributable, in part, to the embryonic lethality of Notch1-deficient mice and the lack of availability of mice with tissue-specific deletion of Notch1 in SMC. It is clear that there is a temporal regulation of both receptors using experimental models of vascular injury. However, the exact contribution of each receptor and in what cell type (VSMC, EC adventitial fibroblast) these changes are manifest remain unclear. Studies using Notch1/−/− mice revealed that these mutant cells proliferate at a reduced rate compared with wild-type cells, whereas the overexpression of Hey 147 in VSMC led to increased VSMC proliferation associated with reduced levels of the cyclin-dependent kinase inhibitors p21waf1/cip1 (Cdkn1a) or p27kip1 (Cdkn1b). In the latter study, Hey2 protein directly interacts with the p27kip1 promoter to repress transcription.

Concluding Remarks
Phenotypic plasticity of VSMC has greatly enhanced our understanding of transcriptional control of VSMC differentiation. To date, an extensive sophisticated transcriptional program governing VSMC phenotypic switching under both physiological and pathophysiological conditions is emerging. It is tightly regulated at the level of chromatin through a complex, synergistic combination of DNA-binding transcription factors (eg, SRF, accessory cofactors for the DNA-binding proteins (eg, myocardin/Elk-1), the direct interaction of DNA and transcription factor complexes (eg, the SRF-CArG interaction), and histone modifications present within promoter chromatin (eg, SMC-specific H3K4Me2 and H4 acetylation at CArG boxes). The combination of molecular interactions around SRF provides multiple means by which important gene regulatory networks may signal to chromatin to dynamically control gene expression. Future studies will likely focus on identification of trans factors and cis elements important for programming histone modifications into VSMC gene promoters during development and following injury. Further investigation will undoubtedly yield important insights into the VSMC component of vascular development and the multitude of disease processes in which VSMC pathology is a prominent component.
The rapid increase in the number of publications that focus on the role of Notch signaling in regulating vascular cell function both in vitro and in vivo reflects the degree of interest in understanding the role of this pathway and highlights the fact that we still have much to learn. It is evident that Notch plays a pivotal role in the differentiation, physiology and function of VSMC through exploitation of the recently defined sophisticated transcriptional program. Despite initial contradictory results, current experimental evidence strongly suggests that its role in controlling VSMC phenotype is time and context-dependent. The differentiation state of the cell, the location within the vascular system (artery, vein, or capillary) and the physical environment (flow induced strain and/or shear stress) all contribute to the final response of VSMC to Notch.

Because VSMC phenotypic switching involves a complex mechanism that has evolved in higher organisms as a means to optimize repair following vascular injury, the extensive plasticity has also made these cells susceptible to maladaptive environmental changes and phenotypic switching in a number of major disease states. Thus, elucidation of mechanisms by which Notch exploits these processes is of critical importance for understanding not only normal VSMC development but also the etiology of major human vascular diseases.

Sources of Funding
The financial support for this article is from Science Foundation Ireland, the Wellcome Trust, Health Research Board of Ireland, and the NIH.

Disclosures
None.

References


39. Schroeter EH, Kisslinger JA, Kopan R. Notch-1 signalling requires JD. MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors.


41. Fryer CJ, White JB, Jones KA. Mastermind recruits CycC:CDK8 to transcriptional co-activator for NOTCH receptors.


43. Fryer CJ, Lamar E, Turbachova I, Kintner C, Jones KA. Mastermind mediates chromatin-specific transcription and turnover of the Notch intracellular domain is ubiquitinated and negatively regulated by the intracellular domain of Notch1 via RBP-Jκ.


Notch and Vascular Smooth Muscle Cell Phenotype
David Morrow, Shaunta Guha, Catherine Sweeney, Yvonne Birney, Tony Walshe, Colm O'Brien, Dermot Walls, Eileen M. Redmond and Paul A. Cahill

Circ Res. 2008;103:1370-1382
doi: 10.1161/CIRCRESAHA.108.187534
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/103/12/1370

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/