Cardiovascular and Hematopoietic Defects Associated With Notch1 Activation in Embryonic Tie2-Expressing Populations

Deepak A. Venkatesh, Kyung-Sook Park, Anne Harrington, Laura Miceli-Libby, Jeong K. Yoon, Lucy Liaw

Abstract—Notch signaling is critical for the development and maintenance of the cardiovasculature, with loss-of-function studies defining roles of Notch1 in the endothelial/hematopoietic lineages. No in vivo studies have addressed complementary gain-of-function strategies within these tissues to define consequences of Notch activation. We developed a transgenic model of Cre recombinase–mediated activation of a constitutively active mouse Notch1 allele (N1ICD+) and studied transgene activation in Tie2-expressing lineages. The in vivo phenotype was compared to effects of Notch1 activation on endothelial tubulogenesis, paracrine regulation of smooth muscle cell proliferation, and hematopoiesis. N1ICD+ embryos showed midgestation lethality with defects in angiogenic remodeling of embryonic and yolk sac vasculature, cardiac development, smooth muscle cell investment of vessels, and hematopoietic differentiation. Angiogenic defects corresponded with impaired endothelial tubulogenesis in vitro following Notch1 activation and paracrine inhibition of smooth muscle cells when grown with Notch1-activated endothelial cells. Flow cytometric analysis of hematopoietic and endothelial precursor populations demonstrated a significant loss of CD71+/Ter119+ populations with an active N1ICD+ allele and a corresponding increase in c-Kit+/CD71 and Flk1+ populations, suggesting a developmental block during the transition between c-Kit– and Ter119-expressing erythroblasts. Cardiovascular lineages are sensitive to an imbalance in Notch signaling, with aberrant activation reflecting a vascular phenotype comparable to a loss-of-function Notch1 mutation. (Circ Res. 2008;103:423-431.)

Key Words: angiogenesis ■ endothelium ■ blood vessels ■ heart development

Formation of the vasculature begins with specification of angiogenic precursors and blood islands in the visceral yolk sac, where there is a close association between primitive hematopoietic cells and developing endothelium.1 Blood vessels develop by aggregation of angioblasts into a primitive network.2 At embryonic day (E)7.0 to E7.5, the yolk sac vasculature develops, starting as scattered blood islands that fuse to form a vascular network. At E8.5, this network fuses with the embryonic vasculature, allowing for the passage of primitive erythroblasts and hematopoietic stem cells into the circulation. Vessel maturation involves complex remodeling, with proliferation and sprouting of new vessels via angiogenesis. Heart development starts at E7.5 to E8.0, with midline endothelial tubes forming a heart tube, which undergoes folding to generate a primitive heart with endocardium, myocardium, and pericardium. At E9.5, the heart is starting the septation process from the common atrial chamber and the primitive ventricle.

Notch signaling plays a critical role in cardiovascular development. Endothelial-specific deletion of Notch1 results in embryonic lethality with vascular defects,3 and Notch1-null mice have defective vascular remodeling. A Notch ligand, Delta-like 4 (Dll4), is expressed in arterial endothelium,4,5 and haploinsufficiency of Dll4 is associated with vascular defects and embryonic lethality.6 Targeted mutations in Delta1 and Jagged1 cause hemorrhaging5 and vascular remodeling defects,7 respectively. The NOTCH3 gene is mutated8 in the human disorder CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), which is manifested by stroke, vascular dementia, and arteriopathy.

Given the widespread defects associated with dysregulated Notch signaling, it is unclear whether Notch acts cell autonomously in the vasculature and the temporal developmental requirement for Notch signaling. Although Notch1 loss-of-function studies in the mouse were analyzed, there have been no cardiovascular-specific Notch1 gain-of-function studies. We investigate the role of Notch signaling in endothelium, hematopoietic cells, and their precursors by expressing an activated form of Notch1 under the control of the Tie2 promoter. This expression resulted in embryonic lethality, associated with severe cardiovascular abnormalities, showing that increased Notch1 signaling also perturbs normal differentiation and remodeling.
Materials and Methods

Tissue Staining and Histological Analysis

The Tie2Cre strain was verified using the GT(ROSA)26Sor1 Cre reporter strain (JAX 003309). Embryos were dissected at various stages and stained for β-galactosidase activity.9 Sections were stained with hematoxylin/eosin or immunostained with antibodies against myc (Developmental Studies Hybridoma Bank, University of Iowa), platelet endothelial cell adhesion molecule (PECAM)-1 (BD Pharmingen) or smooth muscle actin (SMA). For whole mount immunostaining, specimens were fixed in 4% paraformaldehyde overnight and bleached in H2O2 in methanol. Specimens were incubated with respective antibodies overnight and, for PECAM, biotinylated secondary antibody and ABC detection system (Vector Laboratories) were used. For whole mount SMA and myc staining, Alexa-fluor-488–conjugated goat anti-mouse secondary antibody was used.

Reverse Transcription–Polymerase Chain Reaction

RNA from embryonic tissues was isolated using Tri Reagent (Sigma). Primer sequences are in the expanded Materials and Methods section of the online data supplement, available at http://circres.ahajournals.org.

Cell Culture

Three-dimensional tubulogenesis assays were performed using human umbilical vein endothelial cells (HUVECs).10 For coculture, smooth muscle cells (SMCs) were stably transfected with green fluorescent protein (GFP) and cultured with nonlabeled endothelial cells to allow for cell type distinction. Hematopoietic progenitor assays were performed as described.11 Embryos and yolk sacs were treated with 0.25% collagenase/IMDM (4-isocyanoato-4-methylidenephene)20% plasma-derived serum at 37°C for 1 hour, plated at 0.2×10^5–2×10^5 cells/mL in 60% methylcellulose, and cultured for 7 to 10 days. For flow cytometry, collagenase-disrupted embryonic cells were fixed and stained 30 minutes with phycoerythrin (PE)-Ter119, fluorescein isothiocyanate (FITC)-CD71, allophycocyanin (APC)-cKit, PE-Flk1, FITC-Ly6C, and APC-neutrophil antigen 7/4 (BD Bioscience, San Jose, Calif).

Results

Development of a Conditional N1ICD Transgene

For conditional Notch1ICD expression, we adopted a Cre-loxP strategy (Figure 1A). Mouse Notch1ICD (amino acids 1810 to 2556) with a myc epitope was cloned downstream of a floxed GFP sequence driven by the cytomegalovirus (CMV) enhancer/chicken β-actin promoter (CGMycN1ICD). The transgenic allele expresses GFP constitutively. Upon Cre recombination, the GFP sequence is excised, and N1ICD is conditionally expressed. We developed 2 independent founder lines (FVB/N) that have an identical phenotype. N1ICD was activated using B6.Cg-Tg(Tek-cre)12Flv12 (JAX 004128), referred to as Tie2Cre. To test transgene regulation, we examined N1ICD expression in 293T cells. CMVMycN1ICD plasmid was used as a positive control. Normalized luciferase activities are presented as means ± SD. E and F, Tie2Cre×ROSA Cre reporter crosses were set up, and resultant negative (E) or Tie2Cre-positive (F) E9.5 embryos were prepared for detection of β-galactosidase activity. G and H, Tie2Cre×CMGMycN1ICD crosses were obtained and embryos immunostained with anti-myc to detect the N1ICD myc tag. Positive staining was seen in embryonic vasculature (G) and endothelial cells of the dorsal aorta (H) and yolk sac. Lower insets for H and I show background staining in control aorta and yolk sac. The scale bar in G represents 100 μm for G, 25 μm for H, and 50 μm for I.

Figure 1. Development of a conditional mouse N1ICD transgene. A, The N1ICD construct is shown. B, CGMycN1ICD was transfected with or without a CMV-Cre expression plasmid in 293T cells, and expression was monitored by immunoblot (B) or immunofluorescence staining (C). CMVMycN1ICD and CMVSGFP plasmids were used as positive controls for MycN1ICD and GFP expression, respectively. Myc fluorescence is red. D, A CBF-1–luciferase construct was cotransfected with CGMycN1ICD and CMV-Cre plasmids into 293T cells. CMVMycN1ICD plasmid was used as a positive control. Normalized luciferase activities are presented as means ± SD. E and F, Tie2Cre×ROSA Cre reporter crosses were set up, and resultant negative (E) or Tie2Cre-positive (F) E9.5 embryos were prepared for detection of β-galactosidase activity. G and H, Tie2Cre×CMGMycN1ICD crosses were obtained and embryos immunostained with anti-myc to detect the N1ICD myc tag. Positive staining was seen in embryonic vasculature (G) and endothelial cells of the dorsal aorta (H) and yolk sac. Lower insets for H and I show background staining in control aorta and yolk sac. The scale bar in G represents 100 μm for G, 25 μm for H, and 50 μm for I.
CMGycN1ICD (Figure 1B). When CMV-Cre was cotransfected with CMGycN1ICD, we observed robust N1ICD expression with loss of GFP, by immunoblot and immunostaining (Figure 1C). Following Notch1 activation, a CBF-1 reporter was transactivated as expected (Figure 1D).

Activation of Notch1 in Tie-2–Expressing Cells Leads to Embryonic Lethality and Cardiovascular Defects

The Tie2Cre strain was crossed with the ROSA26 Cre reporter strain, and β-galactosidase activity was confirmed in the vasculature (Figure 1E through 1I). We activated the Notch1ICD transgene with Tie2Cre; embryos double positive for Cre and N1ICD are designated as N1ICD+. Immunostaining for the N1ICD epitope tag (myc) showed an appreciable level of expression in the vasculature (Figure 1G), especially in aortic endothelium and yolk sac (Figure 1H and 1I). When Tie2Cre males were bred to N1ICD females, no pups were positive for both transgenes, suggesting embryonic lethality. In early developmental stages, double positive embryos for the Cre and N1ICD alleles were present. Double positive embryos (N1ICD+) at E9.5 showed severe vascular defects and did not survive past E10.5. In normal E9.5, embryos, a well-organized vasculature is present, with large branching intracranial arteries and easily detectable dorsal aorta and outflow tract (Figure 2A and 2C). In contrast, N1ICD+ embryos had disorganized vessels with low vascular density (Figure 2B and 2E). Staining of control embryos with anti-PECAM showed organized vasculogenesis with a dense network of superficial and deep blood vessels (Figure 2C). In contrast, N1ICD+ embryos had a coarse, immature vasculature, indicating impaired angiogenic remodeling (Figure 2E). Vascular complexity, particularly in head capillaries, was reduced in N1ICD+ embryos (Figure 2C and 2E, upper insets). Control embryos had undergone vascular pruning and maturation, resulting in a complex vascular network. Conversely, vessels in N1ICD+ embryos were all of similar size and lacked branching. The intersomitic vessels formed through angiogenic sprouting were present in the N1ICD+ embryos but failed to organize and perfuse the somites (Figure 2C and 2E, lower insets). This corresponded with increased TUNEL-positive cells undergoing apoptosis within the somites in N1ICD+ embryos (Figure 2D and 2F). Histological examination showed that although major arteries were present in N1ICD− embryos, the aortae were smaller. There was also a lack of vascularization in the developmentally delayed neural tube (Figure 2G and 2H), associated with increased apoptosis (Figure 2I and 2J).

N1ICD+ Embryos Have Severely Impaired Cardiac Development

Tie2 is expressed in the endocardium, myocardium, and atrioventricular cushions, and activation of Notch disrupts heart morphogenesis (Figure 3). At the linear heart tube stage (E8.0), hearts from control and N1ICD− embryos were similar. At E9.5, N1ICD+ embryos had an absence of constriction at the atrioventricular canal, dilated ventricles, inflated pericardial sacs, and edema (Figure 3B). The constricted atrioventricular canal is likely to increase chamber pressure, contributing to the dilation and edema. At this stage, the control heart has looped, with atria ascending dorsal to the primitive ventricle and bulbus cordis region (Figure 3C). However, 100% of the hearts from N1ICD− embryos failed to undergo looping, with loss of atrial/inflow tract migration rostrally (Figure 3B and 3D). Histological analysis showed a significant reduction of trabeculation and a loss of structural distinction between atria and ventricles in hearts from N1ICD− embryos (Figure 3E and 3F). The mutants displayed cardiac dysgenesis with profound hypoplastic ventricles, and the myocardium was thinned in the presumptive ventricular region. There is less evidence for...
endocardial cushion formation in the outflow track and atrioventricular canal in N1ICD embryos. Surprisingly, TUNEL and cleaved caspase-3 assays showed no cellular apoptosis in E9.5 N1ICD embryos, despite a severely thinned ventricular wall. This finding suggested that a proliferation defect could be a cause of hypoplasia.

Remodeling and Angiogenic Defects in Notch1ICD Yolk Sacs

The yolk sacs of control and N1ICD at E8.5 had identifiable blood islands, and embryos were similarly developed. Because blood islands are formed by mesodermal condensation, their presence in N1ICD yolk sacs demonstrate initial vasculogenesis. By E9.5, however, N1ICD embryos appeared to lack blood and yolk sac blood supply (Figure 4A and 4B). A vascular plexus with branched vessels is detectable in control yolk sacs at E9.5, with blood-filled vasculature (Figure 4A). In most N1ICD yolk sacs, however, the primitive vascular plexus was unchanged or degenerating. When blood islands fused, they formed round cisternae in N1ICD yolk sacs, rather than a capillary plexus (Figure 4C through 4F). Despite rapid degeneration of the vasculature in N1ICD yolk sacs, establishment of blood flow between the yolk sac and embryo is supported by some circulating red blood cells within N1ICD embryos. Because embryonic erythropoiesis initiates after E11, these erythrocytes originated from the yolk sac. These defects in vascular morphogenesis were modeled using an in vitro bead assay with endothelial cells expressing GFP (control) or N1ICD (Figure 4G). Similar to the in vivo phenotype, endothelial cells with activated Notch signaling had decreased 5-bromodeoxyuridine incorporation (data not shown) and decreased branching morphogenesis, supporting a cell-intrinsic function of Notch signaling in endothelial cells.

Figure 3. Heart defects in Notch1ICD embryos. Freshly dissected E9.5 wild-type (A) or N1ICD (B) embryos are shown. Isolated hearts from N1ICD embryos displayed abnormal ventricular looping and underdeveloped cardiac chambers (D), compared to the normally developed heart (C). The scale bar in D represents 100 μm for A and B, 70 μm for C, and 60 μm for D. E and F, Heart sections from control (E) or N1ICD (F) embryos were hematoxylin/eosin-stained to show ventricular trabeculation. The scale bar in F represents 25 μm for E and F.

Figure 4. Defects in yolk sac vasculature in Notch1ICD embryos. Embryos were collected from control (A, C, and E) or N1ICD embryos (B, D, and F) at E9.5. Whole mount views of embryos with intact yolk sacs show lack of conducting arteries (arrows, A) and orange peel–like appearance of N1ICD yolk sacs (B). C and D, PECAM-1-staining shows normal vascular structure (C) vs the fused primitive vascular network in N1ICD yolk sacs (D). E and F, hematoxylin/eosin-stained sections contrast normal blood filled vessels in control (E) with a gross enlargement between endoderm and mesoderm layers in N1ICD embryos (F), resulting in lacunae-like spaces. Endothelial cells were present in the N1ICD yolk sacs (F, arrows, inset). The scale bar in F represents 50 μm. G, Notch1ICD inhibits endothelial sprouting in vitro. Fibronectin-coated microcarrier beads were seeded with HUVEC-GFP or HUVEC-Notch1ICD, embedded in fibrin, and grown for 3 days. Normal branching was inhibited by Notch1ICD. Total sprouts and sprouts >150 μm (long sprouts) were counted and measured and shown as means±SD.
The cardiac and vascular defects prompted evaluation of genes involved in heart development and angiogenesis. Notch target genes HRT1 and HRT2 are both expressed in control and N1ICD/H11001 embryos. Overexpression of HRT1 results in repression of TBX2/BMP2,13 which are essential for atrioventricular canal development. Although these genes were not altered, BMP10, PEG-1, and Gata6 were decreased in transgenic embryos. Notch-null mutant mouse have similar trabecular defects associated with decreased BMP10. These data suggest that Notch activation results in specific cardiac defects rather than global developmental delays. Increased expression of p27kip1 in the hearts from N1ICD/H11001 embryos supports the idea that decreased proliferation contributes to hypoplasia. We also analyzed expression of chemokines, whose genetic mutations in mice affect hematopoiesis and myelopoiesis. Although the expression of several chemokines was not significantly different in isolated hearts, C-X-C chemokine receptor (CXCR4) and C-X-C ligand (CXCL12) in N1ICD embryos were decreased, consistent with impairment of angiogenesis. Interestingly, there were few changes in expression of angiogenic markers vascular endothelial growth factor receptor and Ang/Tie in the yolk sacs of N1ICD embryos, despite severe deficiencies in vascular remodeling (Figure 5B). However, CXCR4 was strongly expressed in yolk sacs from N1ICD embryos, suggesting the possibility of enhanced CXCR4 expression in hematopoietic progenitor populations that would correspond to lack of hematopoietic differentiation.

Vessels in N1ICD Embryos Lack Smooth Muscle Cells
Endothelial cells are surrounded by mural cells that respond to hemodynamic stress. Hemorrhaging in N1ICD embryos suggested lack of structural integrity, possibly attributable to lack of smooth muscle investment. Immunostaining was performed using anti-SMA. In control E9.5 embryos, the dorsal aorta and branchial arch arteries were surrounded by SMA-positive cells (Figure 6). However, arteries in N1ICD embryos had few SMA-positive cells (Figure 6A and 6B), consistent with the massive hemorrhaging in N1ICD embryos. In addition, control E9.5 yolk sac vasculature shows SMA-positive layers in only controls. C, Yolk sacs from control (left) or N1ICD (right) embryos were stained for SMA. Control yolk sacs had vessels with mural cell investment, but there was no SMA staining in the rudimentary network in yolk sacs from N1ICD embryos (right). The scale bar in F represents 100 μm for A and 140 μm for C.

Figure 5. Gene expression in transgenic N1ICD+ embryos. Semiquantitative RT-PCR analysis was performed on embryos (A) and yolk sacs (B) for expression of genes critical for hematopoiesis and angiogenesis.

Figure 6. Notch1 activation in Tie2 populations inhibits smooth muscle investment of vessels. Immunostaining for smooth muscle α-actin (SMA) was performed on E9.5 embryos. A, SMA expression in the developing heart was comparable in control (left) and N1ICD+ (right) embryos. White lines indicate magnified region in B. B, SMA-positive cells are identifiable surrounding the aorta in controls (arrow, left), whereas none was seen in N1ICD+ embryos (arrow, right). In the N1ICD+ embryos, some fluorescence is seen in the dorsally located somites. The insets for both images show sections through the aorta, showing SMA-positive layers in only controls. C, Yolk sacs from control (left) or N1ICD (right) embryos were stained for SMA. Control yolk sacs had vessels with mural cell investment, but there was no SMA staining in the rudimentary network in yolk sacs from N1ICD+ embryos (right). The scale bar in F represents 100 μm for A and 140 μm for C.

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Notch1 Activation in Tie2 Lineages Inhibits Hematopoiesis

Notch signaling influences differentiation and apoptosis of hematopoietic cells, and there were obviously reduced but visible blood cells in the N1ICD embryo, reflecting primitive hematopoiesis. We used flow cytometry to assess erythroid, myeloid, and endothelial progenitor differentiation by expression of c-Kit, TER119, CD71, Flk1, Ly6C, and neutrophil antigen 7/4. During embryogenesis, hematopoietic stem cells express Sca1, followed by c-Kit, which are both lost during differentiation (Figure 8A). The erythroid-specific antigen TER119 is expressed in proerythroblasts and all subsequent erythroid precursors. In contrast, CD71 is expressed by immature erythroid precursors and decreases during maturation. In wild-type E9.5 embryos, TER119 cells expressed CD71 at various levels, CD71high to CD71medium-low, indicating erythroblast differentiation beyond the late erythroblast stage (Figure 8B). In contrast, the TER119 and CD71 expression profile of N1ICD cells was strikingly different. TER119high CD71medium-low cells were reduced in N1ICD embryonic tissue, but c-Kithigh and CD71high/medium cells were significantly increased, suggesting that erythropoiesis is disrupted or halted at the proerythroblast stage. In comparison to controls at E9.5, the N1ICD embryos had a 1.3- to 1.9-fold reduction in the Ter119/CD71 population in both the yolk sac and embryo (Figure 8B) and a corresponding 4.6- to 3.5-fold increase in the c-Kit and, particularly, the CD71/c-Kit population in N1ICD yolk sacs and embryos (Figure 8C).

Quantification of the myeloid lineage using Ly6C and the neutrophil antigen 7/4 showed no significant differences between groups (supplemental Figure I). In the context of angiogenesis, we analyzed Flk and c-Kit double positive population and also found no differences. However, progenitors expressing Flk1 alone were significantly increased in N1ICD yolk sacs and embryos (Figure 8D).

We further analyzed in vitro differentiation of progenitors. Cells from E9.5 embryos and yolk sacs were cultured in semisolid medium under conditions that allow growth and differentiation of erythroid (CFU-E and BFU-E), myeloid (CFU-GM), and multilineage (CFU-mix) hematopoietic progenitors. Hematopoietic colony-forming cells were generated from wild-type embryos, whereas few rare colonies were obtained from N1ICD littermates under the same conditions, with significantly decreased BFU-E, CFU-GM/G/M, and CFU-mix colony-forming cells from N1ICD yolk sacs and embryos (Figure 8E). We speculated that N1ICD embryos contained lower numbers of hematopoietic stem cells that may be undetectable in direct colony-forming cell cultures. We expanded the number of progenitors by incubating cells from single wild-type compared with pools of 2 or 3 N1ICD littermates for 8 days. No hematopoietic colonies derived from N1ICD cultures. We then compared expression of several genes critical for primitive hematopoiesis (Figure 8F).15,16 We observed an increase in early markers for hematopoietic cells (flk-1, c-Kit, SCL, Lmo2, Runx-1), and a decrease in mature erythroid markers β major globin and Gata-1. These findings indicate that constitutively active Notch1 signaling perturbs the differentiation process of lineage-committed hematopoietic progenitors.

Discussion

Vascular development is a complex process controlled by multiple signaling pathways. Notch-signaling components are widely expressed embryonically, including in early vasculature.17 The importance of Notch signaling for vascular development has been clarified using loss-of-function models in mice, and our novel gain-of-function Notch allele is complementary to these targeting strategies. General and endothelial cell-specific gene targeting of Notch pathway components identified important roles in regulation of vascular morphogenesis and angiogenesis.3,18-20

We demonstrate that activation of Notch1 signaling in Tie2-expressing populations leads to severe defects in remodeling and maturation in the embryonic and yolk sac vasculature. Phenotypic effects were first observed around E9.5, during remodeling of the initial vascular plexus.7 Although PECAM endothelial cells were evident, the vascular network in N1ICD embryos did not undergo remodeling and stabilization. This defect was associated with a lack of SMC

Figure 7. Coculture of smooth muscle cells with endothelial cells with activated Notch signaling suppresses cell proliferation. HUVECs were transduced with LacZ (control) or N1ICD adenovirus and grown for coculture with GFP-expressing human aortic SMCs. After 3 days of coculture, cells were stained with DRAQ5, and SMCs were identified based on GFP fluorescence (A). DRAQ5-based cell cycle analysis was performed on GFP-expressing SMCs (B). C, Cultures were stained with 4,6-diamidino-2-phenylindole (DAPI) (blue, nuclei) and SMA (red) to distinguish between SMCs (GFP and SMA) and endothelial cells (only nuclear staining).
Figure 8. Impaired erythroid differentiation in N1ICD+ embryos. A, Representation of erythroid differentiation and marker expression. Flow cytometry was used to analyze cell surface antigens from embryos or yolk sacs at E9.5. B, CD71+ and Ter119+ populations are shown and graphed are double CD71+/Ter199+ populations in each group. C, CD71+ and c-Kit+ populations are shown, and graphed are the percentages of CD71+/c-Kit+ double positive cells, showing increased c-Kit+ progenitors in N1ICD+ groups. D, Analysis of Flk1+ and c-Kit+ double positive population did not show significant differences but progenitors positive for Flk1 alone were significantly increased in N1ICD+ groups (graphed). E, Methylcellulose colony-forming assays were used to quantify BFU-E and macrophage-forming colonies from yolk sacs and embryos. Shown are representative photomicrographs of BFU-E colonies 8 days after plating. E, RT-PCR was performed using primers as indicated.
investment and disruption of hematopoietic development, particularly erythropoiesis. These phenotypes are consistent with the embryonic expression of Tie2. Tie2 is predominantly expressed in the endothelial cell lineage, hemopoietic stem cells, and some mesenchymal cells with properties of mural cell precursors. Because induction of this transgene is a Cre recombinase–mediated genomic alteration, transgene expression does not require persistent Tie2 expression. Transient Tie-2 expression in a stem or progenitor cell population is predicted to confer transgene expression in resulting cells of that lineage, depending on recombination efficiency.

The primary cardiovascular defects are consistent with activation of Notch1 in the endothelial/hematopoietic lineages, where Notch1 is expressed embryonically. The consequence of temporal Notch1 activation on cellular differentiation has been studied in embryonic stem cell models and other in vitro systems. Activated Notch1 inhibited differentiation of Flk1\(^{+}\) mesodermal cells, and activation in mesenchymal cells inhibited cardiac, endothelial, and hematopoietic cells, favoring mural cell differentiation. Inhibition of Notch in HUVECs increases proliferation and branching, suggesting a normal function to suppress growth and limit branch point generation, which is consistent with our phenotypes following Notch1 activation. Endothelial cells overexpressing Dll4 have reduced vascular endothelial growth factor–A–stimulated proliferation and migration, and Notch activation downregulates p21, suppresses cell cycle progression, and promotes an epithelial to mesenchymal transformation.

Although both loss and gain-of-function approaches have been assessed for other Notch receptors, including Notch4, cardiovascular activation of Notch1 has not been previously evaluated. Our Notch gain-of-function phenotype is similar to Tie2Cre-mediated deletion of the Notch1 allele, which leads to lethality at E10.5 with vascular defects. In both cases, an endothelial vascular plexus forms; however, subsequent angiogenesis and remodeling of the initial network is defective, leading to loss of vascular integrity and hemorrhage. These similarities in Notch loss-of-function and gain-of-function phenotypes suggest dosage sensitivity and that disruption of the precise balance of Notch signaling impairs development. A similar phenotype in Notch4-activated embryos suggests some overlap in individual Notch receptor signaling. More information about selective Notch downstream targets is necessary to molecularly define the differences between these in vivo phenotypes.

Corresponding to the angiogenic defect, N1ICD\(^{+}\) embryos lacked SMC investment. Proper mural investment of the vasculature requires multiple signaling pathways, including platelet-derived growth factor, transforming growth factor (TGF)\(^{+}\), Ang1/Tie2, and potentially Notch signaling. These signals may be intrinsic to a mesenchymal precursor, if Tie2 is expressed in mural cell progenitors, or a result of paracrine signaling from the endothelium. Although Tie2 may be expressed by precursors of mural cells, higher Tie2 expression in the endothelial lineage suggests that this is the major population affected. This latter idea is supported by the phenotypes of other genetic manipulations that alter endothelial development or vascular network maturation. For example, disruption of the angiopoietin-1/Tie2 and endoglin/TGF\(^{+}\) pathways lead to defects in SMC investment that are likely secondary effects of endothelial dysfunction. Indeed, our in vitro analysis of endothelial cell interaction with SMCs shows that activation of Notch signaling within the endothelial compartment affects SMC proliferation. This effect is not attributable to endogenous activation of Notch signaling in SMCs, because we previously showed that Notch signaling in SMCs induced by endothelial cell coculture regulates gene expression but not proliferation. One of the models that we are currently testing is that Notch is a regulator of endoglin/TGF\(^{+}\) signaling in endothelium, and Notch suppression of this pathway may contribute to loss of paracrine signaling to the SMCs.

In N1ICD\(^{+}\) embryos, cardiac abnormalities were similar to activation of Notch4 in embryonic endothelium or deletion of Nsk2-5, which is expressed in the myocardium. The latter defect was attributed to the attenuated development of the myocardium rather than a specific defect in endocardial development because reduced contact between the myocardium and endocardium is a feature of a more primitive pretrabecular ventricle. The idea that Notch affects ventricular development is supported by the trabeculation-defective phenotype of standard and endocardial-specific Notch1 and RBP\(^{+}\) mutants. Interestingly, constitutive cardiac Notch4 activation driven by Mesp1-CRE leads to impaired ventricular myocardium maturation and inhibition of cardiomyocyte differentiation.

Notch1 is essential for early hematopoietic development and regulates adult hematopoiesis. Our results show that Tie2-specific activation of Notch1 inhibits differentiation of early erythroid cells (c-Kit\(^{+}\)) to proerythroid cells (Ter119\(^{+}\)), supporting a functional effect of Notch signaling on erythroid progenitor cells. Similarly, the increase in Flk1\(^{+}\) single populations suggests a potential block in endothelial differentiation, consistent with vascular remodeling defects in the N1ICD\(^{+}\) embryos. Transcription factors regulating hematopoiesis have been characterized, and many are dysregulated in N1ICD\(^{+}\) embryos. We observed accumulation of early markers, Flk1, SCI and Lmo2, and c-Kit, and decreases in proerythroblast maturation markers like Gata1 and \(\beta\)-globin major. Our findings, thus, have widespread implications for the sensitivity of cells of the cardiovascular and erythroid lineage to Notch signaling and provide a model to determine gene targets downstream of Notch1 signaling during embryonic development of the cardiovascular and blood systems.

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Disclosures

None.

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Supplemental Materials and Methods

β-galactosidase staining

The Tie2Cre strain was verified using the GT(ROSA)26Sor<sup>m1Sor</sup> Cre reporter strain (JAX 003309). Embryos and yolk sacs were dissected between embryonic day (E) 8.5 and E9.5, fixed in 0.2% glutaraldehyde/PBS, washed in detergent rinse (phosphate buffer, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP40), and stained overnight at 37°C (0.1M phosphate buffer, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 0.02% NP-40, 2mM MgCl₂, 0.01% sodium deoxycholate and 1mg/ml X-gal).

Whole mount and histological analyses

Tissues were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 5-6µm. Sections were either stained with hematoxylin and eosin (H&E) or immunostained with anti-myc (9E10, Developmental Studies Hybridoma Bank, Univ. Iowa) or anti- PECAM-1. For whole mount immunostaining, samples were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated, and bleached in 3% H₂O₂ in methanol. Samples were rehydrated and placed in PBSMT solution (3% nonfat milk/0.1% Triton X-100/PBS). Specimens were incubated with anti-mouse PECAM (1:100; rat MEC 13.3. BD Pharmingen) or anti-SMA (1.200; mouse clone 1A4, Sigma) in PBSMT overnight at 4°C. For whole mount PECAM staining, biotinylated secondary antibodies (1:400, Sigma) and the avidin/streptavidin-based detection system (Vector Laboratories) were used, with 3,3’ diaminobenzidinetetrahydrochloride (DAB) as the substrate. For whole mount SMA and nyc immunohistochemistry, Alexa-fluor-488 conjugated goat anti-mouse secondary antibody (1:400, Molecular Probes) was used.
Cell culture

Three dimensional tubulogenesis assays were performed using human umbilical vein endothelial cells (HUVEC). For analysis of hematopoiesis, tissues were treated with 0.25% collagenase/IMDM/20% plasma-derived serum (PDS) at 37°C for 1h with trituration. Cells were plated in triplicate at 0.2-2x10^5 cells/ml in 60% methylcellulose supplemented with 10% PDS (Antech, Tyler TX), 5% protein-free hybridoma medium (Gibco/BRL, Grand Island, NY), GM-CSF (2.5ng/ml), SCF (60ng/ml), IL-6 (20ng/ml), IL-3 (20ng/ml), glutamine (2mM) at 37°C. BFU-E, CFU-E, macrophage, mast cell, and granulocyte/macrophage colonies were counted after 7-10 days.

Gene expression analysis by RT-PCR.

At E9.5, whole embryos, yolk sacs, and dissected pooled hearts were collected and RNA was isolated using Tri Reagent (Sigma). We performed expression analysis of p27, p15, p53, VEGFR1-R3, Ang1-2, TBX2, TBX5, BMP2, BMP10, Gata1, Gata6, PEG1, FLK1, cKit, SCL, Lmo2, RNX-1, globins and cyclophilin. Specific primers were used to amplify fragments using equal amounts of cDNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer 1</th>
<th>Primer 2</th>
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<td>TBX2</td>
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<td>p27</td>
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<td>TCCAGGGGCTTATGATTCTG</td>
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</tbody>
</table>
All other primer sequences were taken from Thijssen et al. 2004, Exp Cell Research 299: 286-293.

**Flow Cytometry**

For flow cytometry, collagenase-disrupted tissues were fixed in 70% EtOH and washed, resuspended in 0.2ml 0.1% BSA/PBS, incubated at 4°C with rat IgG (1µg) (Jackson Immunoresearch Westgrove, PA), and stained with PE-Ter119 (1µg) and FITC-CD71 (1µg) APC-conjugated cKit. (BD Biosciences San Jose, CA). Cells were analyzed in a FACS Calibur (BD Bioscience).

**Supplemental Figure**

**Figure S1. Comparison of myeloid cells in N1ICD⁺ embryos.** Flow cytometry was used to analyze cell surface antigens from embryos or yolk sacs at E9.5. Neutrophil antigen 7/4 and Ly6C antigen were detected. There were no significant differences in expression of these markers between groups.