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Essential Role of Endothelial Notch1 in Angiogenesis

Florian P. Limbourg, MD[*], **Kyosuke Takeshita, MD, PhD**[*], **Freddy Radtke, PhD**, **Roderick T. Bronson, DVM**, **Michael T. Chin, MD, PhD**, and **James K. Liao, MD**
Vascular Medicine Research Unit (F.P.L., K.T., M.T.C., J.K.L.), Brigham & Women's Hospital and Harvard Medical School, Boston, Mass; Ludwig Institute for Cancer Research (F.R.), Epalinges, Switzerland; and Rodent Histopathology Core (R.T.B.), Harvard Medical School, Boston, Mass. Dr Limbourg is currently at Medizinische Hochschule Hannover, Kardiologie und Angiologie, Hannover, Germany

Abstract

Background—Notch signaling influences binary cell fate decisions in a variety of tissues. The Notch1 receptor is widely expressed during embryogenesis and is essential for embryonic development. Loss of global Notch1 function results in early embryonic lethality, but the cell type responsible for this defect is not known. Here, we identify the endothelium as the primary target tissue affected by Notch1 signaling.

Methods and Results—We generated an endothelium-specific deletion of Notch1 using Tie2Cre and conditional Notch1$^{flox/flox}$ mice. Mutant embryos lacking endothelial Notch1 died at approximately embryonic day 10.5 with profound vascular defects in placenta, yolk sac, and embryo proper, whereas heterozygous deletion had no effect. In yolk sacs of mutant embryos, endothelial cells formed a primary vascular plexus indicative of intact vasculogenesis but failed to induce the secondary vascular remodeling required to form a mature network of well-organized large and small blood vessels, which demonstrates a defect in angiogenesis. These vascular defects were also evident in the placenta, where blood vessels failed to invade the placental labyrinth, and in the embryo proper, where defective blood vessel maturation led to pericardial and intersomitic hemorrhage. Enhanced activation of caspase-3 was detected in endothelial and neural cells of mutant mice, which resulted in enhanced apoptotic degeneration of somites and the neural tube.

Conclusions—These findings recapitulate the vascular phenotype of global Notch1$^{-/-}$ mutants and indicate an essential cell-autonomous role of Notch1 signaling in the endothelium during vascular development. These results may have important clinical implications with regard to Notch1 signaling in adult angiogenesis.

Keywords

vasculature; genetics; defects; angiogenesis; endothelium

The vascular system is the first organ to form during mammalian embryogenesis. Hemangioblasts congregate in blood islands and then differentiate into endothelial cells to form the primitive vascular plexus and hematopoietic cells to form the circulating blood. This early phase of vascular development, in which these endothelial cell progenitors differentiate and coalesce into a primitive network of undifferentiated blood vessels in the embryo and extraembryonic membranes, is termed vasculogenesis. At later stages of vascular development, this primary vascular plexus is then remodeled into a well-organized network of large and small

Correspondence to James K. Liao, Brigham & Women's Hospital, 65 Landsdowne St, Room 275, Cambridge, MA 02139. E-mail jliao@rics.bwh.harvard.edu.
[*]Drs Limbourg and Takeshita contributed equally to this article.

vessels through sprouting, branching, and network formation in a process termed angiogenesis.[1] This process is controlled by a variety of signaling molecules and their downstream pathways, such as vascular endothelial growth factor (VEGF) and its receptors,[2] transforming growth factor-β and its receptors,[3] angiopoeitin 1 and its receptor Tie2,[4] ephrin-B ligands and EphB receptors,[5] and the Notch ligands delta and jagged.[6,7]

Notch signaling regulates embryonic patterning and binary cell fate decisions and plays a critical role in mammalian embryogenesis and vascular development. Notch genes encode large transmembrane receptors that interact with membrane-bound ligands of the delta, jagged, and serrate family.[8] Although Notch1 is expressed in the developing vasculature and extravascular tissue,[9] Notch4 is restricted to the vascular endothelium.[10] Targeted deletion of Notch1 results in embryonic lethality with severe neuronal and somitic defects.[11,12] In addition, Notch1$^{-/-}$ mutants show defects in angiogenic vascular remodeling in the embryo, yolk sac, and placenta.[13] Although deletion of Notch4 does not affect vascular development, Notch1-Notch4 double-null embryos display more severe vascular phenotypes than embryos deficient in Notch1 alone.[13]

In the vascular system, the arterial and venous vessels are distinct both morphologically and at the molecular level. There are striking differences in gene expression patterns between arterial and venous endothelial cells even before the onset of circulation, which suggests that specific genetic programs regulate arterial versus venous cell fate. For example, the ephrin-B2 and EphB4 ligand-receptor pair differentially marks the developing arteries and veins.[5] In zebra fish development, Notch signaling has been shown to regulate arterial versus venous differentiation downstream of sonic hedgehog and VEGF,[14] and possibly through activation of gridlock, which encodes a hairy related basic helix-loop-helix-transcription factor.[15] In human arterial endothelial cells, VEGF induced upregulation of Notch1 and its ligand, Dll4, in vitro.[16] More recently, the mammalian Hey1 and Hey2 orthologs of gridlock, were shown to play a role in vascular development downstream of Notch.[17] The combined loss of Hey1 and Hey2 leads to a failure of vascular remodeling with impaired arterial differentiation similar to that observed in Notch1$^{-/-}$ mutants. Furthermore, expression of both Hey1 and Hey2 was decreased in Notch1$^{-/-}$ mutants. Also, deletion of the Notch ligand Dll4, which is expressed in arterial endothelial cells, recently has been shown to result in defects in arterial differentiation and arteriovenous malformations.[18,19] These results suggest a crucial role for Notch signaling in vascular development.

Given the widespread tissue distribution of Notch1 during development, it is unclear whether loss of Notch1 expression in endothelium specifically accounts for the observed vascular defects in Notch1$^{-/-}$ mutants or whether loss of expression in another cell lineage affects an inductive signal to the vasculature necessary for proper angiogenic remodeling. The first evidence for an essential role of Notch signaling in the endothelium came from studies of gain-of-function mutations of Notch, in which constitutively active Notch4 was knocked into the Flk-1 locus.[10] Constitutive Notch4 activity in the Flk-1^{+} cells leads to abnormal vessel structure and patterning defects; however, because Flk-1^{+} cells are progenitors for muscle, hematopoietic, and vascular endothelial cells,[20] the essential role of Notch signaling in the endothelium during vascular development cannot be established definitively by these experiments. To address definitively the role of endothelial Notch1 in vascular development, we generated a conditional deletion of Notch1 in the endothelium with a conditional allele and a Cre recombinase transgene under the control of the Tie2 promoter.

Methods

Mice

Notch1$^{flox/flox}$ mice (N1$^{flox/flox}$) and Tie2-Cre transgenic mice (Tie2Cre$^{+/-}$) were described previously,[21-23] and genotyping was performed as described. Rosa R26R mice were obtained from Jackson Laboratories, Bar Harbor, Me. The mice were maintained in the Harvard Medical School animal facilities. The Standing Committee on Animals at Harvard Medical School approved all protocols pertaining to experimentation with animals.

Histological Analysis and Immunohistochemistry of Mouse Tissue

Mouse tissue was fixed in Bouin's fixative. Tissues were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin by standard methods for routine morphological analysis. Immunohistochemistry for cleaved-caspase3 was also performed by standard procedures. Primary antibody against cleaved-caspase3 (Cell Signaling Technology) was used at a dilution of 1:100. Proliferating cell nuclear antigen (PCNA) staining was performed with a PCNA staining kit according to the manufacturer's instructions (Zymed).

Whole-Mount Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated into methanol, and bleached in 5% H_2O_2 and 95% methanol. Embryos were rehydrated and placed in a solution of 3% nonfat milk, 0.1% Triton X-100, and PBS (PBSMT) for 2 hours with 1 change. Embryos were incubated with anti-mouse platelet and endothelial cell adhesion molecule (PECAM; 1:200, BD Bioscience) in PBSMT overnight at 4°C. They were then washed several times and incubated with a 1:200 dilution of horseradish peroxidase-conjugated secondary antibody (Zymed) overnight. Embryos were washed several times in PBSMT and rinsed in PBS containing 0.1% Triton X-100, followed by the addition of 0.3 mg/mL DAB substrate (Sigma) in PBS containing 0.5% $NiCl_2$, 0.1% Triton X-100, and 0.2% BSA. This was allowed to stand for 5 minutes at room temperature. Finally, embryos were washed in PBS containing 0.1% Triton X-100 and 0.2% BSA, postfixed in PFA/glutaraldehyde, and transferred into 50% glycerol in PBS and then 70% glycerol in PBS.

LacZ Staining

To examine *lacZ* expression, mouse embryos were dissected between embryonic day (E) 8.25 and day E10.5, fixed in 0.25% glutaraldehyde/PBS for 5 minutes, rinsed twice with PBS, and stained overnight at 37°C in X-Gal buffer (1.3 mg/mL potassium ferrocyanide, 1 mg/mL potassium ferricyanide, 0.2% Triton X-100, 1 mmol/L $MgCl_2$, and 1 mg/mL X-Gal in PBS).

Results

To determine the role of endothelial Notch1 in vascular development, we generated mice with a conditional deletion of Notch1 in endothelial cells. Two genetically different Tie2Cre$^{+/-}$ mice strains,[22,23] which express the Cre recombinase under the control of the endothelium-specific Tie2 receptor promoter,[24] were mated to mice containing a conditional *notch1* allele flanked by loxP sites.[21] N1$^{flox/+}$/Tie2Cre$^{+/-}$ animals were born with the expected Mendelian ratio and showed normal life spans (data not shown). Male offspring were mated to N1$^{flox/flox}$ females to generate N1$^{flox/flox}$/Tie2Cre$^{+/-}$ animals (Notch1 endothelial knockout [NEKO]).

The distribution of offspring genotypes revealed a complete lack of NEKO pups at birth (observed 0, expected 14; χ^2<0.001). During embryonic development, we observed normal Mendelian ratios until day E10.5 but no NEKO survivors after day E11.5 (Table). In addition, the use of 2 different Tie2Cre strains resulted in similar outcomes (data not shown). These

findings indicate an essential role for Notch1 signaling in the endothelium during embryonic development.

Analysis at day E8.5 demonstrated no difference between NEKO and $N1^{flox/+}$/$Tie2Cre^{+/-}$ embryos in size, stage, and overall appearance (data not shown). At day E9.5, NEKO embryos showed closure of the anterior neural tube but displayed marked pericardial effusion and growth arrest at the 16- to 20-somite stage, indicative of cardiovascular failure. NEKO embryos showed a pronounced delay in posterior development (Figures 1A and 1B), with some individuals exhibiting incomplete embryonic turning (Figure 1G). Because the phenotypes of $N1^{flox/+}$/$Tie2Cre^{+/-}$ and $N1^{flox/flox}$/$Tie2Cre^{-/-}$ were indistinguishable at this and later stages, we chose $N1^{flox/+}$/$Tie2Cre^{+/-}$ animals as controls for further analysis.

The observed mutant phenotype closely mimicked the observed phenotype for the global Notch1 knockout mouse[11-13] and suggested the presence of vascular defects. Whole-mount PECAM-1 immunostaining at day E9.5 demonstrated the presence of vascular networks consisting of endothelial cells in both control and NEKO mutant embryos, indicative of intact vasculogenesis; however, compared with $N1^{flox/+}$/$Tie2Cre^{+/-}$ animals (Figure 1C), in NEKO mutants (Figure 1D) there was a marked reduction in vessel organization and a persistent, immature vascular plexus, which suggests a block in vascular maturation and angiogenic remodeling.

To verify the endothelial specificity of Notch1 deletion and to evaluate further the vascular phenotype, we took advantage of a genetic Cre reporter, the ROSA R26R line, which activates bacterial lacZ expression in a Cre-dependent manner. To this end, we generated $N1^{flox/flox}$/ROSA $R26R^{+/+}$ animals, which were mated to $N1^{flox/+}$/$Tie2Cre^{+/-}$ mice. No LacZ staining was detected in mice without ROSA R26R alleles (Figure 1E). At day E9.5, $N1^{flox/+}$/$Tie2Cre^{+/-}$/ROSA $R26R^{+/-}$ control animals showed lacZ staining throughout the vasculature, without evidence of extravascular expression (Figure 1F). NEKO ROSA $R26R^{+/-}$ mice showed a grossly disorganized vascular plexus without the proper vascular differentiation in the head and trunk observed in control animals (Figure 1G). These observations confirm the specific expression of Cre recombinase in endothelial cells during embryogenesis. Our findings suggest intact vasculogenesis but impaired secondary angiogenic sprouting and remodeling in NEKO mutants.

Primary hematopoiesis and blood vessel development from primitive blood islands first occurs in the yolk sac. Blood vessels form de novo through aggregation of endothelial cells into an initial primary capillary plexus (vasculogenesis). Thereafter, vascular remodeling and maturation forms a differentiated vascular tree of arterial and venous vessels (angiogenesis). The yolk sac of E9.5 control mice had large vitelline blood vessels (Figures 2A, 2C, and 2E). NEKO mutants had pale yolk sacs that lacked obvious large blood vessels (Figures 2B, 2D, and 2F). Visualization of endothelial networks with anti-PECAM antibody revealed the presence of a primary vascular plexus in NEKO mutants (Figure 2F); however, the mutant embryos failed to remodel the primary vascular plexus to form large and small blood vessels of the mature yolk sac. Histological analysis of the vitelline plexus in control mice showed intact differentiation into small capillaries and large vitelline collecting vessels (Figures 2C and 2E), whereas in NEKO mice, vessels were dilated and disorganized, lacking the typical differentiated pattern (Figures 2D and 2F). Vascular defects were also observed in the labyrinthine layer of the placentas. Normally, the allantois attaches to the chorionic plate, which is the initial step in the formation of a functional placenta, followed by vascular invasion of the labyrinthine layer. In control embryos, blood vessels that contained nucleated erythrocytes invaded and interdigitated into the labyrinthine layer of the developing placenta (Figures 2G and 2I). In NEKO mutants, blood vessels remained at the periphery and did not invade (Figures 2H and 2J). These findings provide further evidence of a cell-autonomous defect in

angiogenesis. Of note, all NEKO mutants had circulating red blood cells visible during preparation, in histological sections and as evidenced by hemorrhage, despite the observed vascular defects. Because embryonic erythropoiesis does not begin until after day E11.5, these erythrocytes observed at day E9.5 must originate from the yolk sac as the primary source of hematopoiesis. This indicates that blood flow has been established between the yolk sac and embryo proper.

In the embryo proper, $N1^{flox/+}$/$Tie2Cre^{+/-}$ animals displayed regular branching differentiation of the vascular tree into small and large blood vessels in the body and head of the developing embryo (Figures 3A and 3C). NEKO animals, in contrast, exhibited a coarse vascular plexus that lacked differentiation into large and small vessels and showed a decreased number of branching blood vessels throughout the embryo (Figures 3B and 3D). Interestingly, the anterior cardinal vein in NEKO embryos also appeared hypoplastic compared with controls (compare Figures 3A and 3B, Figures 4A and 4B). In addition, starting at day E9.5, there was intraembryonic hemorrhage into the pericardium (Figures 3E and 3F) and tails (Figures 3G and 3H) of NEKO mutants. These findings suggest a defect in vascular differentiation in NEKO animals, which leads to abnormal vessel formation and vascular hemorrhage.

Global deletion of Notch1 results in defective embryonic morphogenesis, including defects in vascular development, neurogenesis, and somitogenesis. Surprisingly, the NEKO mice displayed embryonic defects in tissues distinct from the vasculature. Specifically, the forebrain neural tube in NEKO individuals was grossly degenerated, with abundant pyknotic and fragmented nuclei that suggested apoptosis when compared with controls, (Figures 4A, 4B, and 4C). In addition, the heart displayed delayed looping beginning at day E9.5 and a thin myocardial wall. Furthermore, in NEKO animals, somites and their accompanying intersomitic blood vessels were poorly defined, with signs of apoptosis (Figure 4D and 4E) compared with controls.

To address the possible mechanism of vascular failure, we examined proliferation and apoptosis in endothelial cells. Immunostaining for PCNA, a marker for cell proliferation, did not demonstrate any significant difference in endothelial proliferation (data not shown). Immunostaining for activated caspase-3, which is specifically activated during apoptosis, revealed widespread apoptosis in neural cells and the inner endothelial lining of the aorta in NEKO animals (Figure 4G), whereas no apoptosis could be detected in wild-type animals (Figure 4F). These findings indicate that lack of Notch1 in endothelial cells leads to endothelial/neural apoptosis rather than defective endothelial cell proliferation, which results in secondary angiogenic failure and end-organ damage.

Discussion

Vascular development requires the precise temporal and spatial regulation of signaling cascades in different tissues. Notch signaling components are widely expressed in the developing embryo, including in endothelial cells and the early vasculature.[9] General knockout strategies in the mouse have been used to show that the Notch1 signaling pathway regulates vascular morphogenesis at the level of angiogenesis.[13,17] Angiogenesis, although absolutely dependent on the vascular endothelium, may also be induced in the vasculature by extrinsic signals from nonvascular tissues during development and in disease states. Accordingly, the vascular defects observed in the global Notch1 knockout could be explained by either an endothelial defect or by failure to generate a differentiation signal by extravascular tissue. To distinguish between these possibilities, we generated a knockout of Notch1 in endothelial cells using a Tie2 promoter-based strategy.[22,23] Genetic cell-fate tracking studies have shown that within the vasculature, Tie2 marks endothelial cells with high specificity.[20,25]

The present results demonstrate an essential cell-autonomous role of endothelial Notch1 in embryogenesis through regulation of angiogenesis. NEKO mutant embryos showed vascular defects in placenta, yolk sac, and embryo proper, whereas heterozygous deletion of Notch1 in endothelial cells generally resulted in no phenotype. In the yolk sacs of mutant embryos, endothelial cells formed a primary vascular plexus, which indicates that vasculogenesis proceeded normally but failed to induce the secondary vascular remodeling required to form an organized network of large and small blood vessels, demonstrating a defect in angiogenesis. These vascular defects were also evident in the placenta, where blood vessels failed to invade the placental labyrinth. In the embryo proper, defective angiogenesis in the vascular domains of the head and intersomitic region was followed by hemorrhage. These findings essentially recapitulate the vascular phenotype of Notch1$^{-/-}$ mutants,[13,17] pointing to the essential role of endothelial Notch1 signaling in angiogenesis during development, and suggest that the target tissue of global Notch1 KO mice with respect to embryonic lethality is the endothelium.

The first evidence for an essential role of Notch signaling in the vasculature came from studies of gain-of-function mutations of Notch, in which constitutively active Notch4 was knocked into the Flk-1 locus.[10] The constitutive activation of Notch4 in the vasculature leads to abnormal vessel structure and patterning that is similar to the abnormalities observed in the present study. Because Flk-1^{+} cells are progenitors for muscle, hematopoietic, and vascular endothelial cells,[20] however, the contribution of endothelial Notch signaling could not be established definitively. In vitro, overexpression of activated Notch4 in endothelial cells inhibited angiogenesis through β1-integrin-mediated cell-matrix interaction and inhibition of endothelial sprouting.[26] The present findings demonstrate a critical role for Notch signaling in the endothelium during vascular development. In combination, these results also demonstrate a requirement not only for endothelial expression but also for precise regulation of Notch activity, because both increased and decreased activation of Notch signaling result in impaired vascular development.

The downstream target genes of Notch1 that mediate its effects on vascular remodeling in the developing mammalian embryo are only partially understood. The Hey family of transcription factors are candidate effector genes of Notch1 signaling in the endothelium on the basis of their ability to be induced by Notch1 in vitro,[27,28] the recapitulation of the Notch1 null phenotype by the Hey1/Hey2 double-knockout mouse,[17] and the decrease in Hey1 and Hey2 expression observed in Notch1 global null mice.[17] Some inconsistencies remain, however, in that Hey1 and Hey2 reportedly have nonoverlapping expression patterns,[29,30] deletion of Hey1 alone does not result in any detectable phenotype,[17] and deletion of Hey2 has not been reported to affect the vasculature.[31-33] Clearly, the identification and characterization of downstream effectors of Notch signaling that are crucial in embryonic vascular remodeling and arterial differentiation require further investigation.

In addition to vascular defects, Notch1$^{-/-}$ mutants show abundant apoptotic cell death throughout the embryo. In the nervous system, apoptosis occurred despite intact neurogenesis. Notch1$^{-/-}$ embryos also showed delayed separation of somites and a misalignment across the midline, although no internal defect in somitogenesis could be detected.[11,12] The present study indicates that some of these changes occur secondary to an endothelial defect. Vascular insufficiency that results in inadequate delivery of nutrients, growth factors, oxygen, or other critical molecules could explain some features of the observed phenotype. An alternative possibility is that the forming vascular system, which is the first functional system to be established in the embryo, delivers developmental cues through signaling between endothelial cells and organ parenchyma, and that these signals help form various organ systems. This intriguing possibility has some precedent, because Notch signaling recently has been shown to be critical for crosstalk of endothelial cells with neural stem cells.[34]

Our assessment of endothelial function in NEKO embryos suggests an effect on endothelial cell apoptosis, as assessed by activated caspase-3 staining. The increased endothelial cell apoptosis observed may easily explain the defects seen in angiogenesis and the remodeling found in these animals. Our additional assessment of endothelial cell proliferation by PCNA staining showed no significant difference between wild-type and conditional Notch1 knockout animals. The molecular mechanism by which Notch signaling regulates caspase-3 activity remains to be determined.

One potential limitation of the present study is that Tie2 expression has also been observed in early hematopoietic progenitors, which give rise to hematopoietic, lymphoid, and vascular cell lineages.[23,35] Therefore, we cannot completely rule out a possible confounding contribution of a hematopoietic or lymphoid cell type to the observed phenotype. The vascular abnormalities identified, however, coupled with the abnormal endothelial apoptosis observed, suggest that the primary defect is in endothelial rather than hematopoietic cells.

In summary, we have shown that mice that lack endothelial Notch1 recapitulate the vascular defects and embryonic lethality of global Notch1-deficient mice. These findings demonstrate the essential role of Notch1 signaling in angiogenesis and vascular development. Further studies investigating the downstream effectors of Notch1 signaling in endothelial cells are required to understand the role of Notch1 signaling in vascular remodeling and angiogenesis in adult mice.

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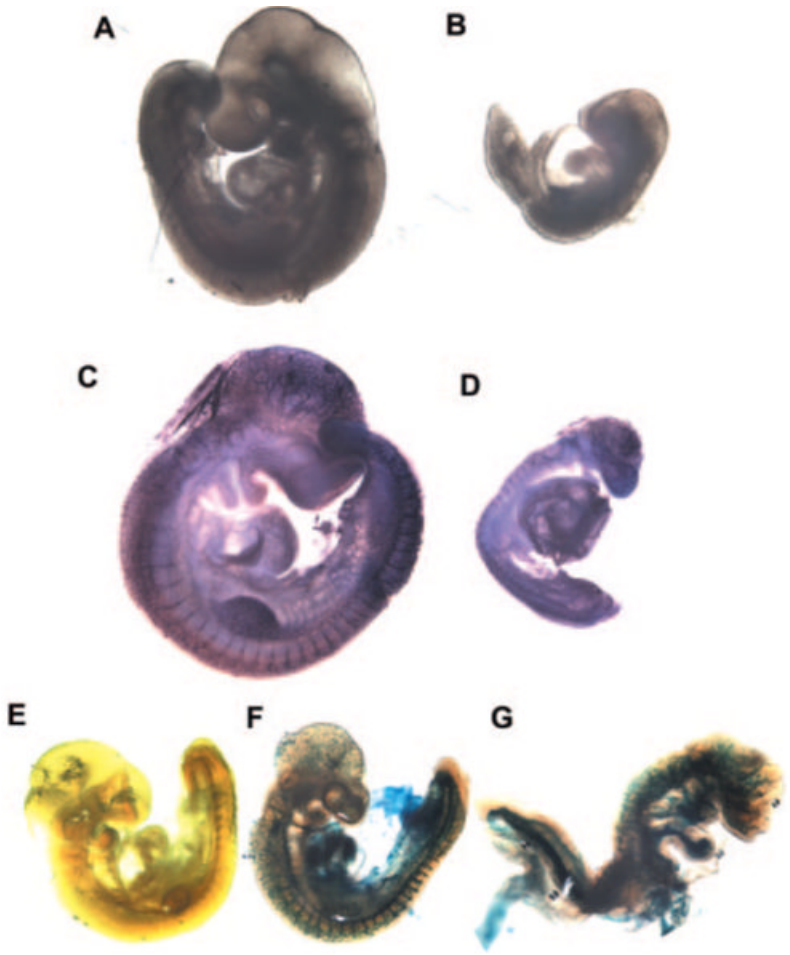

Figure 1.

Growth retardation and vascular defects in NEKO animals at day E9.5. A and B, Whole-mount preparation of control Notch1 ($N1^{flox/+}$/$Tie2Cre^{+/-}$) mice (A) and mice lacking endothelial Notch1 (NEKO, $N1^{flox/flox}$/$Tie2Cre^{+/-}$; B). C and D, Whole-mount PECAM-1-stained control (C) or NEKO (D) embryos. E through G, Whole-mount LacZ-stained control $N1^{flox/+}$/$Tie2Cre^{+/-}$/$LacZ^{-/-}$ (E) and $N1^{flox/+}$/$Tie2Cre^{+/-}$/$LacZ^{+/-}$ (F) or $N1^{flox/flox}$/$Tie2Cre^{+/-}$/$LacZ^{+/-}$ (G) mutant embryos. Note failure to complete embryonic turning in NEKO embryos. Original magnification ×40.

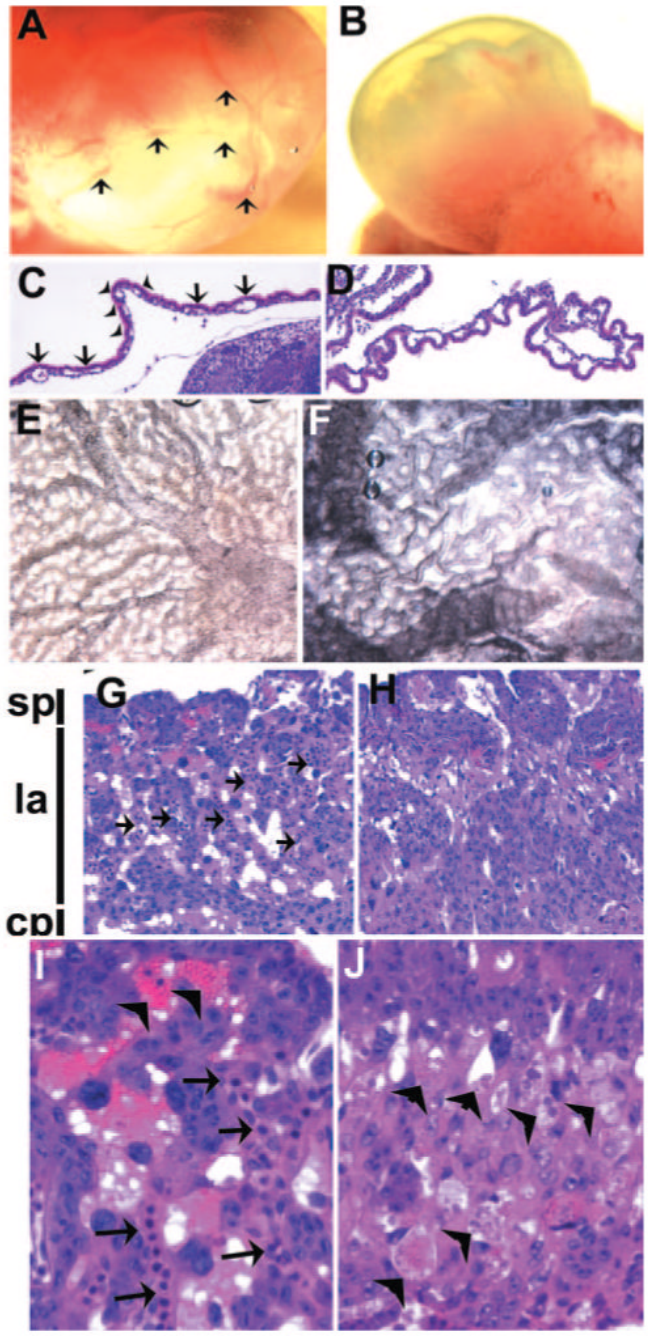

Figure 2.

Angiogenic remodeling in yolk sac and placenta depends on endothelial Notch1. A through D, Yolk sac whole-mount preparation (A) and histological sections of control animals (C) and of NEKO mutants (B, D). Arrows denote major vitelline vessels; arrowheads represent smaller capillaries. There is proper remodeling of vitelline vessels in control animals, which is absent in NEKO mutants. E and F, PECAM-1-stained vasculature of yolk sacs from controls (E) and NEKO embryos (F). G through J, Hematoxylin-and-eosin-stained placental sections of control animals (G, original magnification ×40; I, original magnification ×400) and of NEKO mice (H, original magnification ×40; J, original magnification ×400). Arrows denote invading fetal

vessels filled with nucleated erythrocytes. Arrowheads show maternal blood sinus. sp indicates spongiotrophoblast; la, labyrinth; and cp, chorionic plate.

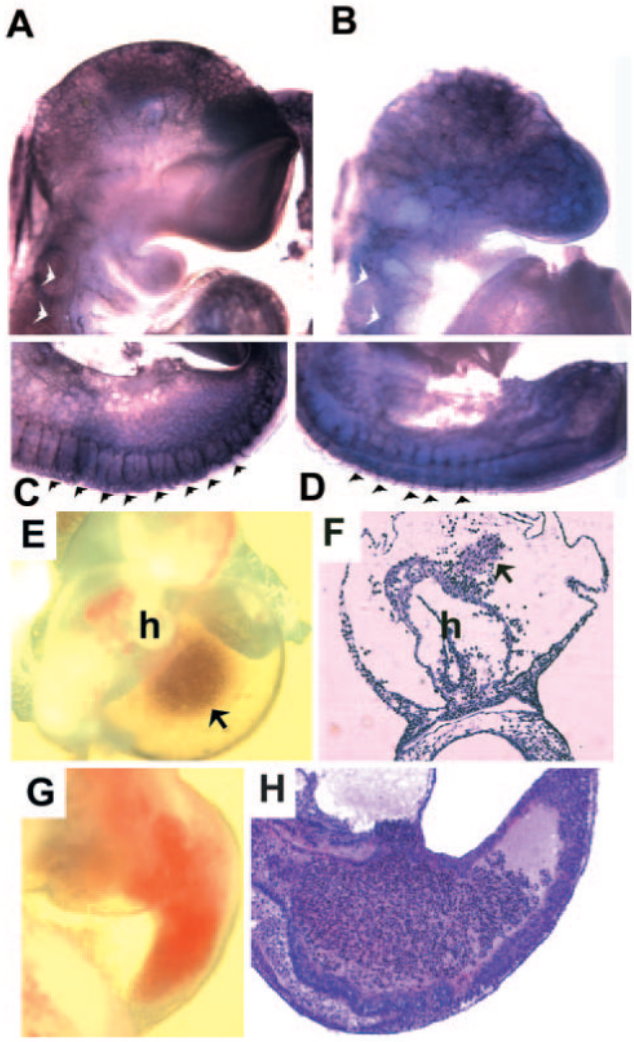

Figure 3.

Impaired angiogenesis and hemorrhage in NEKO mice. A through D, Whole-mount PECAM-1 staining of head (A, B) and tails (C, D) in control and NEKO mutants. There is hypoplastic development of cardinal vein (arrow in B) and decreased sprouting of vessels (arrow in D). White arrowheads in A and B denote cardinal vein. Black arrowheads in C and D denote intersomitic vessels. E through H, Whole-mount view and hematoxylin-and-eosin-stained cross sections of heart and pericardium (E, F) and tails (G, H). Arrows denote hemorrhage. h indicates heart.

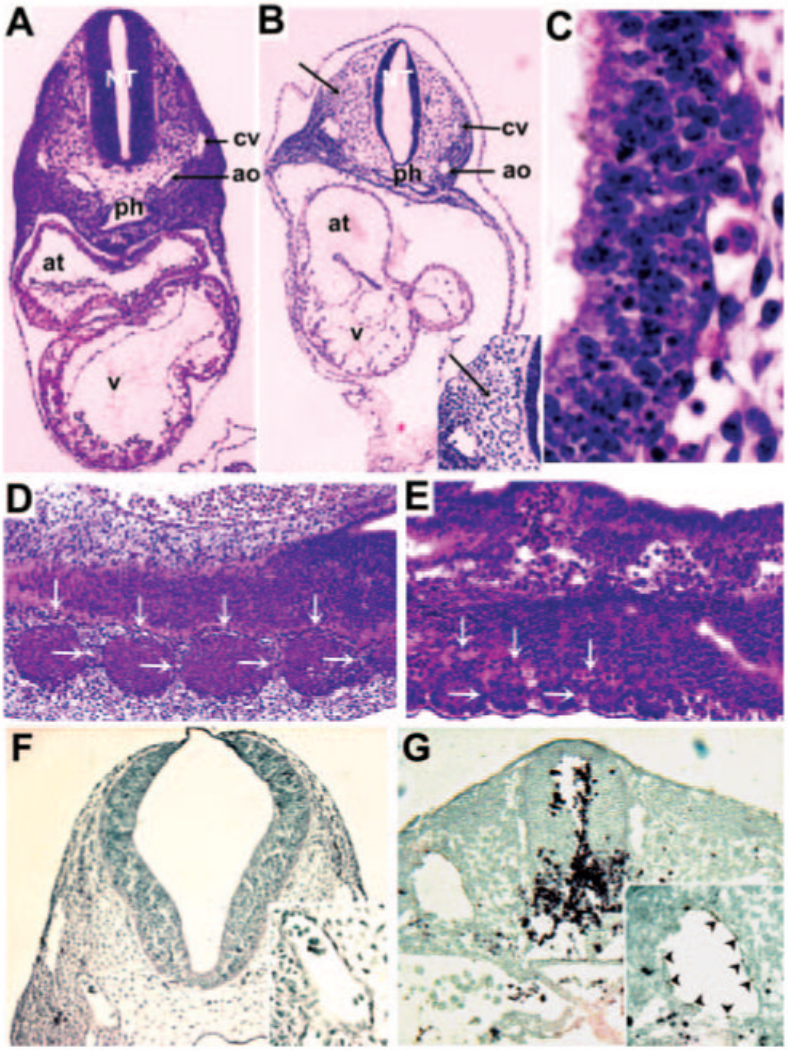

Figure 4.

Neuronal and somitic defects in NEKO mice. A and B, Hematoxylin-and-eosin-stained cross sections through day E9.5 embryos of control (A) and NEKO (B) mice; original magnification ×40. Inset shows hypoplastic cardinal vein at higher magnification. C, Higher magnification (×400) of NEKO mice showing pyknotic nuclei in neural tube. D and E, Hematoxylin-and-eosinstained sagittal sections through embryo trunk at day E9.5 of control (D) and NEKO (E) embryos. Arrows denote intersomitic vessels. F and G, Activated caspase-3 staining of control (F) and NEKO (G) mice. Insets represent aortas at higher magnification. ao indicates aorta; at, atrium; cv, cardinal vein; nt, neural tube; ph, pharynx; and v, ventricle.

Genotype Distribution of Embryos and Newborn Mice

	Tie2Cre$^{+/-}$		Tie2Cre$^{-/-}$	
Time Point	**Notch1$^{flox/flox}$**	**Notch1$^{flox/+}$**	**Notch1$^{flox/flox}$**	**Notch1$^{flox/+}$**
E8.5	5 (4.75)	6 (4.75)	2 (4.75)	6 (4.75)
E9.5	19 (24.25)	35 (24.25)	21 (24.25)	22 (24.25)
E10.5	16 (16)	18 (16)	15 (16)	15 (16)
E11.5	5* (11.5)	13 (11.5)	14 (11.5)	14 (11.5)
E12.5†	0 (13.25)	19 (13.25)	18 (13.25)	16 (13.25)
At birth†	0 (14)	17 (14)	24 (14)	15 (14)

Expected numbers in parentheses.

*Asystolic embryos.

†χ^2<0.005.