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## Critical Role of Endothelial Notch1 Signaling in Postnatal Angiogenesis

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### Abstract

Notch receptors are important mediators of cell fate during embryogenesis, but their role in adult physiology, particularly in postnatal angiogenesis, remains unknown. Of the Notch receptors, only Notch1 and Notch4 are expressed in vascular endothelial cells. Here we show that blood flow recovery and postnatal neovascularization in response to hindlimb ischemia in haploinsufficient global or endothelial-specific Notch1<sup>+/-</sup> mice, but not Notch4<sup>-/-</sup> mice, were impaired compared with wild-type mice. The expression of vascular endothelial growth factor (VEGF) in response to ischemia was comparable between wild-type and Notch mutant mice, suggesting that Notch1 is downstream of VEGF signaling. Treatment of endothelial cells with VEGF increases presenilin proteolytic processing,  $\gamma$ -secretase activity, Notch1 cleavage, and Hes-1 (hairy enhancer of split homolog-1) expression, all of which were blocked by treating endothelial cells with inhibitors of phosphatidylinositol 3-kinase/protein kinase Akt or infecting endothelial cells with a dominant-negative Akt mutant. Indeed, inhibition of  $\gamma$ -secretase activity leads to decreased angiogenesis and inhibits VEGF-induced endothelial cell proliferation, migration, and survival. Overexpression of the active Notch1 intercellular domain rescued the inhibitory effects of  $\gamma$ -secretase inhibitors on VEGF-induced angiogenesis. These findings indicate that the phosphatidylinositol 3-kinase/Akt pathway mediates  $\gamma$ -secretase and Notch1 activation by VEGF and that Notch1 is critical for VEGF-induced postnatal angiogenesis. These results suggest that Notch1 may be a novel therapeutic target for improving angiogenic response and blood flow recovery in ischemic limbs.

### Keywords

angiogenesis; endothelium; ischemia; vasculature

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Notch receptors are transmembrane receptors that interact with membrane-bound ligands, Delta and Serrate/Jagged.<sup>1</sup> Ligand binding induces proteolytic cleavage of Notch and subsequent nuclear translocation of the Notch intracellular domain (NICD),<sup>2</sup> where it interacts with RBP-J protein, and the complex functions as a transcription factor for downstream target

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Disclosures

None.

genes such as Hes-1 (hairy enhancer of split homolog-1).<sup>3</sup> Recent studies suggest that Notch signaling plays a critical role in embryonic development through cell fate determination.<sup>4-6</sup> In particular, the loss of Notch1 or haploinsufficiency of the Notch ligand Dll4 leads to impaired vascular development and somitogenesis, enhanced apoptosis of endothelial cells in the placenta and yolk sac, and embryonic lethality.<sup>5,7,8</sup> Thus, Notch1 is essential for angiogenic vascular remodeling and embryonic development.

Of the Notch receptors, the vascular endothelium expresses only Notch1 and Notch4.<sup>9</sup> Endothelial-specific Notch1<sup>-/-</sup> mice exhibit a similar phenotype to global Notch1<sup>-/-</sup> mice, suggesting that endothelial Notch1 is critical for embryonic vascular development and viability.<sup>7,10</sup> In contrast, Notch4<sup>-/-</sup> mice are viable without any observable vascular defects. However, Notch1<sup>-/-</sup>/Notch4<sup>-/-</sup> mice exhibit a more severe vascular phenotype than Notch1<sup>-/-</sup> mice, suggesting that Notch1 and Notch4 may have overlapping roles in vascular remodeling and morphogenesis during development.<sup>5</sup> The role of Notch1 and Notch4 signaling in postnatal vascular angiogenesis and remodeling remains to be determined.

Postnatal angiogenesis or neovascularization in response to hypoxia, limb ischemia, or wound healing is mediated, in part, by vascular endothelial growth factor (VEGF). VEGF is upregulated in response to hypoxia<sup>11</sup> and promotes angiogenesis in ischemic tissues.<sup>12</sup> Although VEGF also increases the expression of Notch receptors and their ligands, it is not known whether Notch receptors are necessary for VEGF-mediated angiogenic response.<sup>13</sup> The purpose of this study was to determine whether Notch1 and/or Notch4 are essential for postnatal angiogenesis and, if so, to determine the signaling pathway(s) that leads to Notch cleavage and signaling in vascular endothelial cells.

## Materials and Methods

### Animals

N1<sup>+/-</sup>, N4<sup>-/-</sup>, and wild-type (WT) mice were generated as described.<sup>5,14</sup> The ecN1<sup>+/-</sup> mice were generated by crossing Notch1<sup>+/*lox*</sup> and Tie2-Cre transgenic mice (Tie2Cre<sup>+/-</sup>).<sup>7</sup> All of the animals used for the experiments were 10-week-old male mice on pure C57Bl/6 background. All experimental protocols were approved by the Standing Committee on Animal Care at Harvard and Tufts Medical School (Boston, Mass).

### Immunohistochemistry

Immunohistochemistry was performed on the thigh adductor muscle using standard protocol (see expanded Materials and Methods section in the online data supplement, available at <http://circres.ahajournals.org>).

### Isolation of Murine Endothelial Cells

Isolation of endothelial cells from the lungs and hearts of WT, N1<sup>+/-</sup>, ecN1<sup>+/-</sup>, and N4<sup>-/-</sup> mice was performed using 2-sorting with antibody-coated magnetic beads (Dynal beads coated with antibodies for platelet endothelial cell adhesion molecule (PECAM)-1 and intercellular adhesion molecule-2. (See the online data supplement.)

### Cell Culture and Gene Transfer

Human umbilical endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) were cultured as described (see the online data supplement). HUVECs were infected with Ad.caAkt (myr-Akt), Ad.dn-Akt (AAA-Akt) (provided by Kenneth Walsh, Boston University, Mass), Ad.NICD containing GFP reporter (provided by Ken-ichi Tezuka, Gifu University, Japan), or Ad.GFP.

### Hindlimb Ischemia Model

Hindlimb ischemia was achieved by extensive excision of the unilateral femoral artery under anesthesia as described.<sup>15</sup> Blood flow was monitored with a laser Doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments) before and on postoperative days 0, 7, 14, 21, and 28, as described. To control for data variations caused by ambient light and temperature, LDBF was expressed as the ratio of ischemic to nonischemic (contralateral) limb.

### Measurements of Capillary Density

Capillary density in the thigh adductor skeletal muscles was analyzed 28 days after femoral artery excision. Three pieces of ischemic muscles were harvested from each animal, sliced, and fixed in 4% paraformaldehyde. Capillary endothelial cells were identified by immunohistochemical staining with biotinylated isolectinB4 antibody (Vector Laboratories).<sup>16</sup> Fifteen random microscopic fields from 3 different sections per mouse were examined. Capillary density was expressed as the number of capillaries per high-power field (hpf) (magnification,  $\times 400$ ).

### Assay for $\gamma$ -Secretase Activity

The assay for  $\gamma$ -secretase activity was performed as described.<sup>17</sup> Briefly, solubilized membrane preparations were incubated with 8  $\mu\text{mol/L}$  intramolecularly quenched fluorogenic peptide probe harboring the substrate for  $\gamma$ -secretase (Peptide International Inc). Fluorescence was measured using a plate reader (SPECTRA Fluor, TECAM) with excitation wavelength at 355 nm and emission wavelength at 440 nm.

### Cell Proliferation, Adhesion, and Migration Assays

Cell-proliferation assay was performed as described.<sup>18</sup> The number of cells stained with crystal violet was quantified by measurement of absorbance at 590 nm with a microplate reader (SPECTRA Fluor, TECAM). Cellular DNA synthesis was assessed by [<sup>3</sup>H]-thymidine uptake as described.<sup>19</sup> Cell adhesion assay was performed as described.<sup>20</sup> Cell migration was estimated in a modified Boyden chamber (Neuro Probe) with a gelatin-coated 8- $\mu\text{m}$ -pore polycarbonate filter as described.<sup>20</sup> Annexin V staining was used for assessment of apoptotic cells. All protocols are available as in the online data supplement.

### Assessment of Tube Formation

Endothelial cells were infected with Ad.GFP or Ad.NICD at MOI of 50 for 24 hours. Cells were seeded on Matrigel-coated plates at a density of  $2 \times 10^4$  cells per well in medium 199 with 1% FBS with and without VEGF and/or *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) and then incubated for 18 hours at 37°C. Photomicrographs were taken at  $\times 40$  magnification. Total tube length was measured using the NIH Image program and expressed as percentage of control (Ad.GFP).

### Endothelial Explant Assay

An ex vivo angiogenesis assay was performed as described (see the online data supplement).<sup>21</sup> The greatest distance from the aortic ring body to the end of the vascular sprouts (sprout length) was measured by NIH Image program at 3 distinct points per ring and in 3 different rings per treatment group.<sup>22</sup> Results are expressed in arbitrary units (1 U represents 40 pixels per millimeter).

### Statistics

All values are expressed as mean $\pm$ SD. Differences among 3 groups were analyzed by 1-way ANOVA followed by Scheffe's comparison for 2 groups. The comparative incidence of limb

amputation was evaluated by  $\chi^2$  test. A probability value of  $<0.05$  was taken to be significant difference.

## Results

### Notch1 Mediates Blood Flow Recovery and Angiogenesis Following Ischemia

To determine the role of Notch1 and Notch4 in postnatal angiogenesis, we subjected WT, heterozygous Notch1 KO ( $N1^{+/-}$ ), heterozygous endothelial-specific Notch1 KO ( $ecN1^{+/-}$ ), and homozygous Notch4 KO ( $N4^{-/-}$ ) mice to hindlimb ischemia. The expression of Notch1 in endothelial cells of  $N1^{+/-}$  or  $ecN1^{+/-}$  mice was approximately half of that of WT and  $N4^{-/-}$  mice (data not shown). There were no observable differences in appearance, body weight, or heart rate between WT and these mutant Notch mice, either before, during, or after hindlimb ischemia. The  $N4^{-/-}$  mice, however, exhibited a slightly higher systolic blood pressure compared with WT mice ( $116\pm 6$  mm Hg versus  $110\pm 5$  mm Hg,  $P<0.05$ ;  $n=12$  in each group). All of the mice survived hindlimb ischemia surgery, and their phenotypes with respect to limb ischemia were analyzed.

Following hindlimb ischemia, the expression of VEGF in the ischemic adductor muscles was induced diffusely at 7 days, and the level of expression was comparable between WT and Notch mutant mice (Figure 1A). Despite comparable levels of VEGF, distal limb necrosis at 28 days was observed in 4% of WT mice, 42% of  $N1^{+/-}$  mice, and 50% of  $ecN1^{+/-}$  mice (Figure 1B). Distal limb necrosis at 28 days occurred in 6% of  $N4^{-/-}$  mice (data not shown), which is comparable to that of WT mice. These findings indicate that increased distal limb necrosis following hindlimb ischemia in  $N1^{+/-}$  and  $ecN1^{+/-}$  mice was not attributable to differences in VEGF upregulation and that Notch1 rather than Notch4 protects against ischemia-induced limb necrosis.

Basal and postischemic blood flows as measured by LDBF imaging were comparable between mice from each group. Compared with the nonischemic limb (control), blood flow recovery to the ischemic limb at 14 and 28 days was also comparable between WT and  $N4^{-/-}$  mice (ratio of ischemic to control limb:  $0.92\pm 0.08$  and  $0.93\pm 0.08$ , respectively,  $P=NS$ ) (Figure 2A). These findings indicate that Notch4 does not contribute to blood flow recovery in response to ischemia. In contrast, blood flow recovery in  $N1^{+/-}$  and  $ecN1^{+/-}$  mice was substantially impaired at 14 days and remained impaired at 28 days (ratio of ischemic to control limb:  $0.47\pm 0.12$  and  $0.40\pm 0.15$ , respectively,  $P<0.0001$  compared WT;  $n=12$  for each at 28 d). These results indicate that endothelial Notch1 is critical to blood flow recovery in response to ischemia.

The improvement in blood flow recovery corresponded to increased tissue capillary density (Figure 2B). The capillary densities in the nonischemic adductor muscles (control) were comparable between WT and Notch mutant mice (WT,  $58\pm 5$ ;  $N1^{+/-}$ ,  $58\pm 7$ ;  $ecN1^{+/-}$ ,  $56\pm 5$ ; and  $N4^{-/-}$ ,  $57\pm 5$  capillaries per hpf;  $P=NS$  for all compared with WT). Furthermore, following hindlimb ischemia, the capillary density per hpf ( $\times 400$  magnification) in the ischemic adductor muscles of WT and  $N4^{-/-}$  mice were increased to a comparable extent compared with that of control nonischemic capillary density ( $102\pm 11$  and  $101\pm 11$  capillaries per hpf, respectively;  $P<0.001$  compared with control for both;  $P=NS$  between WT and  $N4^{-/-}$  mice). However, the capillary densities in the ischemic adductor muscles of  $N1^{+/-}$  and  $ecN1^{+/-}$  mice were substantially lower ( $70\pm 7.8$  and  $68\pm 7.9$  capillaries per hpf, respectively,  $P<0.001$  compared with WT;  $n=8$  for each at 28 days). These findings indicate that Notch1, but not Notch4, is critical for the post-ischemic angiogenic response.

## Induction of Endothelial Notch1 Expression and Signaling in Ischemic Limb

The expression of Notch1 in control limbs was barely detectable despite presence of PECAM-1-stained endothelial cells. However, following ischemia, Notch1 was expressed in some endothelial cells of ischemic limbs on day 7 (Figure 3A). This corresponded to increased Hes-1 expression in the endothelium of ischemic, but not control, limbs (Figure 3B). In contrast, there was constitutive expression of Notch4 in the endothelium (ie, PECAM-1-positive stained cells), which was comparable in control and ischemic limbs (data not shown). Similarly, in aortas from WT mice, microvessel sprouting was  $228 \pm 19$  U (1 U=40 pixels per millimeter on NIH Image program) (Figure 3C). However, the sprouting of microvessels from the aortas of  $N1^{+/-}$  and  $ecN1^{+/-}$  mice was substantially reduced ( $45 \pm 6$  and  $56 \pm 6$  U, respectively,  $P < 0.01$  for both compared with WT). These findings indicate that ischemia induces the expression and activation of Notch1 and that Notch1 mediates microvessel sprouting.

## Akt Mediates VEGF-Induced Notch1 Cleavage and Activation

Treatment of endothelial cells with VEGF (25 ng/mL) increased Akt phosphorylation within 15 to 30 minutes (Figure 4A). This corresponded with the temporal cleavage of Notch1 and expression of Hes-1. VEGF, however, did not alter the expression of Akt, the Notch ligand, Jagged-1, or Notch1. Cotreatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 or the Akt inhibitor SH-5 inhibited VEGF-induced Akt activation, Notch1 cleavage, and Hes-1 expression (Figure 4B). Interestingly, the NOS inhibitor  $N^G$ -nitro-L-arginine methyl ester partially decreased VEGF-induced Akt phosphorylation but had little or no effect on VEGF-induced Notch1 activation. In contrast, the  $\gamma$ -secretase inhibitor DAPT had no effect on VEGF-induced Akt phosphorylation but completely blocked VEGF-induced Notch1 cleavage and Hes-1 expression. These findings suggest that  $\gamma$ -secretase mediates Notch1 cleavage and activation by Akt.

To verify that Akt mediates Notch1 activation, endothelial cells were infected with adenovirus carrying empty vector (control, Ad.GFP), dominant-negative Akt mutant (Ad.dnAkt), or constitutively active Akt mutant (Ad.caAkt) in the presence or absence of VEGF stimulation (Figure 4C). In endothelial cells infected with Ad.GFP, VEGF increased Akt and glycogen synthase kinase (GSK)-3 phosphorylation, Notch1 cleavage, and Hes-1 expression. In contrast, in endothelial cells infected with Ad.dnAkt, VEGF was unable to activate Akt or Notch1. Infection with Ad.caAkt alone increased Akt and GSK-3 phosphorylation as well as induced Notch1 cleavage and Hes-1 expression. These results indicate that Akt mediates VEGF-induced Notch1 cleavage and activation.

## Activation of $\gamma$ -Secretase by Akt Mediates VEGF-Induced Notch1 Cleavage

Treatment with either LY294002 or SH-5 decreased  $\gamma$ -secretase activity below basal levels and completely blocked VEGF-induced  $\gamma$ -secretase activity (Figure 5A). Indeed, the activation of  $\gamma$ -secretase by VEGF was inhibited in a concentration-dependent manner by the  $\gamma$ -secretase inhibitor DAPT (Figure 5B). Interestingly, endogenous  $\gamma$ -secretase activity was also partially inhibited by DAPT (81 $\pm$ 4% and 62 $\pm$ 8% of control for 2 and 20  $\mu$ mol/L of DAPT, respectively,  $P < 0.05$  compared with control for both), suggesting that  $\leq 20$   $\mu$ mol/L DAPT is incapable of inhibiting a substantial amount of basal  $\gamma$ -secretase activity. The increase in VEGF-induced  $\gamma$ -secretase activity corresponded with increase in Notch1 cleavage, which was blocked by DAPT ( $IC_{50} \approx 5$   $\mu$ mol/L) (Figure 5C).

Because the activation of  $\gamma$ -secretase involves the proteolytic cleavage of 1 of its core components, presenilin, we investigated whether Akt mediates presenilin proteolytic processing by VEGF. Treatment with VEGF enhanced the cleavage of presenilin from the holoenzyme (55 kDa) to the C-terminal fragment (22 kDa) (Figure 5D). Infection of endothelial cells with Ad.dnAkt blocked VEGF-induced proteolytic cleavage of presenilin, whereas



infection of endothelial cells with Ad.caAkt increased presenilin proteolytic cleavage. These findings indicate that PI3K/Akt pathway mediates the activation of  $\gamma$ -secretase and Notch1 by VEGF in endothelial cells.

### Notch1 Mediates VEGF-Induced Endothelial Cell Proliferation and Migration

In the absence of VEGF stimulation, treatment with DAPT did not affect endothelial cell proliferation as determined by cell number and DNA synthesis ( $^3\text{H}$ -thymidine incorporation) (Figure 6A and 6B). These findings suggest that basal  $\gamma$ -secretase activity, which is inhibited partially by DAPT, does not mediate endothelial cell proliferation. Treatment with VEGF produced a 2-fold increase in cell number ( $5620 \pm 150$  to  $9990 \pm 310$  cells,  $P < 0.01$ ) and  $^3\text{H}$ -thymidine incorporation ( $6370 \pm 290$  to  $12400 \pm 590$  cpm/well,  $P < 0.01$ ). Cotreatment with DAPT (2 and 20  $\mu\text{mol/L}$ ) inhibited VEGF-induced endothelial cell proliferation by 58% and 71%, respectively ( $P < 0.001$  for both compared with VEGF alone). These findings suggest that  $\gamma$ -secretase contributes to VEGF-induced endothelial cell proliferation.

Similarly, basal adhesion and migration in a modified Boyden chamber were not affected by inhibition of basal  $\gamma$ -secretase activity with DAPT (Figure 6C and 6D). Treatment with VEGF produced a 2- to 3-fold increase in cell adhesion ( $5,620 \pm 150$  to  $9990 \pm 310$  cells,  $P < 0.01$ ) and migration ( $100 \pm 3\%$  to  $280 \pm 15\%$ ,  $P < 0.01$ ). Cotreatment with DAPT inhibited endothelial cell adhesion and migration in a concentration-dependent manner. These findings indicate that VEGF-induced increase in  $\gamma$ -secretase activity contributes to endothelial cell adhesion and migration.

### Notch1 Mediates the Antiapoptotic Effects of VEGF on Endothelial Cells

To determine whether Notch1 plays an antiapoptotic role in endothelial cells, apoptosis, as determined by the presence of cleaved nuclei by DAPI staining and expression of Annexin V, was assessed in endothelial cells exposed to serum deprivation. Following 16 hours of serum deprivation, approximately 15% to 17% of endothelial cells undergo apoptosis, which was unaffected by treatment with DAPT (Figure 7A). Treatment with VEGF improved endothelial cell survival and decreased the number of apoptotic cells from 16.4% to 1.8% of total cells ( $P < 0.01$ ). However, cotreatment with DAPT (2 and 20  $\mu\text{mol/L}$ ) reversed the protective effects of VEGF and increased the number of apoptotic cells from 1.8% to 8.1% and 14.2% of total cells, respectively ( $P < 0.01$  for both compared with VEGF alone). Similarly, cleaved caspase3, an indicator of the apoptotic process, was present in endothelial cells deprived of serum (Figure 7B). Treatment with VEGF decreased the amount of cleaved caspase3, suggesting decreased apoptosis. In a concentration-dependent manner, cotreatment with DAPT blocked VEGF-induced decrease in caspase3 cleavage. These findings suggest that Notch1 signaling mediates the antiapoptotic effects of VEGF on endothelial cells.

### Notch1 Mediates VEGF-Induced Angiogenesis

Matrigel-based in vitro angiogenesis assay was performed to determine whether Notch1 contributes to VEGF-induced angiogenesis. Under basal conditions, endothelial cells infected with Ad.GFP formed tubular networks, the lengths of which were increased substantially following exposure to VEGF ( $183 \pm 6\%$  versus  $100 \pm 5\%$ ,  $P < 0.01$  compared with Ad.GFP) (Figure 7C and 7D). Cotreatment with DAPT blocked VEGF-induced tubular networks by 75% ( $121 \pm 4\%$  of Ad.GFP,  $P < 0.05$  compared with Ad.GFP+VEGF). Infection of endothelial cells that have been treated with VEGF and DAPT with an adenovirus containing the Notch intracellular domain (Ad.NICD) reversed the inhibitory effect of DAPT on VEGF-induced tube formation ( $160 \pm 7\%$  of Ad.GFP,  $P < 0.05$  compared with VEGF+DAPT). These results indicate that Notch1 mediates VEGF-induced angiogenesis.

## Discussion

Members of the Notch receptor family are highly conserved type I transmembrane receptors that determine cell fate in embryogenesis.<sup>6</sup> What has not been clearly established is the role of Notch receptors in postnatal physiology. In this study, we have shown that endothelial Notch1 plays a critical role in vascular remodeling by mediating the VEGF-induced angiogenic response to limb ischemia. We found that Notch1 contributes to VEGF-induced endothelial cell proliferation, migration, and survival. Furthermore, the loss of Notch1 in the endothelium, as opposed to the loss of Notch1 in other cell types, was primarily responsible for the impaired angiogenic response as blood flow recovery and capillary densities were comparably reduced in endothelial-specific *ecN1<sup>+/-</sup>* and global *N1<sup>+/-</sup>* mice. Although Notch4 is also expressed in endothelial cells,<sup>9</sup> Notch4 does not appear to be necessary for postnatal angiogenesis as both limb necrosis and blood flow recovery in *Notch4<sup>-/-</sup>* mice were similar to that of WT mice. Our results, therefore, indicate that impairment of endothelial Notch1 worsens the severity of limb ischemia with respect to tissue necrosis and suggest that agents or conditions, which improve endothelial Notch1 signaling, may be beneficial in contributing to therapeutic angiogenesis.

Growth factors such as VEGF, hepatic growth factor (HGF), and fibroblast growth factor (FGF) are upregulated in ischemic tissues, leading to increased angiogenesis and improved blood flow. VEGF is critical for vascular development and neovascularization, and VEGF-deficient mice exhibit impaired angiogenesis following limb ischemia.<sup>23</sup> However, the differences in angiogenesis and blood flow recovery between WT mice and Notch1 mutant mice in our study could not be attributed to increases in VEGF expression, because VEGF in the ischemic skeletal muscle was similar between the different groups of mice. These findings suggest that Notch1 must lie downstream of VEGF signaling. This relationship between VEGF and Notch1 is consistent across different cell types and species. For example, in zebrafish, VEGF, which acts downstream of sonic hedgehog, regulate arterial cell fate determination through the Notch pathway.<sup>4</sup> VEGF also activates other downstream signaling molecules including phospholipase C- $\gamma$ , Src, p38MAPK, Ras, Raf, FAK, and PI3K/Akt, leading to endothelial cell proliferation, migration, and survival.<sup>24</sup> In particular, the activation of Akt in endothelial cells leads to cell survival, proliferation, migration, nitric oxide production, and microvascular tube formation in response to VEGF stimulation.<sup>25</sup> Thus, Notch1 signaling could contribute to many of the Akt-mediated effects of VEGF. Indeed, we found that Notch1 expression on the endothelium was induced following ischemic injury and that VEGF-induces Notch1 cleavage and signaling through an Akt-dependent mechanism (Figure 8). Taken together, these findings suggest that Notch1 is an important downstream target of VEGF in vascular endothelial cells.

The Notch receptors are activated when bound to ligands belonging to the Delta or Serrate/Jagged family.<sup>1</sup> Ligand-bound Notch receptors are proteolytically cleaved at their intramembrane site,<sup>26</sup> resulting in the translocation of the Notch intracellular domain (NICD) into the nucleus. Indeed, mutant mice, which cannot cleave Notch1, exhibit a similar phenotype and embryonic lethality as Notch1-deficient mice.<sup>27</sup> The NICD interacts with DNA-binding proteins including RBP-J $\kappa$ , Su(H), and Lag-1, and the multiprotein complex regulates transcription of target genes such as Hes-1 that are involved in cell fate determination.<sup>28</sup> Hes-1 is a basic helix-loop-helix protein, which is essential for neurogenesis, myogenesis, hematopoiesis, self-renewal of neural stem cells, and angiogenesis. How Notch signaling regulates angiogenesis remains to be determined. However, recent studies suggest that endothelial Notch signaling upregulates the expression of Ephrin-B2<sup>29</sup> and Ephrin-B2 is required for directional migration and cell-matrix interaction.<sup>30</sup> Furthermore, Notch may regulate angiogenesis through effects on Hey2/CHF1. Gridlock, the homolog of Hey2/CHF1 in zebrafish, is thought to be downstream of Notch signaling and is involved in arterio-venous differentiation. Thus, although the precise mechanism by which Notch1 regulates angiogenesis is not known, it is likely that several downstream targets mediate this process.



The multiprotein enzyme complex  $\gamma$ -secretase, which includes presenilin, nicastrin, Aph-1, and Pen-2, cleaves Notch receptors.<sup>26</sup> The regulation of presenilin, which comprises the active catalytic core of  $\gamma$ -secretase, is still relatively unknown. Besides Notch receptors,  $\gamma$ -secretase is also required for the proteolytic processing of Alzheimer's disease  $\beta$ -amyloid precursor protein.<sup>31</sup> Thus,  $\gamma$ -secretase is an important regulator of both neurogenesis and neuropathobiology. However, the relationship between Notch receptors and Alzheimer's disease remains to be determined. Presenilin is a 55-kDa aspartyl protease that is also proteolytically cleaved to yield amino- and carboxy-terminal fragments of  $\approx 35$  kDa and  $\approx 20$  kDa, respectively.<sup>32</sup> The proteolytic processing of presenilin is required for its maturation and activation of  $\gamma$ -secretase activity.<sup>33</sup> In agreement with these results, we find that Akt mediates VEGF-induced Notch1 signaling via proteolytic processing of presenilin and subsequent activation of  $\gamma$ -secretase. Interestingly, GSK-3, which interacts with presenilin and is also a downstream target of Akt, has been shown to modulate  $\beta$ -amyloid precursor protein cleavage.<sup>34</sup>

In this study, the physiological relevance of endothelial Notch1 in postnatal neovascularization is demonstrated by the impaired angiogenic response in *ecN1<sup>+/-</sup>* mice and the inhibition of VEGF-induced endothelial cell proliferation, migration, and survival by the  $\gamma$ -secretase inhibitor DAPT. Although relatively little is known about the regulation and function of endothelial  $\gamma$ -secretase, a recent study showed that capillary sprouting and vascular remodeling are impaired in presenilin-deficient mice.<sup>35</sup> Furthermore, DAPT has been shown to inhibit endothelial proliferation and angiogenesis without cytotoxicity, leading to suppression of tumor growth.<sup>36</sup> Thus,  $\gamma$ -secretase and Notch1 signaling may be important downstream targets of VEGF in terms of ischemia-induced neovascularization. These findings suggest that modulation of  $\gamma$ -secretase activity and Notch1 signaling in the endothelium may be therapeutically beneficial in patients with ischemic vascular disease.

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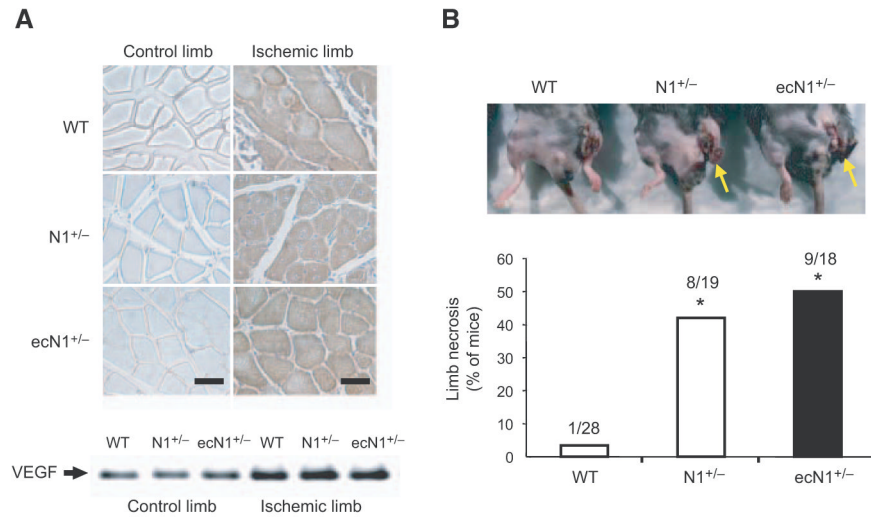
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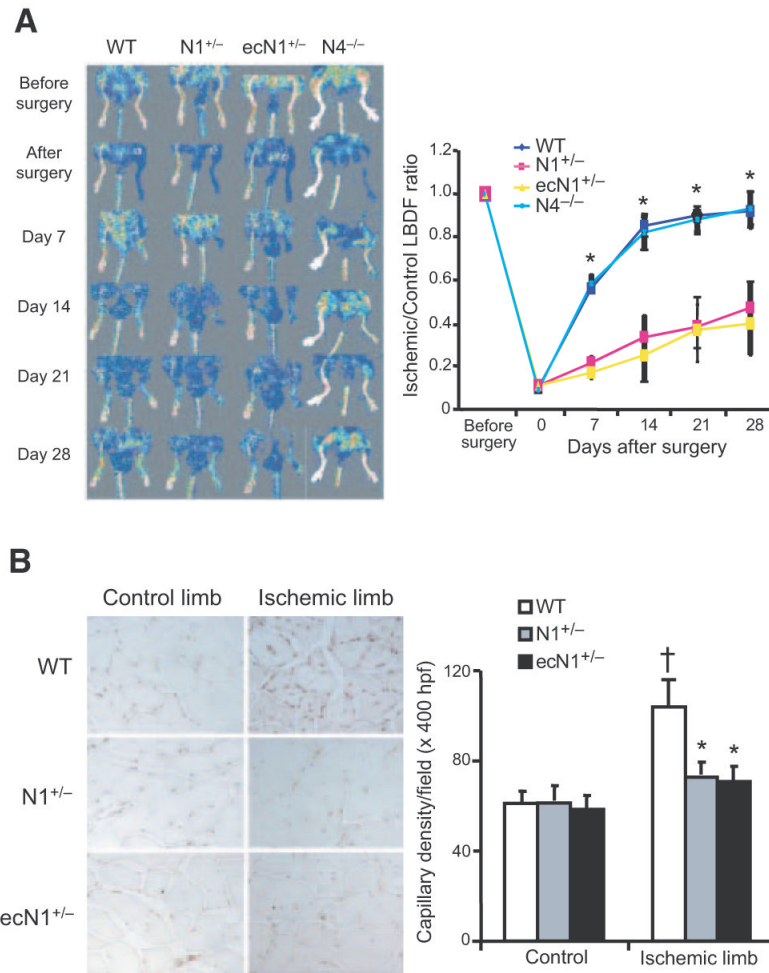
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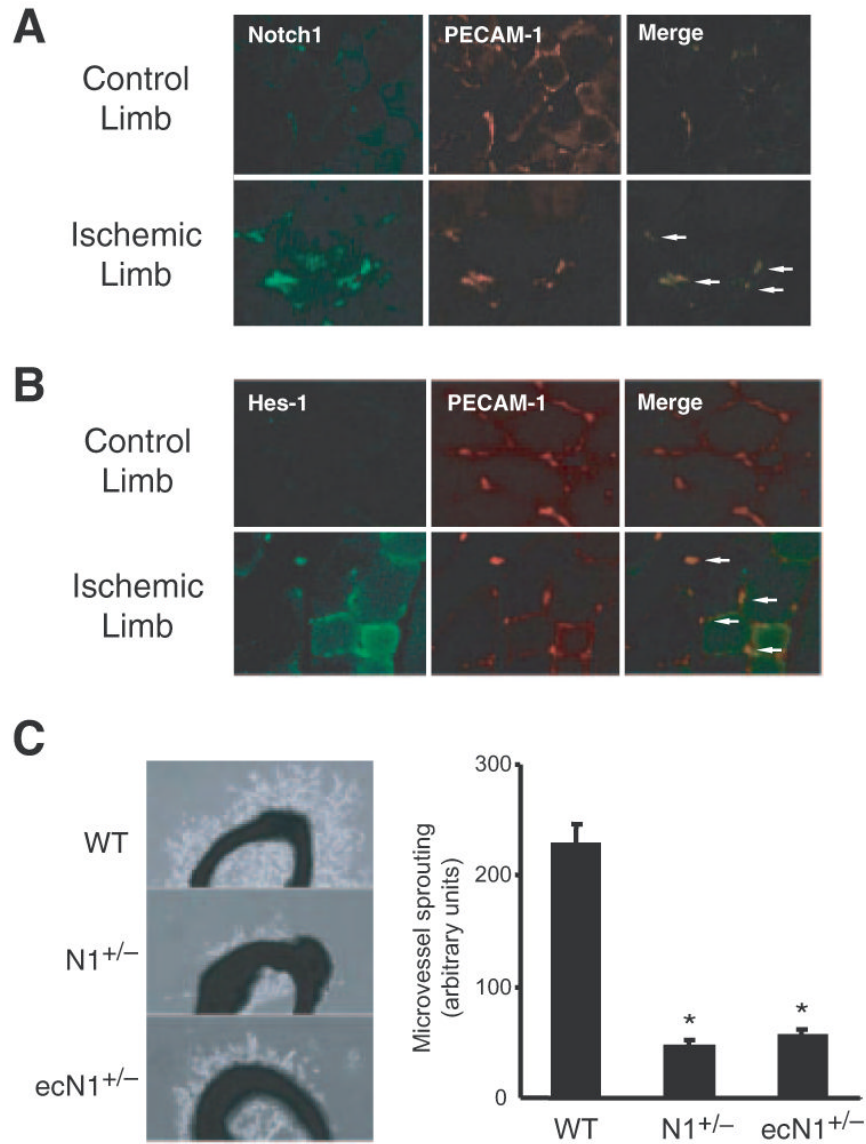


**Figure 1.** Increase limb necrosis in Notch1 mutant mice. **A**, Expression of VEGF in control and ischemic limbs at 7 days was determined by immunohistochemical staining (top) and immunoblot analysis (bottom) of adductor muscle. Bar=25  $\mu$ m. **B**, Photomicrographs of ischemic limb showing distal necrosis at 28 days (top, arrows). The percentage distribution of distal necrosis in WT, N1<sup>+/-</sup>, and ecN1<sup>+/-</sup> mice relative to the total number of mice in each group is shown (bottom). All mice survived hindlimb ischemia surgery and were analyzed. \* $P$ <0.05 compared with WT.



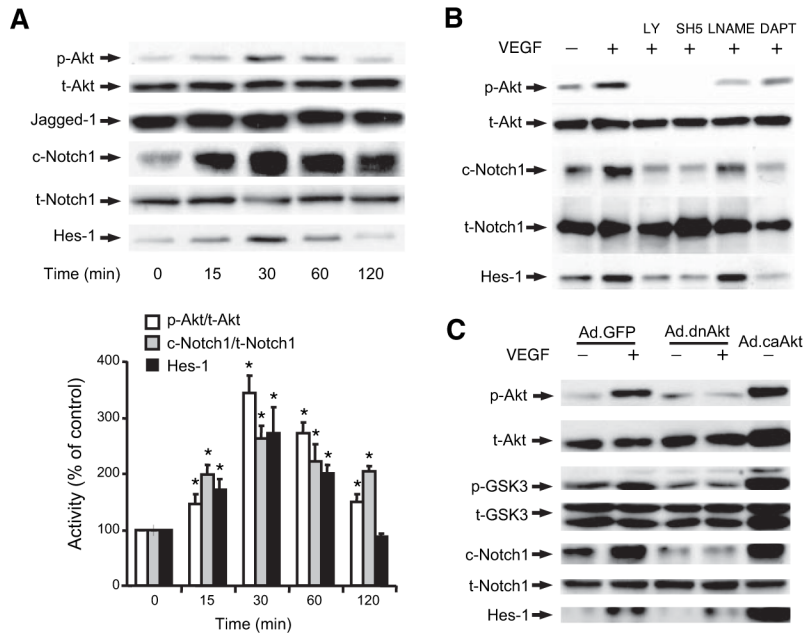
**Figure 2.**

Impaired blood flow recovery and angiogenesis in Notch1 mutant mice. **A**, LBDF showing low-perfusion signal (dark blue) in ischemic hindlimbs of N1<sup>+/-</sup> and ecN1<sup>+/-</sup> mice, whereas high-perfusion signal (red to white) was observed in ischemic hindlimb of WT and N4<sup>-/-</sup> mice (left). Quantitative analysis of blood flow recovery following femoral artery ligation expressed as ischemic to control LDBF ratio in WT, N1<sup>+/-</sup>, ecN1<sup>+/-</sup>, and N4<sup>-/-</sup> mice (right). \* $P < 0.0001$ ,  $n = 12$  in each group. **B**, Isolectin B4 staining (brown-red color) of control (nonischemic) and ischemic adductor muscle tissues from hindlimbs of WT and Notch1 mutant mice (left). The photomicrographs are representative data from 8 individual mice from each group. Bar = 25  $\mu\text{m}$ . Quantitative analysis of capillary density (6 randomly selected fields from each slide; 3 slides per mice; 8 mice each) in WT and Notch1 mutant mice (number per hpf;  $\times 400$  magnification) (right). † $P < 0.001$  compared with WT (nonischemic), \* $P < 0.001$  compared with WT (ischemic).

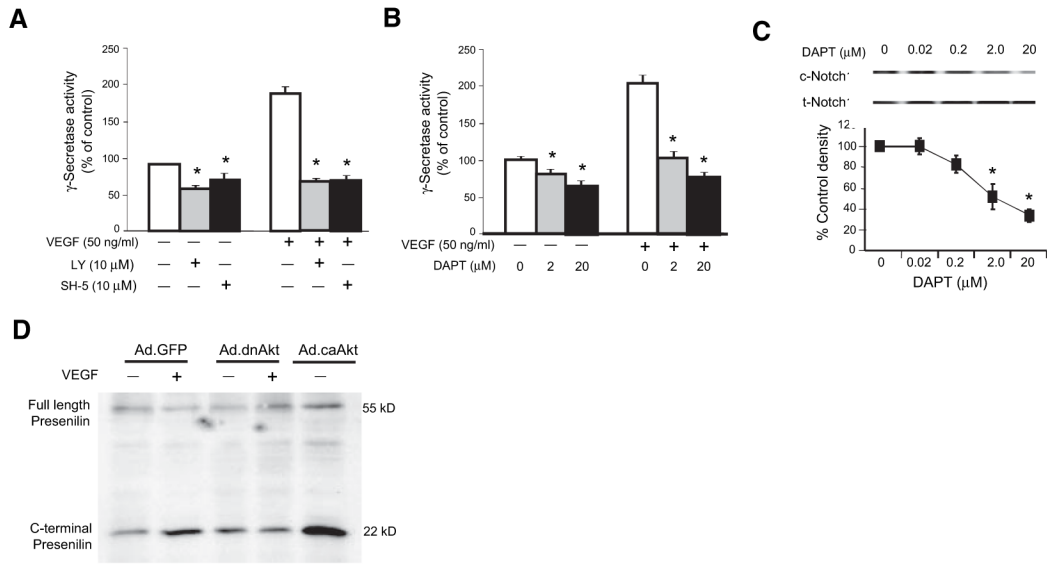


**Figure 3.** Impaired microvessel sprouting in Notch1 mutant mice. A, Immunofluorescence staining for Notch1 (green) and PECAM-1 (red) in skeletal muscle tissues from control and ischemic limbs of WT mice at day7 after the procedure. B, Immunofluorescence staining for Hes-1 (green) and PECAM-1 (red) in skeletal muscle tissues from control and ischemic limbs of WT mice at day7 after the procedure. C, Representative fields ( $\times 40$  magnification) showing microvessel outgrowth in response to VEGF (50 ng/mL) from aortic explants of WT and Notch1 mutant mice (left). Microvessel outgrowth is expressed quantitatively in arbitrary units (1 arbitrary unit represents 40 pixels per millimeter) (right). Data are presented as mean $\pm$ SD, n=8 in each group. \* $P < 0.001$  for N1<sup>+/-</sup> and ecN1<sup>+/-</sup> compared with WT.



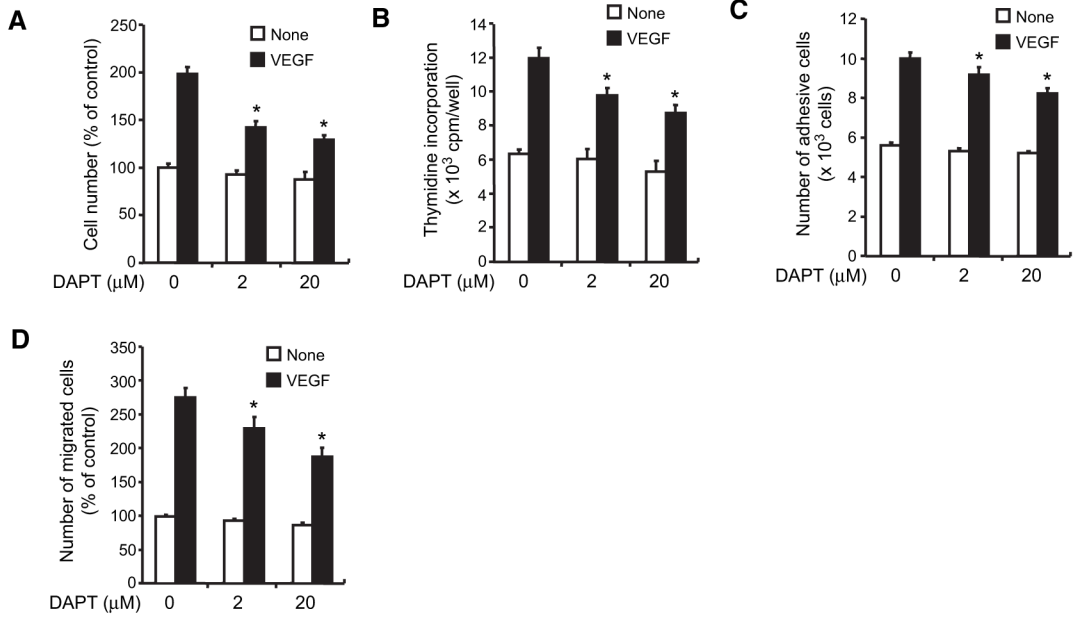


**Figure 4.** Akt mediates Notch1 cleavage and Hes-1 expression by VEGF. **A**, Immunoblots showing time course of Akt and Notch1 activation and Notch1 ligand (Jagged-1) and Hes-1 expression in HUVECs by VEGF (25 ng/mL) (top). Activation of Akt was assessed by Ser473 phosphorylation of Akt (p-Akt) relative to total Akt (t-Akt). Activation of Notch1 was determined by the amount of cleaved Notch1 (c-Notch1) relative to total Notch1 (t-Notch1). Densitometric analysis showing time course of Akt activation (p-Akt/t-Akt), Notch1 activation (c-Notch1/t-Notch1), and Hes-1 expression by VEGF (bottom). Results are presented from 3 independent experiments. \* $P < 0.05$  compared with control (0 time point). **B**, Effects of PI3K inhibitor LY294002 (LY, 10  $\mu\text{mol/L}$ ), Akt inhibitor SH-5 (10  $\mu\text{mol/L}$ ), NOS inhibitor  $N^G$ -nitro- $L$ -arginine methyl ester (LNAME) (1 mmol/L), and  $\gamma$ -secretase inhibitor DAPT (20  $\mu\text{mol/L}$ ) on Akt activation (p-Akt), Notch1 cleavage (c-Notch1), and Hes-1 expression by VEGF (50 ng/mL, 30 minutes). Experiments were performed 3 times with similar results. **C**, Representative immunoblots showing Akt activation (p-Akt), GSK-3 phosphorylation (p-GSK3), Notch cleavage (c-Notch1), and Hes-1 expression in HUVECs infected with adenovirus carrying GFP (Ad.GFP), dominant-negative mutant form of Akt (Ad.dnAkt), and constitutively active mutant form of Akt (Ad.caAkt), in the presence or absence of VEGF stimulation (50 ng/mL, 30 minutes). Three independent experiments yielded similar results.



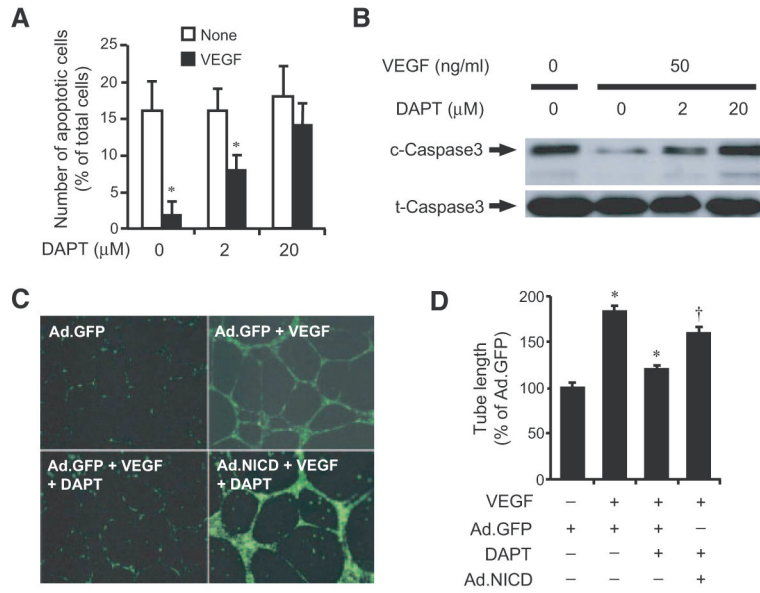
**Figure 5.**

Akt mediates activation of  $\gamma$ -secretase by VEGF. A, Effect of LY294002 (LY) (10  $\mu$ mol/L) or SH-5 (10  $\mu$ mol/L) on  $\gamma$ -secretase activity in HUVECs in the presence or absence of VEGF (50 ng/mL, 30 minutes). \* $P$ <0.05 compared with no treatment or VEGF alone. Results are presented from 3 independent experiments. B, Concentration-dependent effects of DAPT (2 and 20  $\mu$ mol/L) on VEGF-induced (50 ng/mL, 30 minutes)  $\gamma$ -secretase activity. \* $P$ <0.05 compared with no treatment or VEGF alone. Results are presented from 3 independent experiments. C, Representative immunoblots showing the concentration-dependent effects of DAPT (0 to 20  $\mu$ mol/L) on VEGF-induced (50 ng/mL, 30 minutes) Notch1 cleavage (c-Notch1) relative to total Notch1 (t-Notch1) (top). Densitometric analysis of Notch1 activation (c-Notch1/t-Notch1) by VEGF in the presence of increasing concentrations of DAPT (bottom). \* $P$ <0.05 compared with no DAPT. Results are presented from 3 independent experiments. D, Representative immunoblot using C-terminal presenilin antibody showing proteolytic processing of presenilin in HUVECs infected with Ad.GFP, Ad.dnAkt, and Ad.caAkt, in the presence or absence of VEGF stimulation (50 ng/mL, 30 minutes). The protein band at 55 kDa represents presenilin holoenzyme, whereas the 22 kDa band carboxyl-terminal fragment of presenilin. Results are representative of 3 experiments.

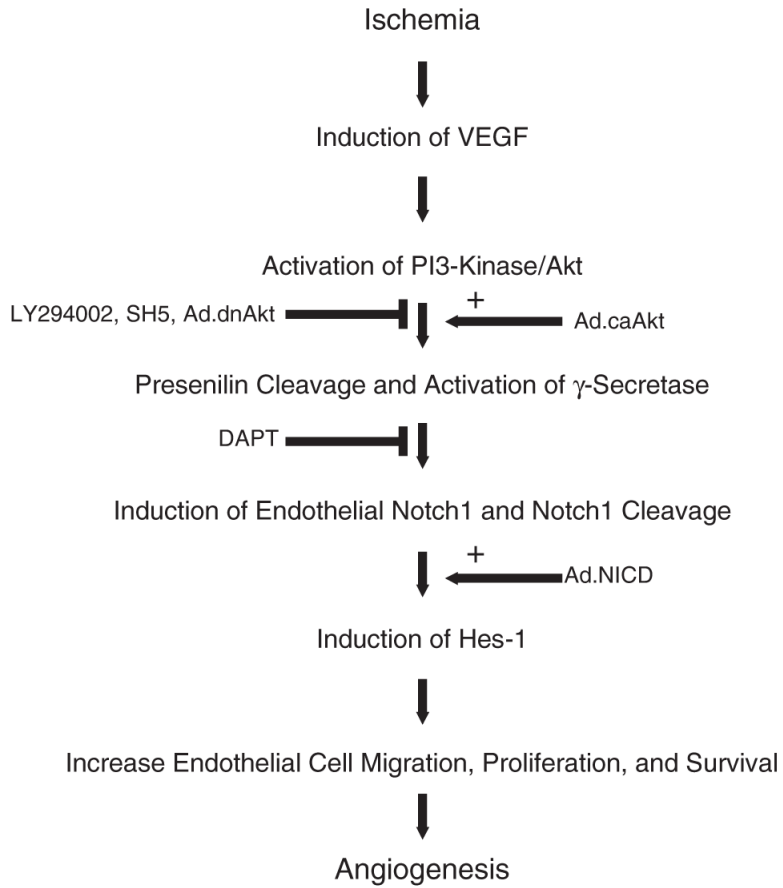


**Figure 6.**

$\gamma$ -Secretase mediates endothelial cell proliferation, adhesion, and migration by VEGF. Concentration-dependent effects of  $\gamma$ -secretase inhibitor DAPT (2 and 20  $\mu$ mol/L) on proliferation (A), [<sup>3</sup>H]-thymidine incorporation (B), adhesion (C), and migration (D) of BAECs in the presence or absence of VEGF (25 ng/mL). \* $P$ <0.001 compared with absence of DAPT. n=8 in each group.



**Figure 7.**  $\gamma$ -Secretase mediates endothelial cell survival and angiogenesis by VEGF. A, Concentration-dependent effects of DAPT (2 and 20  $\mu$ mol/L) on apoptosis of HUVECs in the presence or absence of VEGF (25 ng/mL). Apoptosis was induced by serum starvation for 16 hour and assessed by DAPI and annexin V staining. \* $P$ <0.01 compared with absence of VEGF. n=8 in each group. B, Representative immunoblot showing the concentration-dependent effects of DAPT (2 and 20  $\mu$ mol/L) on cleavage of caspase3 (c-caspase3) relative to total caspase3 (t-caspase3) in HUVECs in the presence or absence of VEGF (50 ng/mL). Three independent experiments yielded similar results. C, Representative microscopic hpfs ( $\times$ 400 magnification) showing Matrigel-based, capillary-like tube formation in HUVECs infected with adenovirus carrying GFP (Ad.GFP) or Notch intracellular domain (Ad.NICD) in the presence or absence of VEGF (50 ng/mL) $\pm$ DAPT (20  $\mu$ mol/L). D, Quantitative analysis of tube formation (tube length) from 8 separate experiments. \* $P$ <0.01 compared with Ad.GFP; † $P$ <0.01 compared with Ad.GFP+VEGF+DAPT.



**Figure 8.** Schematic diagram of endothelial Notch1 signaling in ischemia-induced angiogenesis. Proposed model of Notch1 signaling and angiogenesis following hindlimb ischemia. Akt indicates protein kinase B/Akt; LY294002, PI3K inhibitor; SH5, Akt kinase inhibitor; Ad.dnAkt, adenovirus containing dominantnegative Akt; Ad.caAkt, adenovirus containing constitutively active Akt; Ad.NICD, adenovirus containing Notch intracellular domain.