

Inhibition of endothelial cell proliferation by Notch1 signaling is mediated by repressing MAPK and PI3K/Akt pathways and requires MAML1

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SPECIFIC AIMS

This study aims to elucidate the signaling mechanisms by which the activated Notch pathway inhibits endothelial cell proliferation. We show here that Notch-induced suppression is mediated by the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways.

PRINCIPAL FINDINGS

1. Activation of the Notch1 pathway suppresses endothelial cell proliferation

We have previously documented that enforced activation of Notch1 signaling by transient expression of the intracellular portion of the Notch transmembrane protein (N^{IC}) induces cell growth arrest of human iliac venous and arterial endothelial cells (HIAECs). To study whether stimulation of Notch1 receptors on the cell surface results in proliferation inhibition of HIAECs, we tested two soluble forms of the Notch1 ligands, sDll4 and sJag1, for their effects on cell proliferation. We constructed sDll4 as a chimeric fusion gene, in which the cDNA encoding the extracellular domain was fused to the Fc region of the human IgG1 heavy-chain gene. Expression of the sDll4 protein was confirmed by immunoprecipitation and immunoblotting assays. The biological effects of sDll4 and sJag1 on the activation of cell surface Notch1 were validated by detecting a cleaved form of Notch1 in cells. HIAECs treated with sDll4 or sJag1 displayed reduced proliferation rates (~15–18%) compared to those treated or untreated with controls. This finding implicates suppressed activation of Notch1 signaling on HIAEC proliferation. To determine whether sustained activation of the Notch1 pathway achieves a similar effect, we constructed a lentiviral vector encoding the N^{IC} gene and transfected it into HMVECs and HIAECs. We found that proliferation rates of N^{IC} -transfected cells markedly decreased compared with the rates of either

parental or GFP-transfected control cells. These results are consistent with sDll4-initiated Notch pathway activation, but enforced constitutive activation of the Notch1 pathway has a more profound inhibitory effect on cell proliferation. Taken together, our data demonstrate that activation of the Notch1 pathway inhibits human endothelial cell proliferation in vitro.

2. Activation of Notch1 signaling represses MAPK and PI3K/Akt pathway signaling in endothelial cells

To determine the intracellular signaling pathways that mediate Notch's effect on proliferation inhibition, we tested the potential of activated Notch1 to regulate two proliferation-related signal transduction pathways—the MAPK and PI3K/Akt pathways. Both pathways are activated following vascular endothelial growth factor (VEGF) or serum stimulation in HIAEC and HMVEC. In endothelial cells expressing N^{IC} , phosphorylation of Erk1/2 (p44/42) 30 min after mitogen stimulation was significantly inhibited compared with that in control cells. Similarly, phosphorylation of Akt 1 h after mitogen stimulation was drastically suppressed (**Fig. 1A**). The effects of Notch signaling on regulating MAPK and PI3K/Akt pathways were confirmed by stimulation of HMVECs with sJag1. Phosphorylation of Erk1/2 and Akt was suppressed (**Fig. 1B**). Thus, activation of Notch1 signaling negatively regulates MAPK and Akt pathways in endothelial cells.

3. MAPK and PI3K/Akt pathways are responsible for mediating activated Notch1-induced cell proliferation inhibition

To investigate whether changes in the activity of the PI3K/Akt pathway account for Notch1-induced cell

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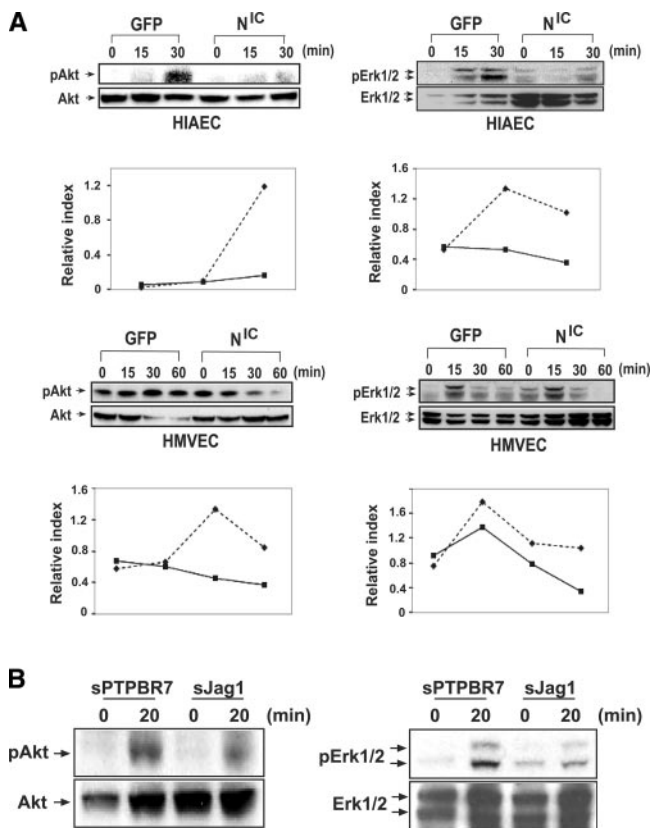


Figure 1. Phosphorylation of MAPK and Akt by Notch1 activation. *A*) Endothelial cells transfected with GFP/lenti or N^{IC}-GFP/lenti were restimulated with 50 ng/ml of h-VEGF after starvation for 16 h. Cells were harvested at different time points (as indicated) and lysed for Western blot analyses. Membranes were blotted with antiphospho-Erk1/2 and -Akt Abs and reblotted with antitotal Erk1/2 and Akt Abs. X-ray films were scanned with a densitometer, and specific bands were quantitatively plotted. A representative result is shown. *B*) Starved HMVECs were treated with sJag1 or sPTPBR7 and restimulated with h-VEGF. Cells were harvested and lysed for Western blot analyses.

proliferation control, we introduced either an active mutant of Akt (Myr-Akt) or GFP (as a control) into HMVEC-N^{IC} cells. The Myr-Akt-transfected cells showed increased phosphorylation of Akt. Cell proliferation assays for HMVEC-N^{IC} demonstrated that their decreased proliferation rate was restored if the Akt activity was enhanced. Similarly, we tested the mediating effect of the MAPK pathway on Notch endothelial cell proliferation inhibition by transfecting an active mutant of H-Ras^{V12} or hemagglutinin (HA)-tagged B-RAF^{V600E} into HMVEC-N^{IC} cells. Expression of H-Ras^{V12} or B-RAF^{V600E} rescued the decreased proliferation rate of HMVEC-N^{IC} cells, implying that the MAPK pathway, like the PI3K/Akt pathway, is indeed responsible for mediating Notch1 signaling-induced inhibition of endothelial cell proliferation. Similar effects were observed in HIAEC cells. Taken together, our results strongly indicate that Notch1 pathway activation inhibits endothelial cell proliferation by concurrently regulating both the MAPK and PI3K/Akt signaling pathways.

4. The role of Notch1 signaling in cell proliferation control is transcription-dependent

To further elucidate the mechanism by which Notch pathway activation suppresses PI3K/Akt and MAPK pathway-mediated endothelial cell proliferation, we tested whether the process is transcription-dependent or -independent. We first tested the effect of HES1 on HMVEC growth. HES1 is a downstream target molecule of the Notch signaling cascade whose expression depends on N^{IC}/CSL/MAML complex-mediated gene transcription. When introduced into HMVEC, HES1 suppressed cell proliferation relative to control. This finding suggests a transcription-dependent mechanism in the execution of Notch signaling. Because MAML is required to transcribe downstream target genes of the Notch pathway, we decided to investigate the potential role of MAML1 in controlling endothelial cell proliferation by introducing a dominant-negative mutant of MAML1/pBabe into HMVEC-N^{IC} cells (DN-MAML1-HMVEC-N^{IC}). We used pBabe as a control (Control-HMVEC-N^{IC}). As expected, DN-MAML1 altered the rate of cell proliferation induced by activated Notch1. This result suggests that the effects of Notch signaling on cell proliferation control are mediated by the Notch/MAML-HES cascade and are transcription-dependent.

5. Effect of Notch signaling on controlling MAPK and PI3K/Akt pathways is MAML1-dependent

To address whether the effects of MAML1 on endothelial cell proliferation control are mediated through the MAPK and PI3K/Akt pathways, we examined whether DN-MAML1 could alter N^{IC}-induced MAPK and PI3K/Akt pathway activities. Compared with Control-HMVEC-N^{IC} cells, phosphorylation of Erk1/2 following mitogen stimulation was significantly re-established in DN-MAML1-HMVEC-N^{IC} cells. Similarly, phosphorylation of Akt following mitogen stimulation was also remarkably reversed. Very similar results were obtained in HIAECs. These data demonstrate that MAML1 is indeed responsible for mediating the effects of activated Notch signaling on cell proliferation control by regulating the MAPK and PI3K/Akt signaling pathways.

CONCLUSIONS AND SIGNIFICANCE

Endothelial cell proliferation is required for vascular spouting and elongation during the new blood vessel formation. When endothelial cells differentiate, which is required for remodeling and maturation of newly formed blood vessels, the cell cycle needs to be arrested. This process is known as differentiation-associated cell cycle arrest. In the matured vasculature, most of the endothelial cells remain in quiescent states. Notch signaling is known to be temporally and spatially involved in the remodeling of newly formed vessels. It has been suggested that Notch signaling is silent during the early stages of angiogenesis (when active endothelial cell proliferation is required) but activated during

vessel maturation (when endothelial cells cease proliferation and likely undergo differentiation-associated cell cycle arrest). Notch pathway activation may also be involved in endothelial cell contact inhibition. If true, this would support the hypothesis that Notch signaling helps control vessel homeostasis by maintaining endothelial cell quiescence.

The current study suggests that the MAPK and PI3K/Akt pathways are under the control of Notch signaling and that both pathways are ultimately responsible for Notch signaling-induced cell proliferation inhibition (Fig. 2). Our work establishes a direct link between the pathways of Notch, MAPK, and PI3K/Akt and provides an explanation for the mechanisms of controlling cell proliferation by Notch signaling in endothelial cells. The signaling cascades delivered from both MAPK and PI3K/Akt pathways regulate cell cycle machinery. It has been demonstrated in endothelial cells that Notch signaling inhibits phosphorylation of Rb and mitogen-induced up-regulation of p21^{Cip1}. Our findings may well bridge the gap between Notch activation and the negative regulation of cell cycle machinery.

Classical Notch signaling is transmitted by means of the Notch-N^{IC}/CSL/MAML cascade. Recent evidence suggests that a transcription-independent mechanism exists for mediating multiple effects of Notch signaling. It is likely that Notch signaling regulates the MAPK and PI3K/Akt pathways at the post-transcriptional concentration because (a) HES1, a post-transcriptional target gene product, can represent the effect of Notch on endothelial cell proliferation control; and (b) MAML1 is an essential element for initiating transcription, DN-MAML1 can antagonize the effect of Notch on MAPK and PI3K/Akt pathway-mediated cellular proliferative or inhibitory signals. Consistently, our preliminary efforts in detecting a direct association between N^{IC} and MAPK or Akt by coimmunoprecipitation were unsuccessful (data not shown). This finding supports the notion that intracellular Notch itself does not directly regulate the MAPK and PI3K/Akt pathways. Because MAPK and PI3K/Akt pathways are simultaneously controlled by the Notch/MAML-HES cascade, and these two parallel pathways are initiated by receptor tyrosine kinases/phosphatases or adaptor-mediated kinases/phosphatases, it is more likely that the regulation of MAPK and PI3K/Akt pathways by the Notch/MAML-HES cascade is mediated by regulating common kinases/phosphatases. It has been reported that Notch

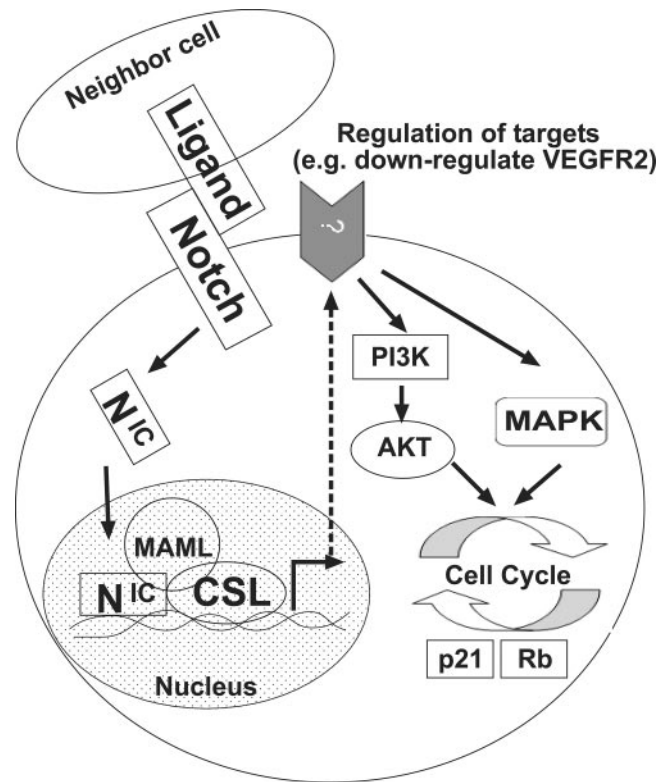


Figure 2. Schematic illustration of a suggested mechanism by which Notch signaling inhibits endothelial cell proliferation. Activation of Notch signaling suppresses MAPK and PI3K/Akt pathways. The Notch/MAML-HES cascade likely regulates MAPK and PI3K/Akt pathways indirectly through the induction/suppression of other targeting gene(s) (e.g., suppressing VEGF-R2). This induction or suppression, in turn, mediates Notch's effect. The signaling cascades from both MAPK and PI3K/Akt pathways ultimately control components of cell cycle machinery, such as Rb and p21^{Cip1}.

activation down-regulates VEGF-R2 expression on endothelial cells. Likely, decreased expression of VEGF-R2 is responsible for initiating weak MAPK and PI3K/Akt pathway activation (considering that VEGF stimulation was used in our experiments). However, since the effects of complete M199 medium are similar to those of VEGF, VEGF-R2 is obviously not the only target molecule mediating Notch-induced suppression. Future studies to identify such Notch signaling-induced target molecules will help define the mechanism underlying the crosstalk between Notch signaling and the MAPK and PI3K/Akt pathways. EJ

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ABSTRACT The requirement for Notch signaling in vasculogenesis and angiogenesis is well documented. In a previous study, we showed that activation of the Notch pathway in endothelial cells induces differentiation-associated growth arrest; however, the underlying mechanism remains to be elucidated. Here, we show that activation of the Notch pathway by either stimulation of cell surface Notch receptors with crosslinked soluble Delta-like 4 (sDll4)/Jagged1 (sJag1) or constitutive expression of the Notch1 intracellular domain (N^{IC}) suppresses endothelial cell proliferation. This suppression is mediated by the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. Following Notch1 activation, both pathways were suppressed in endothelial cells, and alterations in MAPK or PI3K/Akt pathway activity reversed Notch1-induced growth inhibition. Furthermore, we found the effect of Notch1 on endothelial cells to require Mastermind-like (MAML). Overexpression of a dominant-negative mutant of MAML1 antagonized the effects of activated Notch1 on the MAPK and PI3K/Akt pathways. Ectopic expression of Hairy/Enhancer of Split 1 (HES1) consistently reproduced the inhibitory effect of N^{IC} on endothelial cell proliferation. Together, our data demonstrate that the Notch/MAML-HES signaling cascade can regulate both MAPK and PI3K/Akt pathways, which suggests a molecular mechanism for the inhibitory effect of Notch signaling on endothelial cell proliferation.—Liu Z.-J., Xiao M., Balint K., Soma A., Pinnix C. C., Capobianco A. J., Velazquez O. C., Herlyn M. Inhibition of endothelial cell proliferation by Notch1 signaling is mediated by repressing MAPK and PI3K/Akt pathways and requires MAML1. *FASEB J.* 20, E201–E210 (2006)

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THE NOTCH-SIGNALING pathway is involved in development and tissue homeostasis (1). The central components of the Notch pathway are evolutionarily conserved. In mammals, the Notch family consists of four transmembrane receptors (Notch1–Notch4) and five ligands (Jagged1, Jagged2, Delta-like (Dll) 1, Dll3, and

Dll4). Binding of a ligand to its cognate receptor initiates metalloprotease- and γ -secretase-mediated proteolysis, which cleaves Notch and releases its intracellular domain (N^{IC}) from the plasma membrane. Once released, N^{IC} translocates into the nucleus and associates with transcription factors CSL [CBF1, Su(H), Lag-1] and MAML to form a heteromeric complex that mediates the transcription of target genes such as HRT, HEY, or HESR—members of the HES and Hairy-related families of basic helix-loop-helix transcription factors (2). MAML functions as a critical transcriptional coactivator for Notch signaling. Three MAML family members (MAML1–MAML3) have been identified in mammals (3) and found to have non-overlapping tissue distributions. It remains unknown whether MAML gene family members have distinct functions. The N-terminal basic domain of MAML associates with the ankyrin repeat of the Notch receptor and its central and C-terminal domains have transactivation activity. The central MAML activation domain (TAD1) recruits CBP/p300 to promote nucleosome acetylation at Notch enhancers, whereas a glutamine-rich region of MAML (TAD2) enhances phosphorylation of N^{IC} (4).

The involvement of Notch in regulating vascular formation has been suggested from observations of Notch/ligand expression in vascular endothelium and from defects in the vascular phenotypes of targeted mutants in the Notch pathway. Notch1, Jagged1, Jagged2, and Dll1 are expressed in vascular endothelium in vertebrates (5–8), whereas Notch4 is specifically restricted to the vascular endothelium (9). Dll4, a newly identified ligand responsible for activating Notch1 and Notch4, is preferentially expressed in arterial endothelium (10), which suggests a potential role for Dll4 in modulating arterial development (arteriogenesis). Targeted Notch1^{-/-}, Notch1^{-/-}/Notch4^{-/-}, Jagged1^{-/-}, and Dll1^{-/-} mutants all result in vascular defects (11–15). Endothelium-specific deletion of Notch1 using Tie2-Cre and Notch^{flox/flox} mice further

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confirms the essential role of Notch1 in vascular development (16). In addition, the human degenerative vascular disease cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) has been associated with mutations in Notch3 (17). The effect of Notch signaling is dose-dependent; therefore, Notch signaling must be appropriately regulated in order to maintain normal vascular development. Overactivation of the Notch pathway does affect the biological readout, as exemplified by the vascular patterning defects in mouse embryonic endothelium caused by expression of activated Notch4 (18). In zebrafish, development of the aorta requires the *gridlock* gene, a homologue of mammalian HES (19). Moreover, essential roles have been documented for Jagged1 and HEY1 in modulating vessel formation in vitro (20, 21).

The precise mechanisms by which Notch signaling regulates vascular function remain poorly understood. Notch signaling likely exerts its effects on the vasculature in a spatiotemporal manner by influencing endothelial cell fate, such as proliferation, differentiation, and apoptosis. The effects of Notch signaling on cell fate determination usually depend on timing, gene dosage, and cell type/context. We and others have documented the regulation of endothelial cell proliferation and apoptosis by Notch signaling (21, 22). To elucidate the underlying mechanism, we tested the hypothesis that Notch signaling affects endothelial cell proliferation by controlling the MAPK and PI3K/Akt signaling pathways. Both pathways are widely known to govern cell proliferation and survival. Our results show that the effects of Notch signaling on inhibiting endothelial cell proliferation are mediated through the MAPK and PI3K/Akt signaling pathways and that these activities depend on MAML1-mediated transcription of target genes. Thus, we establish a functional link between the Notch pathway and that of MAPK and PI3K/Akt and provide a better understanding of the mechanisms for Notch signaling in suppressing endothelial cell proliferation.

MATERIALS AND METHODS

Reagents

SDS-polyacrylamide gels were purchased from Invitrogen (Carlsbad, CA), h-vascular endothelial growth factor₁₆₅ (VEGF) from NCI-FCRDC (Frederick, MD), and the MTT cell proliferation assay kit was from American Type Culture Collection (ATCC, Manassas, VA). Protein A-Sepharose™ was purchased from Pharmacia (New York, NY). All other chemicals and solutions were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Cells and cell culture

Human microvascular endothelial cells (HMVECs) were cultured on plates coated with type I collagen in endothelial cell medium (EGM™) as described (23). Human iliac artery endothelial cells (HIAECs) were obtained from ATCC (CRL-

2475) and cultured on plates coated with 1% gelatin in Medium 199 (Invitrogen) supplemented with 10% FBS (HyClone, Logan, UT), 10 mM L-glutamine, 100 µg/ml heparin, and endothelial cell growth supplement (ECGS kindly provided by E. Levine, The Wistar Institute, Philadelphia, PA). All endothelial cells were used between passages 6 and 20. COS-7, 293 T, and NIH/3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS. All cells were incubated at 37°C in 98% humidified air containing 5% CO₂.

Construction, expression, purification, and concentration of chimeric soluble proteins

A chimeric gene encoding a fusion protein (sDll4) of the extracellular portion of human Dll4 and the Fc portion of human IgG1 was constructed as follows: The cDNA fragment of the extracellular portion of Dll4 was generated by polymerase chain reaction (PCR) using sense [5'-TAGGATCCATGCGGGCAGCGTCCCGGAGC-3'] and antisense [5'-TACCCGGGCGGCAAGCCCACGGGGAAC-3'] primers in which *Bam*HI and *Sma*I sites (underlined) were created at the ends of the cDNA fragment. Dll4/pcDNA3 (kindly provided by S. Kusano, the University of North Carolina, Chapel Hill, NC) was used as template for PCR. Amplified PCR fragment was purified and subcloned into pCR2.1, which has *Bam*HI and *Sma*I sites. The generated plasmid was termed Ex-Dll4/pCR2.1. The gene for the Fc portion of human IgG1 was prepared by digesting the mFas-Fc plasmid (24) with *Pst*I, from which a 5.8 kb fragment was recovered. After blunting and subsequent digesting with *Not*I, the fragment was ligated with Ex-Dll4/pCR2.1, which was then digested with *Sma*I and *Not*I, to generate sDll4-Fc/pCR2.1. The desired plasmid was confirmed by DNA sequencing. To construct sDll4/Ad5, the gene fragment of sDll4-Fc was digested with *Bam*HI and *Not*I and subcloned into a *Bgl*II- and *Not*I- digested pShuttle-cytomegalovirus (CMV) vector. After *Bgl*II digestion, the cDNA end becomes compatible with that after *Bam*HI digestion. Recombinant adenoviruses were generated by homologous recombination (25). COS-7 cells were infected with sDll4/Ad, sDll4 was purified from the culture supernatant by a protein-A-Sepharose column, and fractions containing high concentrations of sDll4 were collected and dialyzed. To generate sJag1 and sPTPBR7, the plasmids of sJag1 (a gift from T. Kadesch, University of Pennsylvania, Philadelphia, PA) and sPTPBR7 (26) were transfected into COS-7 cells in a 100 mm tissue culture dish by calcium phosphate methods. After overnight culture in 10 ml of complete DMEM, the culture medium was replaced with 8 ml of serum-free DMEM and incubated for an additional 5 d. The culture supernatant was collected and concentrated by Amicon Ultra centrifuge filtration (NMWL, 10 kDa, Millipore, Billerica, MA). Expression of sPTPBR7 was confirmed by immunoblotting. To stimulate the Notch1 receptor more efficiently, we crosslinked soluble ligands with anti-IgG. For MTT assay, we first coated 96-well plates with anti-human IgG antibody (Ab) (10 µg/well), then washed with 100 µl/well of PBS 3 times, purified sDll4 (20 µg/well), or equal volumes (50 µl/well) of concentrated supernatant containing sJag1 or sPTPBR7 were added and plates were incubated at 37°C for 30 min. After washing well with PBS 3 times, 3000 cells/well were seeded on plates in complete M199 medium and cultured for 2 d until MTT assays were performed.

Recombinant lentiviruses, retroviruses, and adenoviruses

For gene transfer, we constructed a variety of viral vectors. GFP/lenti [pHX(prim)-CMV-GFP] was obtained from the

Gene Therapy Program (Division of Medical Genetics, University of Pennsylvania, Philadelphia, PA). Into this vector, the enhanced GFP gene was inserted downstream of the CMV promoter and upstream of the post-transcriptional regulatory element of woodchuck hepatitis virus (WP) and flanked by HIV long terminal repeats (LTRs) (27). To construct N^{IC}-GFP/lenti [pHX(prime)-CMV-N^{IC}-IRES-GFP], a gene fragment encoding N^{IC} (28) was inserted into pIRES2-enhanced GFP (Clontech, Palo Alto, CA) between *EcoRI* and *BamHI* sites. The N^{IC}-IRES-GFP gene fragment was then digested out with *XhoI* and *DraI* and blunted. The GFP gene in the pHX'-CMV-GFP vector was replaced by the N^{IC}-IRES-GFP gene fragment. To achieve this, the pHX'-CMV-GFP vector was digested with *NotI* and *BamHI*. After removing a small fragment containing the GFP gene, we ligated the remaining 2 fragments with a linker [5'-GGCCGCCCGGG-3' annealed with 5'-GATCCCCGGGC-3'] to generate a *SmaI* site between *NotI* and *BamHI* sites. The blunted N^{IC}-IRES-GFP gene fragment was subsequently ligated into the modified pHX'-CMV backbone vector by digesting the *SmaI* site. Orientation of the inserted gene fragment was confirmed by restriction digestion. The N^{IC}-GFP/lentiviral vector was confirmed by DNA sequencing.

Production of pseudotyped lentiviruses was achieved by cotransfecting 293T cells with 3 plasmids (27): a packaging-defective helper construct, pCMVΔR8.2 (Gene Therapy Program), which encodes the HIV-1 gag and pol precursors as well as the regulatory protein Rev; a plasmid, pMD.G (Gene Therapy Program), which codes for a vesicular stomatitis virus glycoprotein (VSV-G) envelope; and a vector construct, pHX'-CMV, which harbors the gene of interest. Briefly, 48 h after transient transfection of 293T cells via calcium phosphate method, viral supernatants were collected, filtered through 0.45 μm low-protein-binding filters (Nalgene, Rochester, NY), and stored in aliquots at -70°C. Titers were determined by transducing NIH/3T3 cells and counting the number of green cells using fluorescence microscopy. Lentiviruses collected 48 h posttransfection displayed titers of around 10⁷ transducing units/ml in NIH/3T3 cells.

Retroviral vector MAML305/pBabe (DN-MAML1) and empty pBabe vector (control) were described previously (29). H-Ras^{V12}/pBabe-puro and hemagglutinin (HA)-tagged B-Raf^{V600E}/pBabe were kindly provided by D. Tuveson (University of Pennsylvania, Philadelphia, PA) and G. Robertson (Penn State College of Medicine, Hershey, PA), respectively. Recombinant retroviruses were generated by transfecting vector into Phoenix (AMPHO) helper-free retrovirus producer lines, obtained from G. P. Nalon (Stanford University, Stanford, CA) via calcium phosphate method. Forty-eight hours after transient transfection, viral supernatants were collected, filtered, and titered. In general, retroviruses displayed titers of around 10⁷ transducing units/ml in NIH/3T3 cells.

For recombinant adenoviruses, Notch1/Ad5 was constructed by insertion of full-length of Notch1 gene (provided by T. Kadesch, University of Pennsylvania) into adenoviral vector and confirmed by DNA sequencing. GFP/Ad5 was obtained from the Gene Therapy Program. DN-p85/Ad5 and Myr-Akt/Ad5 were provided by W. Ogawa (Kobe University, Kobe, Japan) and described elsewhere (30, 31). The HES1/Ad5 (32) was kindly provided by D. W. Ball (Johns Hopkins University School of Medicine, Baltimore, MD), and the sDll4/Ad5 was constructed as described above. All recombinant adenoviruses were propagated in 293 cells and purified using cesium chloride as described (33). Before infection of endothelial cells, adenoviruses were titrated to determine plaque-forming units (pfu) as described (34).

Viral infection of experimental cells

To infect cells with lentiviruses or retroviruses, we exposed them overnight to viruses at a multiplicity of infection (MOI) ranging from 2 to 5 in the presence of 4 μg/ml polybrene. Cells were then washed, cultured with complete medium for 2 additional days, and analyzed for protein expression by Western blot. Cells were pooled and used for subsequent assays. In general, transfection efficiency was higher than 95% with lentiviruses and 90% with retroviruses. For most cells, long-term (more than 3 mo) gene expression could be maintained. For infection of cells with adenovirus, subconfluent endothelial cells were infected with viruses for 2 h at 37°C in serum-free medium. Viral suspensions were then replaced by regular medium. After 48 h, cells were harvested for subsequent analysis as indicated in individual experiments.

Cell proliferation assays

Cell proliferation was measured by MTT cell proliferation assay according to the manufacturer's protocol. Briefly, cells were seeded into 96-well plates at 3000 cells per well. On the day of harvest, 100 μl of spent medium was replaced with an equal volume of fresh medium containing 10 μl of MTT reagent. Plates were incubated at 37°C for 4 h, 100 μl of detergent reagent was added to each well, and plates were further incubated at room temperature in the dark for 2 h. Absorbance was measured at 570 nm.

Matrigel network formation assay

Wells of 24-well plates were coated with 400 μl Matrigel® (growth factor reduced, Becton Dickinson, Franklin Lakes, NJ), diluted at 1:1 with Medium199, and allowed to polymerize at 37°C for 30 min. Cells were seeded onto Matrigel at 1 × 10⁶ cells/well with Medium199 and incubated at 37°C. Networks were observed under an inverted phase-contrast microscope and photographed at various times.

Immunoblotting

Western blots were performed as described (35). Membranes were probed with antibodies to: phospho-MAPK (#9106, New England Biolabs, Beverly, MA), p44/42 MAPK (#9102, New England Biolabs), Akt (#9272, New England Biolabs), phospho-Akt (Ser-473/Thr308, #9916, New England Biolabs), Dll4 (sc-18639, Santa Cruz Biotechnology, Santa Cruz, CA), Notch1 (925, a rabbit polyclonal antiserum against residues 1759–2095) (36), activated Notch1 (ab8925, Abcam, Cambridge, MA), HES1 (kindly provided by T. Sudo, Toray Industries, Inc., Kamakura, Japan), Myc-Tag 9B11 (New England Biolabs), and β-actin (AC-15, Sigma), followed by HRP-conjugated secondary Ab (Jackson ImmunoResearch, West Grove, PA) and subjected to enhanced chemiluminescence (ECL) (Amersham). Membranes were stripped and reblotted as required in the individual experiment. For quantization, X-ray films were scanned with densitometer. The relative index was referred to a ratio of the scores of the specific bands measured by densitometry with regard to the score of the background in the same X-ray film.

RESULTS

Activation of Notch1 pathway suppresses endothelial cell proliferation

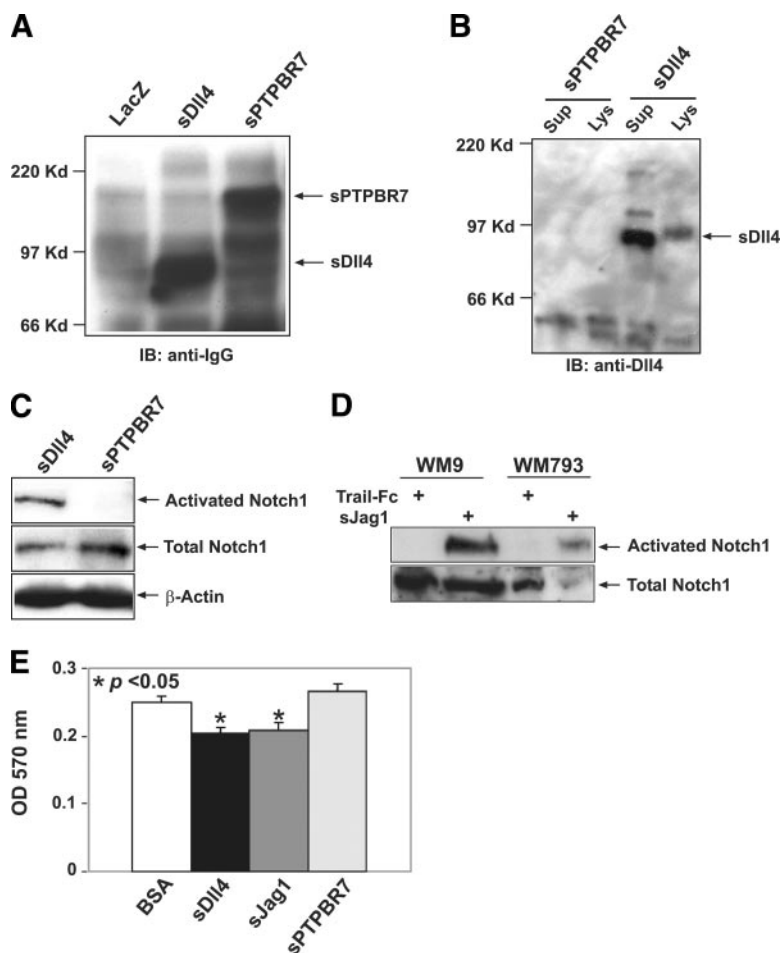
We have previously documented that enforced activation of Notch1 signaling by transient expression of

N^{IC} induces cell growth arrest of HIAECs (28). To study whether stimulation of Notch1 receptors on the cell surface results in cell proliferation inhibition of HIAECs, we tested two soluble forms of the Notch1 ligands, sDll4 and sJag1, for their effects on cell proliferation. We constructed sDll4 as a chimeric fusion gene, in which the cDNA encoding the extracellular domain was fused to hinge, CH2 and CH3 regions of the human IgG1 heavy-chain gene. Expression of sDll4 protein in the supernatant of recombinant adenovirus-infected COS-7 cells was confirmed by immunoprecipitation with protein A-Sepharose beads and subsequent immunoblotting with both anti-IgG and anti-Dll4 antibodies (Fig. 1A–B). sPTPBR7 (26) was used as a control. To confirm the biological effect of sDll4 on the activation of cell surface Notch1, we stimulated HIAECs, which were transfected with Notch1/Ad for overexpression of Notch1 by sDll4 or sPTPBR7, respectively, and crosslinked with anti-IgG Ab. As showed in Fig. 1C, crosslinked sDll4 induced activation of Notch1. Characterization of sJag1 was carried out in melanoma cells (WM9 and WM793) and Trail-Fc (a gift from T. Kadesch, University of Pennsylvania) was used as control (Fig. 1D). The effects of sDll4 and sJag1 on HIAEC proliferation were examined by MTT assay. Treatment cells with concentrated sDll4 or sJag1 weakly but significantly inhibited cell proliferation rates (~15–18%) compared with treated or untreated with controls. This

finding implies a suppressive effect of activated Notch1 signaling on HIAEC proliferation (Fig. 1E). The weak inhibitory effect could reflect the physiological concentration of endogenous Notch1 signaling strength. To determine whether sustained activation of the Notch1 pathway achieves a similar effect, we constructed a lentiviral vector encoding the N^{IC} gene linked to the GFP marker via an internal ribosome entry site (Fig. 2A). COS-7 cells infected with N^{IC} -GFP/lentivirus or GFP/lentivirus (control) confirmed N^{IC} expression (Fig. 2B), demonstrating that GFP is a reliable marker for N^{IC} expression in N^{IC} -GFP/lentiviral vector-transfected cells. When HIAECs were transfected at an MOI of 5, greater than 95% of transfected cells exhibited GFP expression (Fig. 2C) and expression was stable for greater than 6 mo. GFP-transfected cells proliferated the same as nontransfected parental cells. Similar results were obtained with HMVECs. Experiments were performed using pools from each transfected cell line in which the GFP-positive population accounted for >95% of total cells. For transfected endothelial cells, only low passage numbers (<10 after transfection) were used.

We tested the proliferative properties of transfected cells using MTT assays. Proliferation rates of HIAEC- N^{IC} and HMVEC- N^{IC} cells markedly decreased compared with the rates of either parental or control cells, HIAEC-GFP, and HMVEC-GFP (Fig. 2D). These results are

Figure 1. Stimulating Notch1 with soluble ligands inhibits endothelial cell proliferation. A–B) Characterization of sDll4. COS-7 cells were transfected with lacZ/Ad, sDll4/Ad, and sPTPBR7 for 2 d. Culture supernatants (in A and B) and cell lysates (in B) were immunoprecipitated with protein A-Sepharose beads and immunoblotted with anti-human IgG (A) or anti-Dll4 (B). C) Stimulation with sDll4 activates Notch1. HIAECs transfected with Notch1/Ad for 2 d were treated with 1 μ g/ml of sDll4 or sPTPBR7 for 6 h and harvested for Western blot assays. Expression of total Notch1 (925) and activated Notch1 (ab8925) were detected with specific Abs. β -actin was used as a loading control. D) Characterization of sJag1. WM9 and WM793 cells were treated with anti-IgG Ab crosslinked sJag1 or Trail-Fc for 6 h and harvested for Western blot assays. Expression of total Notch1 (925) and activated Notch1 (ab8925) were detected with specific Abs. E) Cell proliferation were detected with MTT assay; 2 μ g/ml of sDll4, sJag1, sPTPBR7 and BSA were added to 96-well plates coated with anti-IgG. 3000 of HIAEC were seeded per well and cultured in complete M199 medium for 2 d and subjected to MTT assays. Results are means \pm SD of three independent experiments. *P* value is shown from Student's sedimentary coefficient *t* tests.



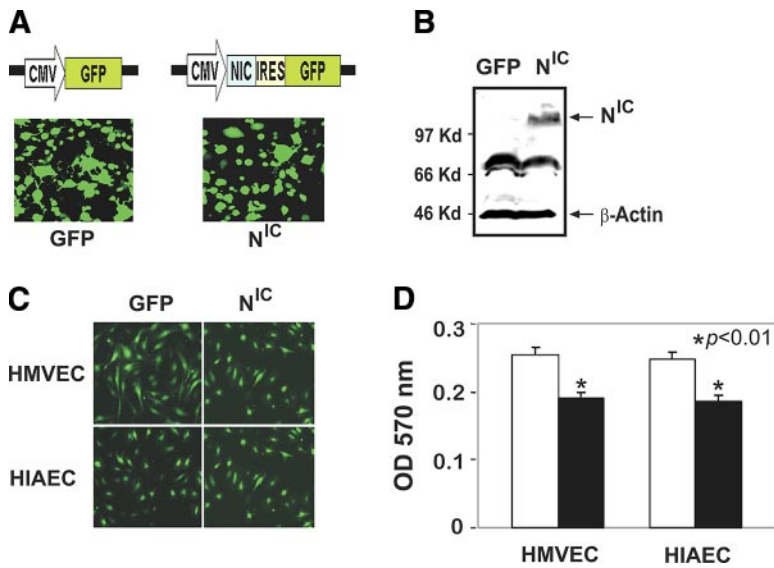


Figure 2. Effect of activated Notch1 on cell proliferation control. *A*) Schematic map of N^{1C}-GFP construct. COS-7 cells transfected with GFP or N^{1C}-GFP lentiviruses were photographed after 7 d using fluorescence microscopy ($\times 20$). *B*) COS-7 cells transfected with GFP or N^{1C}-GFP lentiviral vectors after 48 h were harvested and lysed. Whole cell lysates were subjected to Western blot analyses. Expression of Notch1 is shown. β -actin was used as a loading control. *C*) Seven days after transfection, equal numbers of GFP or N^{1C}-GFP-transduced endothelial cells were replated to determine GFP expression using fluorescence microscopy ($\times 20$). *D*) Endothelial cells were transfected with lentiviral vectors, and proliferation was determined by MTT assays. White bars represent GFP-transfected control cells and black bars indicate N^{1C}-GFP-transfected cells. Results are means \pm SD of three independent experiments. *P* value is shown from Student's sedimentary coefficient *t* tests.

consistent with sDll4-initiated Notch pathway activation, but enforced constitutive activation of the Notch1 pathway has a more profound inhibitory effect on cell proliferation. Taken together, our data demonstrate that activation of the Notch1 pathway inhibits human endothelial cell proliferation in vitro.

Activation of Notch1 signaling represses MAPK and PI3K/Akt pathway signaling in endothelial cells

Since constitutive activation of the Notch1 pathway achieved a more profound suppressive effect on endothelial cell proliferation, we used N^{1C}/lentiviral-transfected stable cell lines in all subsequent attempts to identify the signaling mechanism. To determine the intracellular signaling pathways that mediate Notch's effect on cell proliferation inhibition, we tested the potential of activated Notch1 to regulate 2 proliferation-related signal transduction pathways—the MAPK and PI3K/Akt pathways. Both pathways are activated following vascular endothelial growth factor (VEGF) or serum stimulation in HIAECs (28) and HMVECs (13, 37). In HIAECs expressing N^{1C}, phosphorylation of Akt 30 min after mitogen stimulation [50 ng/ml of recombinant human VEGF (h-VEGF) or complete M199 medium containing 10% of FBS] was significantly inhibited compared with that in control cells. Similarly, phosphorylation of Erk1/2 (p44/42) 15 min after mitogen stimulation was significantly suppressed (Fig. 3A). Because activated Notch1 suppresses endothelial cell proliferation but prolongs cell survival (to maintain growth arrest but not induce senescence as growth-arrest is reversible by resupplement of serum) (28), HIAEC-N^{1C} cells had basal levels of phosphor-Akt and -Erk1/2 compared with HIAEC-GFP control cells under starvation conditions. In HMVECs, very similar results were observed despite higher basal levels of phosphor-Akt and -Erk1/2 after starvation. To confirm the effects of activation of Notch pathway on regulating MAPK and PI3K/Akt pathway activities by using a more natu-

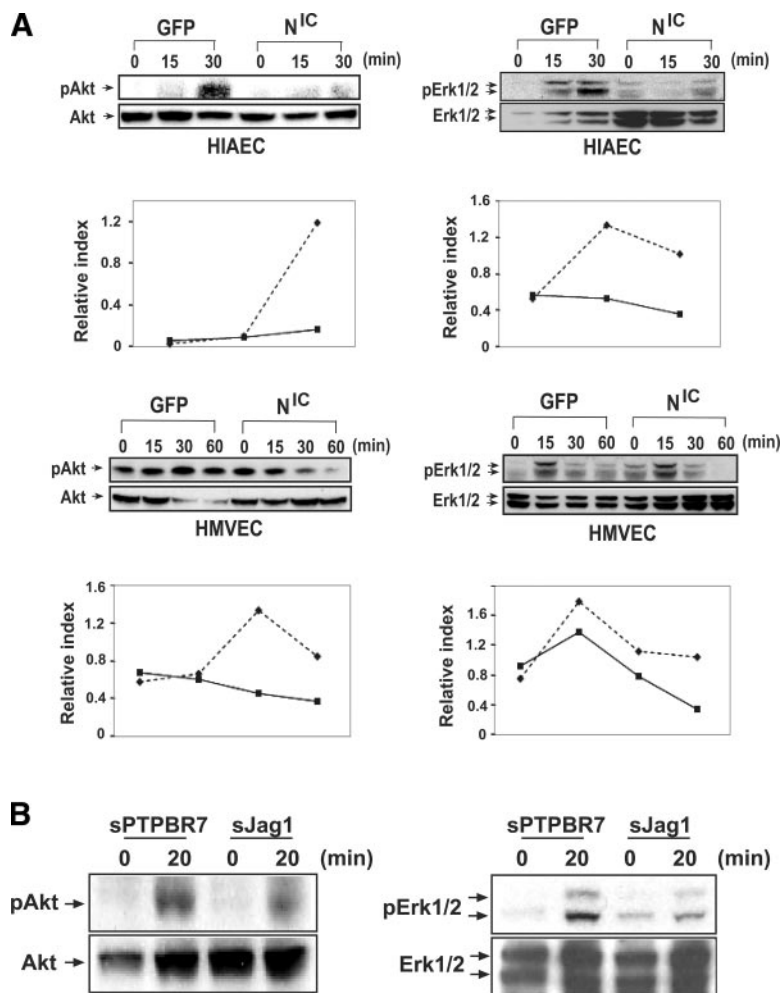
ral Notch activation system, we tested the effect of sJag1 stimulation on the phosphorylation of Erk1/2 and Akt in HMVECs. 1×10^6 HMVECs starved for 20 h in serum-free M199 medium were treated with anti-IgG (2 μ g/ml) crosslinked sJag1 or sPTPBR7 (2 μ g/ml), respectively, 10 min before reapplying 50 ng/ml of h-VEGF. Phosphorylation of Erk1/2 and Akt 20 min after h-VEGF stimulation was suppressed, though slightly (Fig. 3B). This is consistent with the weak effect of sJag1 on endothelial cell proliferation, which might reflect the physiological concentration of endogenous Notch1 signaling strength. Thus, activation of Notch1 signaling negatively regulates MAPK and Akt pathways in endothelial cells.

MAPK and PI3K/Akt pathways are responsible for mediating activated Notch1-induced cell proliferation inhibition

To investigate whether changes in the activity of the PI3K/Akt pathway account for Notch1-induced cell proliferation control, we introduced either an active mutant of Akt (Myr-Akt) (30) or GFP (as a control) into HMVEC-N^{1C} cells using adenoviral vectors for gene transfer. Forty-eight hours after adenovirus infection, cells were harvested for subsequent analyses. The Myr-Akt-transfected cells showed increased phosphorylation of Akt (Fig. 4A, upper panel). Cell proliferation assays for HMVEC-N^{1C} demonstrated that their decreased proliferation rate was restored if the Akt activity was enhanced (Fig. 4A, lower panel).

We tested the mediating effect of the MAPK pathway on Notch endothelial cell growth inhibition by transfecting an active mutant of H-Ras^{V12} or HA-tagged B-RAF^{V600E} into HMVEC-N^{1C} cells. We used Western blots to confirm the expression of H-Ras^{V12} and B-RAF^{V600E} as well as the activation of MAPK pathway in transfected cells (Fig. 4B). Expression of H-Ras^{V12} or B-RAF^{V600E} rescued the decreased proliferation rate of HMVEC-N^{1C} cells, implying that the MAPK pathway,

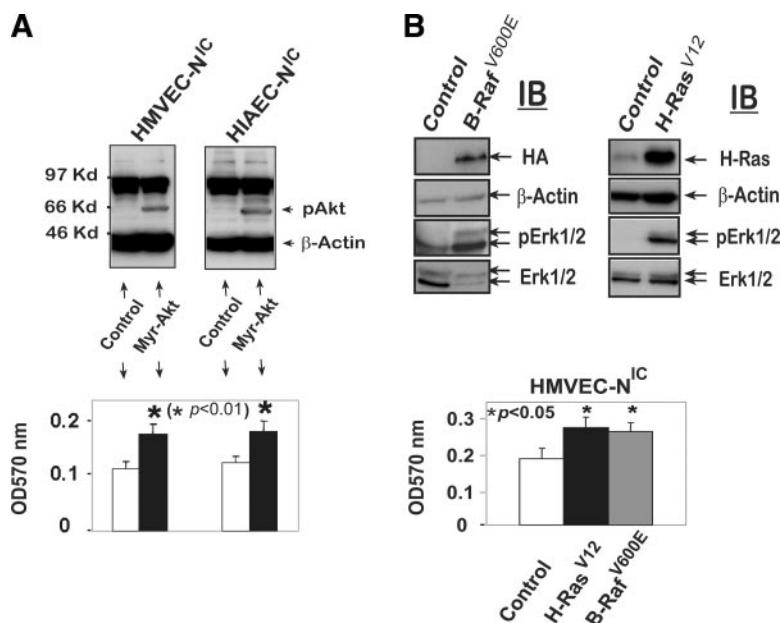
Figure 3. Phosphorylation of MAPK and Akt by Notch1 activation. *A*) Endothelial cells transfected with GFP/lenti or N^{IC}-GFP/lenti were restimulated with 50 ng/ml of h-VEGF after starvation for 16 h. Cells were harvested at different time points as indicated and lysed for Western blot analyses. Membranes are blotted with antiphospho-Erk1/2 and -Akt Abs and reblotted with antitotal Erk1/2 and Akt Abs as described. X-ray films were scanned with densitometer and specific bands were quantitatively plotted. A representative result was shown. *B*) Starved HMVECs were treated with sJag1 or sPTPBR7 and restimulated with h-VEGF. Cells were harvested and lysed for Western blot analyses.



like the PI3K/Akt pathway, is indeed responsible for mediating Notch1 signaling-induced inhibition of endothelial cell proliferation. Similar effects were observed in Myr-Akt- and H-Ras^{V12}- or HA-tagged B-RAF^{V600E}-transfected HIAEC-N^{IC} cells (data not

shown). Introduction of Myr-Akt or H-Ras^{V12} into HMVEC-GFP control cells also increased their proliferation rates (data not shown). This is not surprising as Akt and Ras are not Notch pathway-specific targets but are general signaling molecules required for cell pro-

Figure 4. Altering MAPK or PI3K/Akt activities reverses Notch signaling-induced cell proliferation inhibition. *A*) Activation of Akt by transfection of endothelial cells with Myr-Akt/Ad. GFP-transfected cells were used as control. Enhanced Akt phosphorylation was detected in Myr-Akt-transfected cells by Western blotting. β -actin was used as loading control. Cell proliferation was examined by MTT assays. Results are means \pm SD of three independent experiments. *P* value is shown from Student's *t* tests. *B*) HMVEC-N^{IC} cells were transfected with retroviruses generated with H-Ras^{V12}/pBabe or B-Raf^{V600E}/pBabe or pBabe. Expression of exogenous H-Ras and B-Raf and corresponding activation of endogenous MAPK pathway was confirmed by Western blotting. Cell proliferation was examined by MTT assay 2 d after transfection. Results are means \pm SD of three independent experiments. *P* value is shown from Student's *t* tests. Similar results were observed in HIAEC-N^{IC} cells.



liferation/survival. Our study places MAPK and PI3K/Akt pathways under the control of Notch signaling and suggests certain practically useful “Ex-Notch signaling pathway” targets to municipalize Notch’s effect. Taken together, our results strongly indicate that Notch1 pathway activation inhibits endothelial cell proliferation by concurrently regulating the MAPK and PI3K/Akt signaling pathways.

Roles of Notch1 signaling in cell proliferation control is transcription-dependent

To further elucidate the mechanism by which Notch pathway activation suppresses PI3K/Akt and MAPK pathway-mediated endothelial cell proliferation, we tested whether the process is transcription-dependent or -independent. Mounting evidence points to a transcription-independent mechanism in mediating multiple effects of Notch signaling (38, 39). We first tested the effect of HES1 on HMVEC cell growth. HES1 is a downstream target molecule of the Notch signaling cascade whose expression depends on N^{IC}/CSL/MAML complex-mediated gene transcription. When introduced into HMVEC with an adenoviral vector (50 pfu/cell), HES1 suppressed cell proliferation relative to GFP-transfected or untreated parental cells (Fig. 5A). This finding suggests a transcription-dependent mechanism in the execution of Notch signaling. Because MAML is required to transcribe downstream target genes of the Notch pathway, we decided to investigate a potential role of MAML1 in controlling endothelial cell proliferation by introducing a dominant-negative mutant of MAML1/pBabe [MAML1 (a truncated mutant containing amino acid 1–305) into HMVEC-N^{IC} cells (DN-MAML1-HMVEC-N^{IC}). We used pBabe as a control (Mock-HMVEC-N^{IC}). Cells infected with retroviruses at 2 MOI were pooled after 48 h and used for subsequent assays. Expression of DN-MAML1 in cells was demonstrated by Western blotting (Fig. 5B). As expected, DN-MAML1 partly altered cell proliferation rates induced by activated Notch1 (Fig. 5B). This result suggests that the effects of Notch signaling on cell proliferation control are mediated, at least partly, by the Notch/MAML-HES cascade and are transcription-dependent.

We then examined whether MAML1 is also required for Notch1-mediated vascular network formation. Compared with control cells (pBabe-HMVEC-N^{IC}), constitutive expression of DN-MAML1 in HMVEC-N^{IC} cells retarded their network formation on Matrigel. In pBabe-HMVEC-N^{IC} cells, initial immature network formation started ~2 h after seeding cells on Matrigel and matured networks formed after ~6 h; whereas in DN-MAML1-HMVEC-N^{IC} cells, fewer networks formed at 6 h (Fig. 5C). These results indicate that the effects of Notch signaling on endothelial cell network formation are also mediated through the Notch-MAML cascade. Thus, Notch signaling suppresses endothelial cell proliferation while it enhances endothelial cell differentiation. It supports our previous hypothesis that Notch

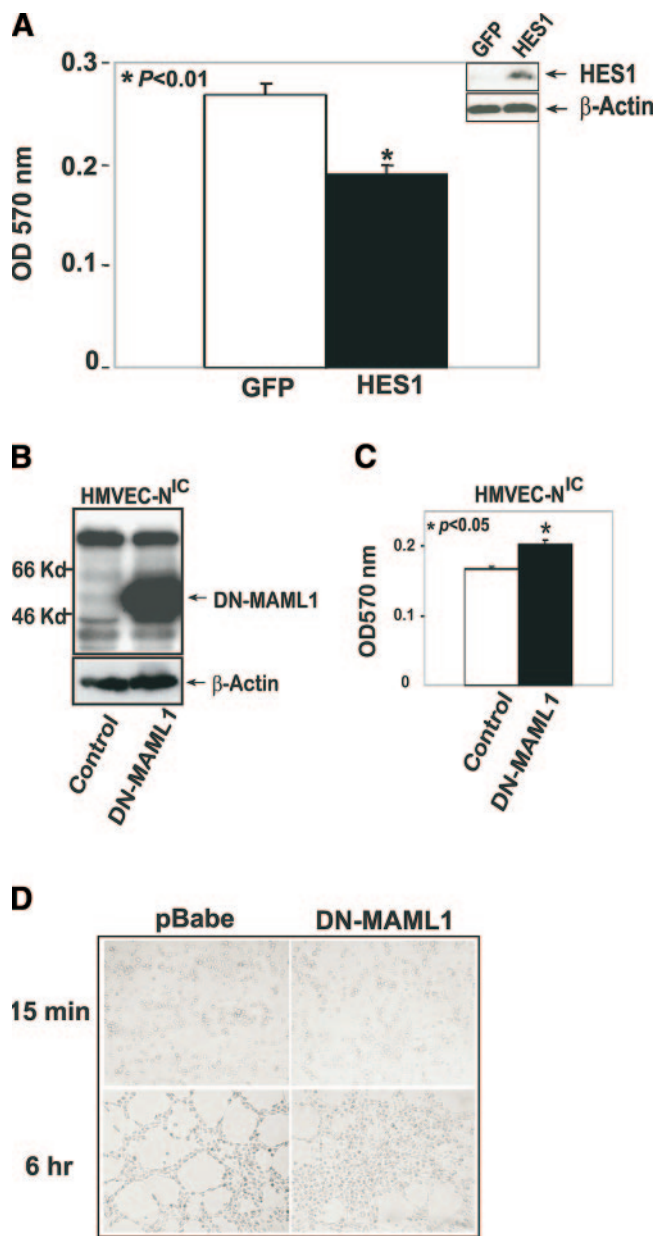


Figure 5. Effect of Notch signaling on cell proliferation control is transcription-dependent. *A*) Expression of HES1 inhibits HMVEC proliferation. Cell proliferation was analyzed by MTT assays 48 h after transfection with HES1 in an adenoviral vector. GFP was used as control. Untransfected parental cells displayed similar proliferative properties as GFP control cells. Results are means \pm SD of three independent experiments. *P* value is shown from Student’s *t*-tests. *Insert:* Expression of exogenous HES1. β -actin levels demonstrate equal loading conditions. *B*) Expression of DN-MAML1 reverses Notch signaling-induced proliferation inhibition. HMVEC-N^{IC} cells were transfected with DN-MAML1/pBabe or pBabe (control), respectively. Expression of Myc-tagged DN-MAML1 was detected on Western blot by 9-B11 Ab. β -actin was used as loading control. *C*) Cell proliferation was examined by MTT assays. Results are means \pm SD of three independent experiments. *P* value is shown from Student’s *t* tests. *D*) Expression of DN-MAML1 suppresses network formation. We transfected HMVEC-N^{IC} cells at 1×10^5 /well with DN-MAML1/pBabe or pBabe and plated them on Matrigel. Vascular network formation was observed and photographed at various time points. Representative photos are shown (10 \times).

signaling induces a differentiation-associated growth arrest, which is essential to angiogenesis (28).

Effect of Notch signaling on controlling MAPK and PI3K/Akt pathways is MAML1-dependent

To address whether the effects of MAML1 on endothelial cell proliferation control are mediated through the MAPK and PI3K/Akt pathways, we examined whether DN-MAML1 could alter N^{IC}-induced MAPK and PI3K/Akt pathway activities. Compared with Mock-HMVEC-N^{IC} control cells, phosphorylation of Erk1/2 following mitogen stimulation (h-VEGF, 50 ng/ml) was significantly re-established in DN-MAML1-HMVEC-N^{IC} cells (Fig. 6). Similarly, phosphorylation of Akt following mitogen stimulation was also remarkably reversed. Very similar results were obtained in HIAEC-N^{IC} cells (data not shown). These data demonstrate that MAML1 is indeed responsible for mediating the effects of activated Notch signaling on cell proliferation control by regulating the MAPK and PI3K/Akt signaling pathways.

DISCUSSION

Endothelial cell proliferation is required for vascular sprouting and elongation during new blood vessel formation. When endothelial cells differentiate, which is required for remodeling and maturation of newly formed blood vessels, the cell cycle needs to be arrested. This cessation is known as differentiation-associated cell cycle arrest (40). In matured vasculature, most of the endothelial cells remain in a quiescent

state. Notch signaling is known to be involved in remodeling newly formed vessels in a temporal and spatial fashion. It has been suggested that Notch signaling is silent during the early stages of angiogenesis (when active endothelial cell proliferation is required) but activated during vessel maturation [when endothelial cells cease proliferation and likely undergo differentiation-associated cell cycle arrest (21, 41)]. Notch pathway activation may also be involved in endothelial cell contact inhibition (22). If true, this supports the hypothesis that Notch signaling helps control vessel homeostasis by maintaining endothelial cell quiescence (21).

The current study suggests that the MAPK and PI3K/Akt pathways are under the control of Notch signaling and that both pathways are responsible for the ultimate outcome of Notch signaling-induced cell proliferation inhibition. Our work establishes a direct link between the pathways of Notch, MAPK, and PI3K/Akt and provides an explanation for the mechanisms of controlling cell growth by Notch signaling in endothelial cells. The signaling cascades delivered from both MAPK and PI3K/Akt pathways regulate cell cycle machinery. It has been demonstrated in endothelial cells that Notch signaling inhibits phosphorylation of Rb (12) and mitogen-induced up-regulation of p21^{Cip1} (22). Our findings may well bridge the gap between Notch activation and negative regulation of cell cycle machinery.

Depending on cell type and context, the Notch pathway can generate either inhibitory or proliferative signals (42, 43). Very similar to its role in endothelial cells, Notch signaling also inhibits keratinocyte proliferation by inducing p21^{Cip1} (44) and induces cell growth arrest in small cell lung cancer (32), prostate cancer (45), and hepatocellular carcinoma (46), which is associated with a cell cycle block at G₁. In contrast, activation of the Notch pathway induces ectopic T cell proliferation in bone marrow (47) and provides cell proliferation cues in acute lymphoblastic T cell leukemia (48), mouse mammary tumors (9), and transformed kidney epithelial cells (49). It is unclear whether suppression or promotion of cell proliferation by Notch signaling in other cell types occurs by inhibiting or activating the MAPK and PI3K/Akt pathways. It would be interesting to test this possibility.

Classical Notch signaling is transmitted by means of the Notch-N^{IC}/CSL/MAML cascade. Recent evidence suggests that a transcription-independent mechanism exists for mediating the multiple effects of Notch signaling (38, 39). It is likely that Notch signaling regulates the MAPK and PI3K/Akt pathways at the post-transcriptional concentration because (a) HES1, a post-transcriptional target gene product, can mimic the effect of Notch on endothelial cell proliferation control; and (b) MAML1 is an essential element for initiating transcription. DN-MAML1 can antagonize the effect of Notch on MAPK- and PI3K/Akt pathway-mediated cellular proliferative or inhibitory signals. Consistently, our preliminary efforts in detecting a direct association between N^{IC} and MAPK or Akt by

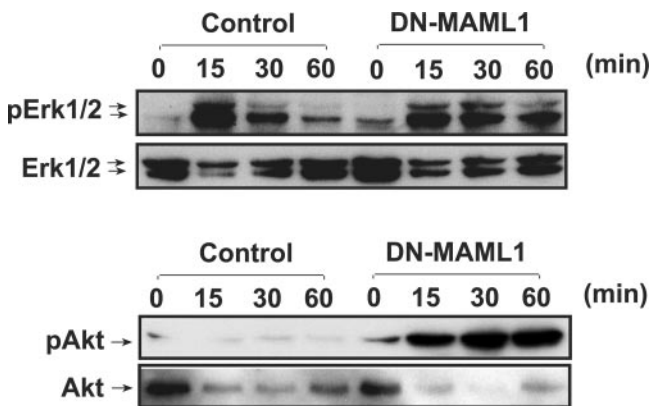


Figure 6. Effect of DN-MAML1 on MAPK and Akt phosphorylation. Reverse N^{IC}-induced suppression of MAPK and Akt phosphorylation by DN-MAML1. HMVEC-N^{IC} cells were transfected with DN-MAML1/pBabe or pBabe (control) for 48 h, respectively. Cells were replated and starved using serum-free basic medium overnight then restimulated with 50 ng/ml of h-VEGF. Cells were harvested at various times and lysed for Western blot analysis. Membranes were blotted with antiphospho-Erk1/2 and -Akt Abs, stripped, and reblotted with antitotal Erk1/2 and Akt Abs. DN-MAML1 reversed N^{IC}-induced suppression of MAPK and Akt phosphorylation. A representative result was shown. Similar results were observed in HIAEC-N^{IC} cells.

coimmunoprecipitation were unsuccessful (data not shown). This supports the notion that intracellular Notch itself does not directly regulate the MAPK and PI3K/Akt pathways. Because MAPK and PI3K/Akt pathways are simultaneously controlled by the Notch/MAML-HES cascade, and these 2 parallel pathways are initiated by receptor tyrosine kinases/phosphatases or adaptor-mediated kinases/phosphatases, it is more likely that regulation of the MAPK and PI3K/Akt pathways by the Notch/MAML-HES cascade is mediated by regulating common kinases/phosphatases. It has been reported that Notch activation down-regulates VEGF-R2 expression on endothelial cells (41). Decreased expression of VEGF-R2 is likely responsible for initiating weak MAPK and PI3K/Akt pathway activation (considering that VEGF stimulation was used in our experiments). However, since complete M199 medium and VEGF achieve similar effects, VEGF-R2 is obviously not the only target molecule to mediate Notch's suppression. Future studies to identify such Notch signaling-induced target molecules will help define the mechanism underlying the crosstalk between Notch signaling and the MAPK and PI3K/Akt pathways. **[F]**

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