



Notch Activation during Endothelial Cell Network Formation *in Vitro* Targets the Basic HLH Transcription Factor HESR-1 and Downregulates VEGFR-2/KDR Expression

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Received August 6, 2001

Angiogenesis is essential for normal homeostasis, wound healing, and tumor growth and involves a switch in endothelial cell (EC) phenotype from quiescence to migration, proliferation and network formation, and back to quiescence. The notch signaling pathway is critically involved in cell fate decisions during development, and mice deficient in several notch/notch ligand genes have vascular phenotypes. Here we show that notch signaling is activated during EC capillary-like network formation *in vitro* and that EC express transcripts for notch 1, notch 4, the notch ligand delta 4, and the putative notch processing enzymes ADAM-10 and presenilin. Expression of dominant negative notch blocks network formation; however, constitutively active notch (NICD) does not induce morphologic changes. Furthermore, both EC network formation and expression of activated notch 1 or notch 4 induce expression of the bHLH transcription factor HESR-1 and downregulate the known HESR-1 target VEGFR-2 (KDR). Notch-mediated reduction in VEGFR-2 expression results in decreased EC proliferation in response to VEGF but not bFGF. These data suggest that HESR-1 may be involved in the phenotypic changes that characterize the progression from EC proliferation and migration to network formation and quiescence. © 2002 Elsevier Science (USA)

Key Words: Notch; HESR-1; VEGFR-2; angiogenesis; endothelial cell; *in vitro*; bHLH transcription factor.

INTRODUCTION

Angiogenesis is the process of new blood vessel formation from existing vessels in response to angiogenic stimuli and involves endothelial cell (EC) degradation of the adjacent extracellular matrix, migration (sprouting) into the surrounding tissue, proliferation, alignment, tube formation, recruitment of parenchymal cells, and a return to quiescence (Carmeliet, 2000; Risau, 1997; Yancopoulos *et al.*, 1998). The importance of angiogenesis in solid tumor growth and survival is well established and solid tumors larger than a few millimeters in size uniformly induce a vascular supply (Folkman, 1990; Folkman and Klagsbrun, 1987). It is now well recognized that compounds that inhibit angiogenesis are effective at blocking tumor growth in animal models (Boehm *et al.*, 1997; Folkman, 1997; Kerbel *et al.*, 2000).

Although angiogenesis is an excellent therapeutic target, little is known regarding the signaling mechanisms involved in regulating the different stages of an angiogenic response. Angiogenic growth factors that

induce proliferation and migration of EC, such as VEGF (Leung *et al.*, 1989) and bFGF (Friesel and Maciag, 1995), have been identified, as have factors such as the angiopoietins (Sato *et al.*, 1995; Suri *et al.*, 1996), which regulate maturation of developing vessels. Recently, the ephrin/Eph system has also been implicated in regulating angiogenesis during development (Wang *et al.*, 1998). An important question that remains to be answered is what determines whether, in response to an angiogenic stimulus, an individual cell remains quiescent to maintain vessel integrity or migrates from the parent vessel to initiate new vessel formation.

The notch signaling pathway is highly conserved between invertebrates and vertebrates and is critical for cell fate determination. In humans, there are at least four notch receptors, notch 1–4, and several ligands, including jagged 1 and 2 and delta 1–4 (Artavanis-Tsakonas *et al.*, 1999). The notch receptor is activated by binding to one of its ligands on an adjacent cell, which induces sequential cleavages, resulting in release of the notch intracellular domain (NICD). ADAM-10, ADAM-17, and presenilin have been implicated in these processing steps (Mumm and Kopan, 2000). Classically, NICD then acts via RBP-j (also known as CBF/Su(H)/LAG-2) (Kato *et al.*, 1997) to induce expression of genes of the HES/E(spl) family (Bailey and Posakony, 1995), which then downregulate tissue-specific genes of the MASH/achaete-scute family. Notch signaling thereby determines the fate of the notch expressing cell by suppressing differentiation (Hing *et al.*, 1994). In this way, the notch pathway can mediate lateral inhibition, where a ligand expressing cell can suppress differentiation of neighboring “notch” cells, thereby contributing to patterning a field of cells. Notch-mediated lateral inhibition, for example, has been demonstrated in patterning of both the eye and sensory bristles in *Drosophila* (Artavanis-Tsakonas *et al.*, 1999).

There is now growing evidence implicating the notch pathway in angiogenesis. For example, the human cardiovascular diseases CADASIL and Alagille Syndrome have been associated with mutations in the *notch 3* and *jagged 1* genes, respectively (Joutel and Tournier-Lasserre, 1998). Additionally, in mice several receptors and ligands are expressed in both the

embryo and adult vasculature (Lewis, 1998), and mice lacking *notch 1* (Krebs *et al.*, 2000; Swiatek *et al.*, 1994) or the ligand *jagged 1* (Xue *et al.*, 1999) have vascular defects. Notch 4, which potentiates the notch 1 phenotype when deleted, is exclusively expressed in the vasculature in embryonic mice (Uyttendaele *et al.*, 1996) along with one of its ligands, delta 4 (Shutter *et al.*, 2000; Villa *et al.*, 2001). Finally, *presenilin* knockout mice exhibit a complex phenotype, including extensive hemorrhaging (Shen *et al.*, 1997).

Previously, we identified HESR-1, a novel bHLH transcription factor that regulates capillary-like network formation *in vitro* (Henderson *et al.*, 2001). Recent studies suggest that HESR-1 is a negative regulator of transcription in EC (Henderson, manuscript in preparation). *In situ* hybridization studies of mouse embryos have identified transcripts in the heart and heart vasculature as well as several other tissues (Chin *et al.*, 2000; Kokubo *et al.*, 1999; Leimeister *et al.*, 1999; Nakagawa *et al.*, 2000). Gridlock (HESR-2) is a closely related family member and is the gene responsible for a mutant phenotype in zebrafish resembling coarctation of the aorta (Zhong *et al.*, 2000). It has recently been shown to be critical for specification of arterial cells in the early embryo (Zhong *et al.*, 2001). HESR-1 is related to the HES/Hairy/E(spl) family, all known notch targets, and HESR-1 is downstream of notch in presomitic and somitic mesoderm in mice (Kokubo *et al.*, 1999). A recent study showed that constitutively active notch can activate the HESR-1 promoter in 10T1/2 and COS cells (Maier and Gessler, 2000; Nakagawa *et al.*, 2000); however, there are no data regarding upstream activators of HESR-1 expression EC. In the present study we sought to determine (1) whether notch is active during capillary-like network formation *in vitro*; (2) whether notch targets HESR-1 in EC; and (3) whether notch activity is necessary for network formation. We report that HESR-1 is indeed activated by notch in EC and that notch activity is essential for EC capillary-like network formation. Furthermore, network formation, acting through notch, downregulates expression of VEGFR-2, a known HESR-1 target gene. These data implicate notch and HESR-1 in the regulation of EC phenotype.

MATERIALS AND METHODS

Cell culture and reagents. M199 medium and fetal bovine serum (FBS) were purchased from GibcoBRL (Grand Island, NY). Endothelial cell growth supplement (ECGS), bFGF, VEGF, and fibronectin were from Collaborative Biomedical (Bedford, MA). Collagenase type I was purchased from Worthington Biomedical Corporation (Lakewood, NJ). Tissue culture flasks and plates were from Falcon Labware/Becton–Dickinson (Franklin Lakes, NJ). The 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2-tetrazolium-5-carboxanilide (XTT) and phenazine methosulfate (PMS) used in the VEGF mitogenesis experiments were purchased from Sigma (St. Louis, MO). Human umbilical vein EC were isolated from umbilical cords (Gimbrone *et al.*, 1974) obtained from regional hospitals under approved protocols. EC were routinely cultured in M199 medium with 20% FBS and supplemented with ECGS at 37°C in 5% CO₂. For all experiments EC were used between passages three and six.

Plasmids. The plasmid containing the intracellular portion of the human notch-1 gene (amino acids 1758 to 2566) cloned into the pBabe puro expression vector (Capobianco *et al.*, 1997) was a gift of Dr. Tony Capobianco. The HA-tagged intracellular portion of the mouse notch 4 (int3) gene cloned into the pLNCX expression vector (Uyttendaele *et al.*, 1998) was a gift from Dr. Jan Kitajewski. The soluble jagged 1 and notch 3 extracellular domains, both cloned into the pAdlox plasmid, were a gift of Dr. Tom Maciag (Small *et al.*, 2001). The HES-1 promoter pGL2 luciferase vector (Jarriault *et al.*, 1995) was a gift of Dr. Raphael Kopan. The pU981-6 plasmid containing multimerized RBP-j binding sites driving luciferase (Minoguchi *et al.*, 1997) was a gift from Dr. Zimmer-Strobl. The VEGFR-2 promoter pGL2 luciferase plasmid (Patterson *et al.*, 1995) was a gift of Dr. Cam Patterson. The full-length HESR-1 promoter (nucleotides –2015 to +1) was amplified from genomic DNA and cloned into the pGL3-enhancer luciferase vector.

Transfections. One million EC were transfected by electroporation with 5 to 10 µg of plasmid DNA in 500 µl of M199 medium with 5% FBS in cuvettes with a 4-mm gap (Invitrogen, Carlsbad, CA) using a Bio-Rad

GenePulser (Bio-Rad, Hercules, CA) at a capacitance of 960 µF and 250 V. Cells were then washed with M199 medium containing 10% FBS, plated, and allowed to recover for several hours. Transfection efficiency was determined by transfecting EC with a GFP expression plasmid and analyzing fluorescence 18 h later by FACS (FACSCalibur, Becton–Dickinson, San Jose, CA). Transfection efficiency was regularly between 40 and 60% for all experiments shown. Transfections were normalized by transfecting cells with 1 µg of a CMV-β-galactosidase plasmid. β-Gal was assayed using Galacto-Light Plus (Bedford, MA).

Network-forming assay. The network-forming assay has been previously described (Henderson *et al.*, 2001). Briefly 3 × 10⁵ EC were seeded in triplicate into a 12-well plate coated with rat tail collagen type I (1.5 mg/ml) and fibronectin (3 µg/ml) in M199 medium, 20% FBS, and ECGS and allowed to attach for at least 1 h. Following this, cells were overlaid with an additional 800 µl of collagen/fibronectin. Once the collagen had polymerized cells were fed with M199 medium containing 10% FBS and 25 ng/ml each of VEGF and bFGF. After 18 h cells were harvested with 2000 U/ml of collagenase, washed, and lysed and luciferase activity was measured with the Luciferase Assay System (Promega, Madison, WI) and read on a Monolit 2010 (Labnet, San Diego, CA).

Quantification of network formation. Following completion of the network-formation assay two images (40× magnification) per well were captured on an IX70 Olympus microscope using Optronics digital capture software (Optronics Inc., Goleta, CA). Images were analyzed using Image Pro Plus image analysis software (Media Cybernetics, Silver Springs, MD) for total network length. All conditions were assayed in triplicate and compared against EC transfected with parent plasmid.

Mitogenesis assay. EC were transfected as described above with the specified plasmids. Three thousand EC were seeded onto gelatinized 96-well plates. Six wells were plated per condition and cells were grown for 72 h in M199 medium containing 10% FBS and 5 ng/ml of bFGF or VEGF or no growth factor. For the last hour 50 µl of XTT/PMS (200:1) was added per well and absorbance then read on a CERES 900C plate reader at 450 nm.

RT-PCR analysis. RNA was isolated from network-forming assays or freshly isolated capillaries (Springhorn *et al.*, 1995) using Trizol (GibcoBRL, Grand Island, NY). PCR was performed using *Taq* polymerase (GibcoBRL, Grand Island, NY) on a PTC-Thermal Cycler (MJ Research, Watertown, NJ) for 30 cycles at annealing temperatures: delta 4 (62°C), ADAM-10 (61°C), notch 4 (66°C), notch 1 (66°C), presenilin (58°C). Primers used were Delta 4 upper 5' GC-CGGGTACCTTCTCGCTCATCATC 3', lower 5' GC-CTCCCAGCCCTCATCACAAGTA 3'; ADAM-10 upper 5' ATCCCATAAATACGGTCTCTCA 3', lower 5' ACGCCATCATCAAATCTCGGTCTG 3'; Notch 4 upper 5' TCCTGGGGCCCCGGGCTGAAGAAAAG 3', lower 5' ACGCCGGATGAGCTGGAGGACGAGA 3'; Notch 1 upper 5' GCGGCCGCTTTGTGCTTCT-GTTC 3'; lower 5' CGGCCGGCGCGTCCTCCTT 3'; presenilin upper 5' AGTCCGCACGCCTCTTGTTTCG 3', lower 5' TGCTCCTGCCGTTCTCTATTGTCA 3'; and GAPDH upper 5' ACCACAGTCCATGCCAT-CAC 3', lower 5' TCCACCCTGTTGCTGTTGCTGTA 3'. Semiquantitative PCR was performed by running the PCR reaction as above but for 25, 26, and 28 cycles and comparing amplicon intensities by gel electrophoresis.

Statistics. All data were analyzed using Sigma Stat (SPSS, Chicago, IL). Mean values from luciferase assays and mitogenesis assays were compared within each experimental group by one-way analysis of variance and Tukey's comparison test, $P < 0.05$.

RESULTS

Notch Is Expressed in Human EC

During development both notch 4 and delta 4 are largely restricted in their expression to the vasculature and notch-1-deficient mice die of vascular defects. We therefore used RT-PCR to investigate the expression of these genes in cultured EC and freshly isolated capillaries. Confirming earlier results of *in situ* hybridization analysis (Shutter *et al.*, 2000; Uyttendaele *et al.*, 1996) we identified transcripts for notch 4 and delta 4 in both cultured EC and freshly isolated EC. In addition,

we also identified transcripts for notch 1, ADAM-10, and presenilin. A representative experiment is shown for notch 1, notch 4, and the control gene GAPDH, in both networks and monolayers (Figs. 1A and 1B). Using semiquantitative RT-PCR techniques we found no difference in expression of these genes between networks and monolayer cultures, although we do have preliminary data suggesting differential expression of jagged 1 under these conditions (A.M.H. and C.C.W.H., unpublished observations). These results indicate that genes involved in notch pathway signaling are expressed in human EC both *in vitro* and *in vivo*; however, we have not yet determined which combination of notch and notch ligand is most important during network formation.

The Notch Pathway Is Active during Network Formation in Vitro

Mice homozygous for a targeted disruption of the notch 1 allele die *in utero* as a consequence of failure to remodel the early vasculature. To determine if the notch pathway is active during network formation *in vitro* we assayed for upregulation of the well-characterized notch target gene HES-1 (Beatus *et al.*, 1999; Jarriault *et al.*, 1995). Human EC cultured in three-dimensional collagen gels form anastomosing capillary-like networks within 18 h of plating (Davis and Camarillo, 1996; Goto *et al.*, 1993; Heffelfinger *et al.*, 2000; Henderson *et al.*, 2001; Montesano *et al.*, 1983), whereas cells growing in two-dimensional culture form a monolayer with cobblestone morphology (Henderson *et al.*, 2001, and data not shown). EC were transfected with a HES-1 promoter luciferase construct and embedded in collagen (three-dimensional) or plated onto collagen (two-dimensional). Luciferase activity was measured 18 h later, by which time formation of the tubular network was complete. Network formation stimulated luciferase production over fourfold when compared to that in two-dimensional monolayer cultures (3395 RLU vs 813 RLU, $P = 0.008$) (Fig. 2A), suggesting that notch was indeed activated. As an independent assay for notch engagement we examined activity of RBP-j (CBF/Su(H)), an immediate downstream target of notch (Kato *et al.*, 1997; Mino-guchi *et al.*, 1997). EC were transfected with a lucif-

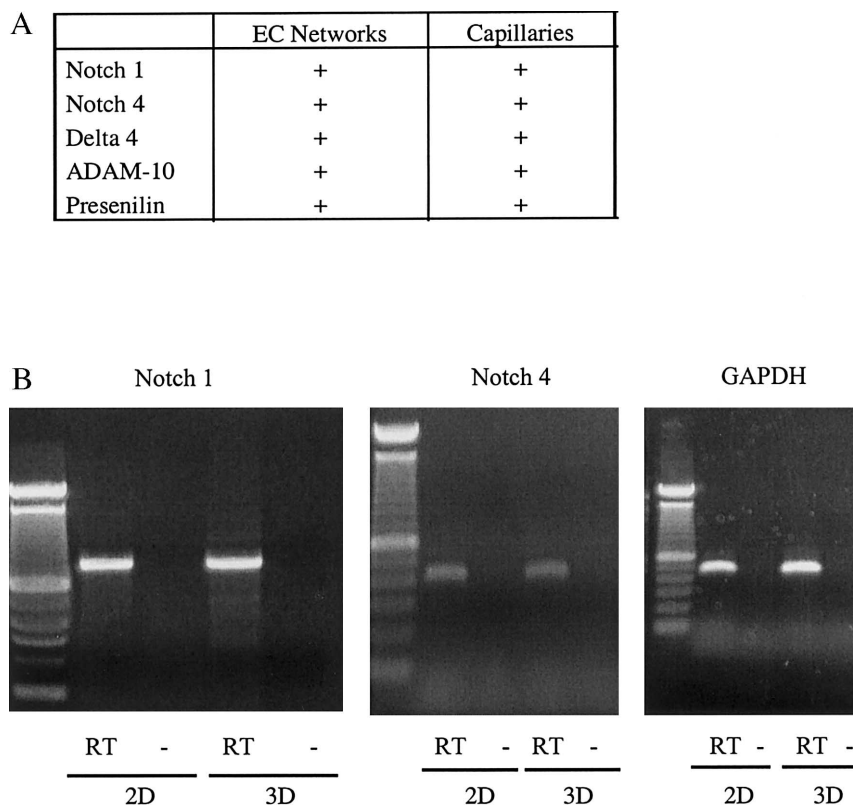


FIG. 1. RT-PCR analysis of notch pathway gene expression in endothelium. EC were cultured in an *in vitro* network-forming assay and harvested for RT-PCR analysis of notch and notch ligand expression. Isolated capillaries were also analyzed. (A) Expression of notch pathway genes in both network-forming EC *in vitro* and in freshly isolated capillaries. (B) PCR products for notch 1, notch 4, and GAPDH in monolayer (2D) versus network (3D) cultures, both with (RT) and without (-) reverse transcriptase.

erase reporter construct driven by multimerized RBP-j binding sites and then embedded in collagen or plated onto collagen. Again, network formation stimulated luciferase production, by approximately 2.5-fold when compared to that of two-dimensional cultures (5880 RLU vs 2180 RLU, $P = 0.007$) (Fig. 2B). These results indicate that the notch pathway is active during network formation *in vitro*.

To determine whether notch signaling is necessary for induction of network formation we used dominant negative forms of jagged 1 (sJag) or notch 3 (N3EC). These consist of the extracellular domain of jagged or notch 3 truncated so as to remove the transmembrane and intracellular domains. EC were transfected with either control plasmid or dominant negative construct and embedded in collagen gels. Cultures were photographed after 18 h and the extent of network formation was quantitated as described under Materials and

Methods. Both sJag and N3EC significantly inhibited network formation, reducing overall network length by 45 and 52%, respectively (Fig. 2C). Neither construct significantly affected morphological characteristics of monolayer cultures (data not shown). We conclude from these experiments that notch signaling is necessary, though not sufficient, for induction of the morphological changes associated with formation of capillary-like networks *in vitro*.

Notch 1 and Notch 4 Target HESR-1 in EC

We previously reported that expression of HESR1 is upregulated during network formation *in vitro* and that blocking its induction using antisense oligonucleotides disrupts this process (Henderson *et al.*, 2001). Furthermore, others have shown that the HESR-1 promoter can be activated by overexpression of notch in

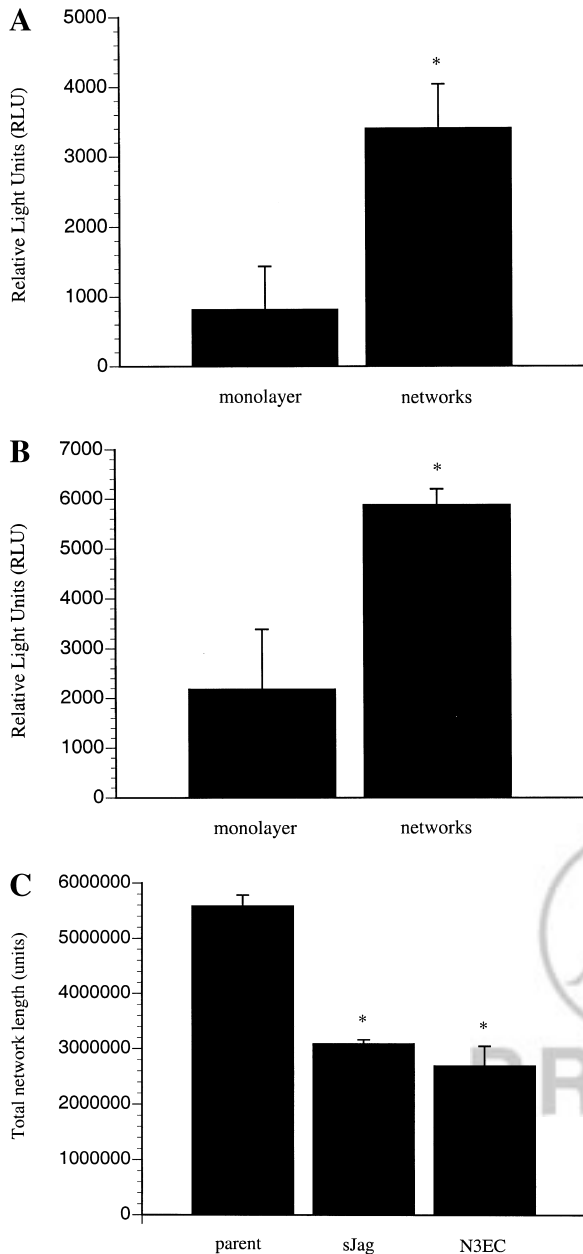


FIG. 2. The notch pathway is active in network-forming EC *in vitro*. (A) To determine if the notch pathway is active during network formation *in vitro* the HES-1 promoter was transfected into EC by electroporation. The transfected cells were grown in collagen gels and luciferase production was measured at 18 h. Network-forming cultures had significantly greater luciferase activity than monolayer cultures. (B) The same as (A) except a promoter containing RBP-j binding sites was used. To determine if the notch pathway is necessary for network formation *in vitro* EC were transfected with parental or the dominant negative notch constructs sJag or N3EC

COS cells (Maier and Gessler, 2000; Nakagawa *et al.*, 2000). To determine whether notch is sufficient to activate expression of HESR-1 in EC during network formation we used constitutively active forms of notch 1 (notch 1 IC) and notch 4 (notch 4 IC), which lack the extracellular domain and mimic ligand-induced notch signaling. When transfected into EC both notch 1 IC and notch 4 IC induced endogenous HESR-1 expression (data not shown) and significantly enhanced the activity of a cotransfected HESR-1 promoter-luciferase construct, inducing two- to fourfold higher levels of luciferase compared to cells transfected with control plasmid (2151 RLU vs 503 RLU for notch 1 IC and 1006 RLU vs 503 for notch 4 IC, $P < 0.001$) (Fig. 3A). Thus both notch 1 and 4 can target HESR-1 in EC. To determine whether notch signaling is necessary for upregulation of HESR1 during network formation we again used dominant negative jagged 1 and notch 3. Both constructs when expressed in network-forming cells dramatically blocked upregulation of endogenous HESR1 transcripts. Compared to control-transfected cultures, HESR1 expression was decreased 80% by sJag 1 and 68% by N3EC (Fig. 3B). Together, these data indicate that notch activation is both necessary and sufficient for upregulation of HESR1 expression in network-forming EC.

Notch Downregulates VEGFR-2 in EC

It is not known how notch functions to regulate blood vessel development; however, based on previous data that HESR-1 can downregulate VEGFR-2 mRNA and protein levels and block EC proliferation (Henderson *et al.*, 2001) and that the closely related HESR-2 (CHF-1) can downregulate VEGF (Chin *et al.*, 2000), we hypothesized that notch may be an upstream regulator of this angiogenic growth factor pathway. To test this hypothesis we assayed VEGFR-2 promoter activity in EC in the presence or the absence

and network formation was assessed by image analysis. (C) EC transfected with either of the dominant negative notch constructs had significantly reduced network formation compared to parental cells. Each graph represents one of three similar experiments. An asterisk indicates statistically significant, $P < 0.005$.

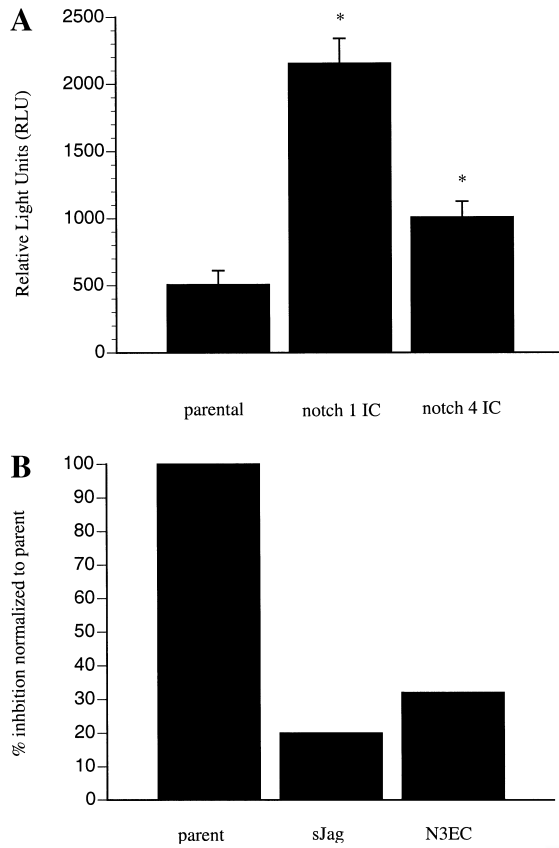


FIG. 3. Notch 1 IC and notch 4 IC upregulate HESR-1 in EC. (A) EC were transfected with a HESR-1 promoter luciferase construct along with either notch 1 IC or notch 4 IC or control expression plasmids. Notch 1 IC and notch 4 IC significantly increased HESR-1 promoter-driven luciferase activity. (B) To determine if blocking the notch pathway could prevent upregulation of HESR-1 we performed RT-PCR analysis on mRNA isolated from EC transfected with either dominant negative notch 3 or jagged and grown in three-dimensional collagen gels. HESR-1 expression was normalized to that of GAPDH. Both dominant negative constructs significantly blocked HESR-1 upregulation. Each graph is one of three similar experiments. An asterisk indicates that the difference from control is statistically significant, $P < 0.05$.

of cotransfected notch 1 IC or notch 4 IC. The VEGFR-2 promoter was constitutively active in proliferation EC, consistent with the expression of endogenous mRNA and protein (Henderson *et al.*, 2001). Both notch 1 IC and notch 4 IC significantly decreased promoter activity (Fig. 4A). Notch 1 IC was consistently more effective, blocking by up to 50%.

To test the physiologic relevance of VEGFR-2 downregulation by notch we transfected EC with notch 1 IC

and notch 4 IC expression vectors and measured proliferation over time in response to the angiogenic growth factors bFGF and VEGF. Our prediction was that notch-induced downregulation of VEGFR-2 would reduce EC responsiveness to VEGF but not to bFGF. This indeed was the case. Cells transfected with notch 1 IC or notch 4 IC were grown for 72 h, at which time the degree of proliferation was assessed using the metabolic dye XTT. In the experiment shown both

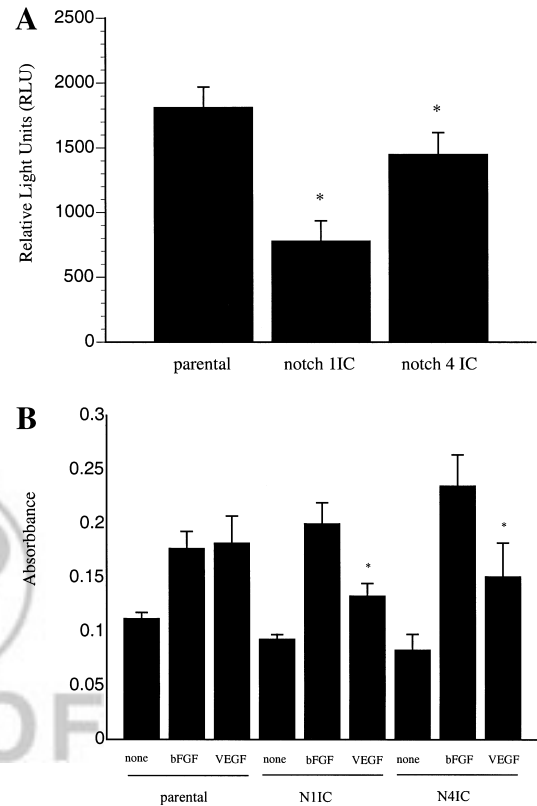


FIG. 4. Notch 1 IC and notch 4 IC downregulate VEGFR-2 promoter-luciferase activity EC. (A) EC were transfected with a VEGFR-2 promoter-luciferase construct along with notch 1 IC or notch 4 IC expression plasmids. Notch 1 IC and notch 4 IC significantly downregulated promoter activity. (B) EC were transfected with notch 1 IC, notch 4 IC, or control expression plasmids and grown for 48 h in the presence of 5 ng/ml of VEGF. XTT was then added for 1 h and proliferation was measured as absorbance of XTT at 450 nm. EC transfected with notch 1 IC or notch 4 IC constructs had significantly lower proliferative responses to VEGF, but not to bFGF, compared to control. Each graph represents one of three similar experiments. An asterisk indicates that the difference between bFGF and VEGF-induced proliferation is statistically significant, $P < 0.005$.

notch 1 IC and notch 4 IC significantly reduced proliferative responses to VEGF, by 32.5 and 35% (0.177 vs 0.182 OD and 0.235 vs 0.151 OD, Fig. 4B), respectively, but not to bFGF. In some experiments notch IC expression also reduced proliferation to bFGF; however, the effect on VEGF-induced proliferation was always significantly greater (data not shown). This decrease may relate to the finding that some of the mitogenic effect of bFGF is mediated by increased VEGF (Seghezzi *et al.*, 1998; Stavri *et al.*, 1995) or there may be direct effects of notch signaling on EC proliferation independent of VEGFR2 expression. The notch-mediated reduction in proliferative response to VEGF is consistent with our previous findings that overexpression of HESR-1 similarly blocks proliferation of EC, as well as migration (Henderson *et al.*, 2001).

VEGFR-2 Expression Is Reduced in Network-Forming EC *In Vitro*

We have determined that network formation activates the notch pathway and that notch IC, a constitutively active form of notch, upregulates HESR-1 and downregulates VEGFR2. Finally, we wished to test the prediction that VEGFR-2 is downregulated during network formation and correlate this with the upregulation of HESR-1. EC were transfected with either the VEGFR-2 promoter reporter, or the HESR-1 promoter reporter, and then plated, either in a network-forming assay or as a monolayer. Luciferase production was then measured over time. Activation of the HESR-1 promoter was rapid, reaching maximal levels by 4 h after plating, consistent with the kinetics of mRNA upregulation (Henderson *et al.*, 2001) (Fig. 5, squares). Activity of the promoter then declined out to 18 h; however, levels were still significantly higher in networks than in monolayer cultures. In contrast, in network-forming cultures VEGFR-2 promoter activity was rapidly downregulated, reaching a plateau at 10 to 15% of expression in two-dimensional cultures by 8 h (Fig. 5, circles). As predicted, this downregulation of VEGFR-2 coincides temporally with the upregulation of HESR1.

In aggregate, our data in this report and in our previous study suggest that in the later stages of new

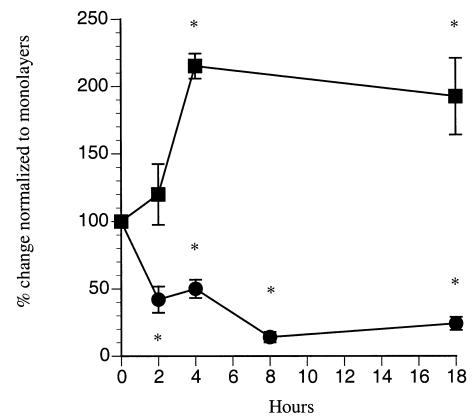


FIG. 5. Upregulation of HESR-1 correlates with downregulation of VEGFR-2 during network formation. EC were transfected with either a VEGFR-2 promoter reporter (circles) or a HESR-1 promoter reporter (squares) and luciferase activity was determined over time in cells growing on collagen or embedded in collagen and forming networks. Data are shown as the percentage of change relative to monolayer culture. Temporally, the upregulation of HESR-1 during tube formation coincides with downregulation of VEGFR-2. One of three similar experiments. An asterisk indicates a statistically significant difference between networks and monolayer cultures at each time point, $P < 0.005$.

vessel formation, when cells are no longer migrating and proliferating, but are forming new networks, notch activity may be a critical switch that reduces VEGFR-2 expression through the action of the negative transcriptional regulator HESR-1.

DISCUSSION

We have used an *in vitro* EC network-forming assay to investigate the role of the notch signaling pathway in EC morphogenesis. We have identified transcripts for notch 1 and 4 and the ligand delta 4 in cultured human EC and freshly isolated capillaries, consistent with published *in situ* hybridization studies (Shutter *et al.*, 2000; Villa *et al.*, 2001) showing largely EC-restricted expression of notch 4 and delta 4 and more general expression of notch 1. We also report the identification of transcripts for the putative notch processing enzymes ADAM-10 and presenilin. Targeted disruption of *notch 1* (Krebs *et al.*, 2000; Swiatek *et al.*, 1994), *notch 1* and *notch 4* (Krebs *et al.*, 2000), or *jagged*

1 (Xue *et al.*, 1999) results in early embryonic death due to disrupted vascular development. In all cases these mice complete vasculogenesis but succumb to defects in vascular remodeling. Moreover, transgenic mice overexpressing *notch 4* also die *in utero* due to incomplete angiogenic remodeling, resulting in fewer small vessels, disorganized vessels, and dilated vessels with reduced vessel wall integrity (Uyttendaele *et al.*, 2001). In addition, mutation of the *presenilin* gene results in numerous defects, including extensive hemorrhaging (Shen *et al.*, 1997).

While these studies indicate a role for notch in angiogenesis, they do not address the mechanism by which notch signaling regulates angiogenesis. Although results from the *notch 4* transgenic mice, where notch 4 IC expression was controlled by an EC-specific promoter, suggest that notch signaling in EC themselves is crucial for vascular patterning, notch signaling in EC has not been directly demonstrated. In an *in vitro* assay that models some aspects of *in vivo* angiogenesis, including EC migration, alignment, and tube formation, we have used two readouts to demonstrate activity of the notch pathway. Both RBP-j, an immediate downstream effector of notch signaling, and the promoter of HES-1, a well-characterized notch target gene, are activated during the process of network formation. Importantly, we have also identified the bHLH transcription factor HESR-1 as a notch target gene in EC and shown that its induction can be blocked by dominant negative notch or jagged. A previous study in 10T1/2 fibroblasts showed that notch 1 IC activation of the HESR-2 (HRT-2) promoter was dependent on a proximal RBP-j binding site (Nakagawa *et al.*, 2000). The HESR-1 promoter contains two such sites, and it is likely that these are targets of notch signals in EC.

We identified HESR-1 in a screen for genes upregulated in EC during the transition from migration/proliferation to network formation, and it has been independently isolated by others (Chin *et al.*, 2000; Kokubo *et al.*, 1999; Leimeister *et al.*, 1999; Nakagawa *et al.*, 2000; Zhong *et al.*, 2000) and shown to be expressed in the developing heart and vasculature, as well as other tissues. We demonstrated expression in mature, quiescent vasculature in the adult but reduced expression in proliferating EC (Henderson *et al.*, 2001).

Blocking upregulation of HESR-1 antisense oligonucleotides blocks network formation. Two closely related family members, HESR-2 and HESR3, have different, but overlapping expression patterns (Leimeister *et al.*, 1999; Nakagawa *et al.*, 2000). HESR-2 (CHF-1, gridlock) in zebrafish is involved in regulation of VEGF expression (Chin *et al.*, 2000), and aortic development (Zhong *et al.*, 2000). Here we show that HESR-1 is a downstream target of notch in EC, suggesting that it may be an effector of notch signaling in EC. Importantly, inhibiting notch signaling inhibits EC network formation *in vitro*, consistent with the ability of HESR-1 antisense oligonucleotides to block formation of networks. What might be the significance of notch-mediated HESR-1 activation in these cells? Activated notch 4 inhibits branching morphogenesis in mammary epithelial cells (Uyttendaele *et al.*, 1998) and mice transgenic for activated notch 4 lack small branching vessels. Many of the stages of angiogenesis, including branching, are driven by VEGF (Conway *et al.*, 2001) and we have previously shown that HESR-1 blocks VEGFR2 expression and reduces EC migration and proliferation. We wondered, therefore, whether notch signaling in EC may be acting to downregulate EC responsiveness to VEGF, allowing establishment of the mature, quiescent phenotype. In support of this we found that overexpression of either notch 1 or notch 4 did indeed block VEGFR-2 promoter activity and reduced the responsiveness of EC to VEGF, but not to bFGF, in a proliferation assay. Of note is the observation that VEGFR-2 is dramatically downregulated in adult EC and is undetectable in most adult tissues (Kappel *et al.*, 1999). We did not find that Notch IC expression was sufficient to promote network formation (data not shown), in contrast to a previous report using immortalized rat brain EC (Uyttendaele *et al.*, 2000). This difference may be due to the use of primary versus immortalized cells or to the use of transient versus stable transfections.

The mechanism by which HESR-1 downregulates VEGFR-2 promoter activity is not known; however, HESR-2 (CHF-1) has been shown to block VEGF promoter activity by binding to ARNT, thereby preventing ARNT/EPAS-1 dimerization and binding to a HIF-1 site (Chin *et al.*, 2000). Interestingly, however, whereas the VEGF and VEGFR-1 (*flt*) promoters are

responsive to hypoxia, through the HIF element, the VEGFR-2 promoter is not (Gerber *et al.*, 1997). This suggests that HESR-1 is acting on this promoter through an alternative mechanism, perhaps involving direct binding to an E-box, of which there are several in the VEGFR-2 promoter. We are currently addressing this question.

In other systems, notch is involved in inductive signaling between nonequivalent cells, whereby ligand expressing cells signal to notch expressing cells to inhibit specific pathways, thus allowing progression to secondary fates (Mumm and Kopan, 2000). While it remains unclear what role notch plays in vascular development and angiogenesis it is tempting to speculate that notch–ligand interaction may determine which cells in a vessel wall respond to an angiogenic stimulus by sprouting, migrating, and forming a new vessel and which remain quiescent. A mechanism whereby sprouting cells inhibit nearby cells from the same phenotype is reminiscent of lateral inhibition (Lewis, 1998), a hallmark of notch signaling. It is not known whether there are preexisting “notch” and “ligand” cells in the vessel wall or if notch signaling is downregulated in some cells in response to angiogenic stimuli, thus allowing expression of the angiogenic EC phenotype.

In conclusion, we have shown that the notch pathway, and a downstream target, the bHLH transcription factor HESR-1, are necessary for capillary-like network formation *in vitro*. Notch activation during network formation downregulates VEGFR-2, a known target of HESR-1, and this results in decreased responsiveness of the EC to VEGF. Notch signaling, therefore, may control the responsiveness of individual cells to an angiogenic signal and thereby regulate which cells in the vessel wall sprout and which remain quiescent. Future studies will address this question directly.

ACKNOWLEDGMENTS

We thank Drs. Capobianco, Kitajewski, Kopan, Patterson, and Zimmer-Strobl for providing plasmids. This work was supported by grants from the National Institutes of Health (RO1 HL60067), the Avon Breast Cancer Foundation, and the U.S. Army Medical Research Materiel Command under DAMD 17-98-1-8291.

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