Feed-forward Signaling by Membrane-bound Ligand Receptor Circuit

THE CASE OF NOTCH DELTA-LIKE 4 LIGAND IN ENDOTHELIAL CELLS*

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Vincenza Caolo‡, Nynke M. S. van den Akker§, Sanne Verbruggen‡, Marjo M. P. C. Donners*, Geertje Swennen §§, Henny Schulten†, Johannes Waltenberger‡, Mark J. Post†, and Daniel G. M. Molin††

From the Departments of ‡Phyiology, §Cardiology, and *Molecular Genetics, Cardiovascular Research Institute Maastricht, Maastricht University, 6229 ER Maastricht, the Netherlands

The DELTA like-4 ligand (DLL4) belongs to the highly conserved NOTCH family and is specifically expressed in the endothelium. DLL4 regulates crucial processes in vascular growth, including endothelial cell (EC) sprouting and arterial specification. Its expression is increased by VEGF-A. In the present study, we show that VEGF-induced DLL4 expression depends on NOTCH activation. VEGF-induced DLL4 expression was prevented by the blockage of NOTCH signaling with γ-secretase or ADAM inhibitors in human cardiac microvascular ECs. Similar to VEGF-A, recombinant DLL4 itself stimulated NOTCH signaling and resulted in up-regulation of DLL4, suggesting a positive feed-forward mechanism. These effects were abrogated by NOTCH inhibitors but not by inhibition of VEGF signaling. NOTCH activation alone suffices to induce DLL4 expression as illustrated by the positive effect of DLL4 expression. The notion that the positive feed-forward mechanism might propagate NOTCH activation to neighboring ECs was supported by our observation that DLL4-eGFP-transfected ECs induced DLL4 expression in nontransfected cells in their vicinity. In summary, our data provide evidence for a mechanism by which VEGF or ligand-induced NOTCH signaling up-regulates DLL4 through a positive feed-forward mechanism. By this mechanism, DLL4 could propagate its own expression and enable synchronization of NOTCH expression and signaling between ECs.

The NOTCH family encompasses a fundamental signaling pathway involving four receptors (NOTCH-1, -2, -3, and -4) and, in vertebrates, five cognate ligands (DLL-1, -3, and -4, and JAGGED-1 and -2). Cell-cell contact is a prerequisite for NOTCH signaling as all members are membrane bound. The first activation step upon receptor-ligand interaction involves cleavage of the extracellular domain of the NOTCH receptor by ADAM (a disintegrin and metalloprotease). Subsequently, the γ-secretase complex instigates a second proteolytic cleavage resulting in the release of the NOTCH intracellular domain (NICD) into the cytoplasm. Next, the NICD translocates to the nucleus, where it associates with the Recombinant signal binding protein for immunoglobulin kappa J region (RBP-Jk) CBF1/RBP-Jk, Su(H), Lag1 (CSL) transcription factor to initiate the transcription of its downstream targets basic helix-loop-helix proteins HES (hairy/enhancer of split) and hairy related transcription factors (HRT, HEY, and HERP) (1).

Among the ligands, DLL4 is specifically expressed in the arterial endothelium and plays a major role during embryonic vascular development. DLL4 haploinsufficiency is embryonically lethal due to severe vascular abnormalities related to a loss of arterial and venous specification (2–7). DLL4 overexpression on the other hand induces arterIALIZATION even in the venous compartment that typically lacks NOTCH expression (8).

NOTCH signaling is important for the development of endothelial cell sprouting. In this process, VEGF induces the formation of new sprouts, whereas DLL4 signaling appears to reduce their formation (9, 10). Because DLL4 is up-regulated by VEGF (11), this signaling pathway might serve as a negative feedback loop with respect to endothelial sprouting. This feedback could be bidirectional as NOTCH reduces VEGF responsiveness through down-regulation of VEGFR-2 (12) and subsequently suppresses the sprouting phenotype in the adjacent stalk cell, thus limiting the number of sprouts. Accordingly, loss of DLL4 function promotes excessive endothelial cell sprouting and endothelial cell migration (13). VEGF-A has also been reported to induce the activation of ADAM-10 and -17 (14), which are the proteases required for the first step in NOTCH activation. This interrelation between VEGF-A and NOTCH signaling suggests that VEGF not only induces the expression of DLL4 but also stimulates NOTCH...
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signaling. However, alternative pathways have been suggested for VEGF-induced DLL4 expression. Up-regulation of DLL4 by VEGF has been considered the result of activated Forkhead box C (FOXC) transcription factors by a PI3K and ERK/MAPK-dependent pathway (15). FOXC2 has been shown to activate the DLL4 and HEY2 promoters, which both contain a forkhead binding element (FBE) (15, 16). In particular, FOXC2 functionally interacts with RBP-Jκ and NICD to activate the HEY2 promoter (15). Similarly to HEY2, the DLL4 promoter presents with multiple RBP-Jκ binding sites (17), suggesting that NOTCH might regulate DLL4 expression as well. Altogether, these observations prompted us to speculate that NOTCH signaling could play a role in up-regulating the expression of its own ligand DLL4, which in turn would activate NOTCH signal transduction, thus creating a positive feed-forward signaling that propagates the activation to adjacent cells.

In the present study, we show that in endothelial cells, VEGF-induced DLL4 up-regulation is NOTCH activation-dependent. VEGF positively affects DLL4 expression and NOTCH signaling mainly by activating VEGFR-2, whereas inhibition of NOTCH signaling abrogated VEGF-induced DLL4 expression. Consistently, immobilized recombinant DLL4 also induced endogenous DLL4 expression in a NOTCH-dependent manner unrelated to VEGF signaling, indicating a feed-forward mechanism downstream of NOTCH. The increase in DLL4, along with transactivation of the DLL4 promoter by NICD-1 and NICD-4 confirmed the role of NOTCH signaling in this process, further suggesting that NOTCH activation is sufficient for DLL4 induction. In this endothelial system, we found no evidence for FOXC2-dependent DLL4 expression by VEGF. Finally, we show that DLL4 transfected endothelial cells were able to increase DLL4 expression of surrounding cells providing a mechanism by which NOTCH signaling is distributed between communicating endothelial cells.

EXPERIMENTAL PROCEDURES

Reagents—Human cardiac microvascular endothelial cells (HCMvECs) (Lonza, Vervies, Belgium) were cultured in EGM-2MV, with 5% FBS (Lonza). Recombinant VEGF-A₁₆₅ was purchased from RELIATech GmbH (Brauschweig, Germany); DLL4 (rDLL4) extracellular domain was from R&D Systems (Abingdon, Oxfordshire, UK); L685,458 (VEGFR-1 inhibitor) (18, 19) were both kindly provided by Professor Manfred Gessler and have been described previously in Ref. 17. To obtain a minimal DLL4 promoter fragment lacking any putative RBPs-Jκ binding site the pGL3-hDLL4 MscI (−931) were kindly provided by Professor Manfred Gessler and have been described previously (23). All transfection experiments were carried out in triplicate. HCMvECs were grown on 0.2% gelatin-coated plates. Transfections were performed with Lipofectamine LTX and PLUS reagent (Invitrogen). After 24 or 48 h of culture, RNA or proteins were isolated and analyzed by RT-qPCR or Western blotting, respectively.

 Luciferase Reporter Assay—HCMvECs seeded on 0.2% gelatin in PBS-coated plates were transiently transfected with 0.6 µg of either inducible RBP-Jκ responsive firefly luciferase construct (SuperArray Bioscience Corp., Frederick, MD). After 24 h of stimulation with VEGF-A₁₆₅ or inhibitors, the cells were harvested using Dual Glo luciferase assay system (Promega, Leiden, the Netherlands), and reporter activity was measured on a PE Victor3 plate reader (PerkinElmer Life Sciences). PAECs were transiently transfected with pcDNA NICD-1, NICD-4, or vector alone along with the 6-kb DLL4 promoter firefly luciferase construct or with the deletional constructs of this promoter (−2616, −1587, −931, or −931-Deletion (D), respectively). The Renilla luciferase reporter plasmid was cotransfected as internal control for defining transfection efficiency. After 24 h of transfection, cells were harvested, and luciferase activity was assessed as reported above.

RNA Isolation and RT-qPCR and Protein Extraction—After each experiment, RNA was isolated by using RNeasy microkit Qiagen (Qiagen, GmbH, Hilden, Germany). A total of 100 ng RNA per sample was subjected to Reverse Transcriptase (RT), qPCR was performed using Superscript III™ Platinum Two-step qRT-PCR kit with SYBR green (Invitrogen) and a primer concentration of 10 µM according to Van den Akker et al. (24). The PCR primers used were as follows: human β-actin sense, 5′-ATCTCCACCTCCTGAACTCC-3′; β-actin
**RESULTS**

VEGFR2-mediated DLL4 Expression Involves NOTCH Signaling in Primary Endothelial Cells—To investigate the effect of VEGFR-1 and VEGFR-2 signaling on DLL4 expression, HCMvECs were exposed to receptor specific ligands, i.e. Placenta Growth Factor-2 (PIGF-2) for VEGFR-1, VEGF-E for VEGFR-2, and VEGF-A_{165} for both receptors (25–27). Both VEGF-A_{165} and VEGF-E increased DLL4 expression, whereas PIGF-2 had no significant effect, suggesting a predominant involvement of VEGFR-2 (Fig. 1A). In accordance, specific inhibition of VEGFR-2 by the IMC-1121b antibody (Fig. 1B) prevented VEGF-A_{165} induced DLL4 expression, whereas VEGFR-1 inhibition with IMC-18F1 had no effect (Fig. 1B). Final confirmation for the dominance of VEGFR-2 signaling came from PAEC cell lines that overexpress either the human VEGFR-1 or VEGFR-2 (22). In line with the HCMvEC results, VEGF-A_{165} induced DLL4 only in VEGFR-2 transfected but not in VEGFR-1-transfected PAECs (Fig. 1C).

VEGFA-induced DLL4 expression in HCMvECs was abrogated by blockage of NOTCH signaling with γ-secretase and ADAM inhibitors (Fig. 1D), suggesting a role for NOTCH signaling. To further substantiate that VEGF-A_{165} activated NOTCH-signaling, RBP-Jκ luciferase activity and NOTCH target gene HES-1 expression were assessed. VEGF-A_{165} induced RBP-Jκ promoter activity and HES-1 expression (Fig. 1, E and F), an effect that was prevented by NOTCH inhibition (Fig. 1, E and F).

**Activated NOTCH Signaling Induces DLL4 Expression by Activating DLL4 Promoter—NOTCH-1 and NOTCH-4 are the main mediators of NOTCH signaling in the endothelium (28, 29). To define the direct effect of NOTCH signaling on DLL4 expression, HCMvECs were transfected with the intracellular domain of NOTCH-1 and -4 (NICD-1 and NICD-4, respectively) or empty vector. Both NICD-1 and -4 induced DLL4 mRNA and protein expression (Fig. 2, A and C, lower panel). Endogenous NOTCH signaling was not required for this stimulation as NICD-1 and -4 overexpression had the same effect in the presence of γ-secretase inhibition (Fig. 2B).

The DLL4 promoter has previously been described to contain a FBE and DLL4 expression was reported to be induced by VEGFA in a FOXC-mediated manner (15, 16). Furthermore, the DLL4 promoter sequence also contains several deletional DLL4 promoter fragments (1587, one (1587-D) RBP-Jκ binding sites resulted in NICD-1 transactivation that was significantly stimulated when co-transfected with NICD-1 and -4 induced DLL4 mRNA and protein expression (Fig. 2, A and C, lower panel). Endogenous NOTCH signaling was not required for this stimulation as NICD-1 and -4 overexpression had the same effect in the presence of γ-secretase inhibition (Fig. 2B).

To discriminate between FOXC and NICD/RBP-Jκ-regulated DLL4 expression, several deletional DLL4 promoter fragments were analyzed for their transactivation activity upon NICD-1 and -4 activation in PAECs. These promoter fragments lacked the FBE site and contained either three (−2616), two (−1587), one (−931), or no (−931-D) RBP-Jκ binding site, respectively (17). The 6-kb DLL4 promoter fragment was significantly stimulated when co-transfected with NICD-1 or NICD-4 (Fig. 3A). FBE deletion in the presence of the three RBP-Jκ sites resulted in NICD-1 transactivation that was comparable with the full-length promoter. Significant transactivation was also apparent after NICD-4 overexpression, although it was less effective than NICD-1 (Fig. 3A).

The shorter −1587 and −931 constructs, containing two and one RBP-Jκ binding sites, respectively, showed reduced but still significant induction by NICD-1. Although NICD-4 transactivated the −1587 fragment that contained two RBP-Jκ binding sites, NICD-4 was unable to induce
promoter activity in the −931 DLL4 promoter fragment that presented with only one RBP-Jκ binding site. As expected, no NICD transactivation effect was observed for the −931-D fragment that lacked both the FBE and all RBP-Jκ binding sites (Fig. 3A).

Like in HCMvECs, NICD-1 and -4 overexpression (Fig. 3C) up-regulated DLL4 mRNA expression (Fig. 3B) in PAECs. Transfection of both HCMvECs and PAECs with a constitutively active FOXC2 expressing vector did not alter DLL4 expression (Fig. 3D), indicating that in endothelial cells, the FOXC2 pathway seems redundant for NOTCH-stimulated DLL4 expression.

**DLL4-induced NOTCH Signaling Up-regulates DLL4 Expression**—To address a potential DLL4-driven positive feed-forward loop, rDLL4 protein was used to stimulate the NOTCH pathway in HCMvECs. Similar to VEGFA, rDLL4 was able to increase the expression of DLL4. NOTCH signaling blockage by γ-secretase and ADAMs inhibitors prevented rDLL4-induced DLL4 expression (Fig. 4A). The activation of NOTCH signaling by rDLL4 was further assessed by RBP-Jκ luciferase promoter analysis. rDLL4 stimulation induced transactivation of RBP-Jκ promoter, which was prevented by NOTCH inhibitors (Fig. 4B). To investigate whether rDLL4-induced DLL4 expression depends on VEGFR-2 signaling, we cultured HMCvECs on rDLL4 along with specific VEGFR-2 or/and VEGFR-1 inhibitors and analyzed DLL4 expression. The inhibition of VEGFR-2, VEGFR-1, or of both receptors did not affect rDLL4-induced DLL4 expression suggesting that VEGF signaling is not required for the positive feed-forward circuit (Fig. 4C).

**DLL4 Induces Propagation of Endogenous DLL4 Expression in Adjacent Cells**—As NOTCH is an intercellular signaling pathway, we hypothesized that NOTCH-induced DLL4 expression taking place in one specific cell would eventually induce NOTCH signaling and subsequently DLL4 expression in its neighboring cell. To test this hypothesis, HCMvECs were transfected with pIRE2-eGFP or pDLL4IRES2-eGFP with or without NOTCH signaling inhibitor (L685,458), and DLL4 expression was analyzed by immunofluorescence staining. The eGFP (green) signal allows to discriminate between...
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**FIGURE 2.** NICD-1- and NICD-4-induced DLL4 expression in HCMvECs. A and B, DLL4 mRNA expression in HCMvECs transiently transfected with NICD-1, NICD-4, or pcDNA empty vector as control with or without L685,458. Data represent mean ± SE (n = 3). One-way ANOVA test was used. *, significantly different from control (mock) p < 0.05. C, Western blot analysis of protein extracts from HCMvECs transfected with NICD-1 and NICD-4. Membranes were probed with anti-NOTCH-1 (upper left panel), anti-NICD-4 (upper right panel), or anti-DLL4 (lower panel).

affected and nontransfected cells (Fig. 5, A–C, GFP). pIRES2-eGFP-transfected control cells presented relatively weak DLL4 expression (red staining) in their surrounding cells (Fig. 5A, DLL4). In contrast, the pDLL4IRES2-eGFP-transfected cells revealed high DLL4 levels, and prominent DLL4 staining was also detected in the nontransfected surrounding cells (Fig. 5B, DLL4). The pDLL4IRES2-eGFP expressing cells treated with L685,458 had high DLL4 expression; however, no DLL4 was detected in the surrounding nontransfected cells (Fig. 5C, DLL4) indicating that inhibition of NOTCH signaling was sufficient to prevent the DLL4-induced positive feed-forward loop.

**DISCUSSION**

The VEGF and NOTCH signaling pathways and their intricate interactions are indispensable for regulating blood vessel formation. VEGF-A has been reported to modulate the expression of NOTCH members in arterial endothelial cells. VEGFR-2 acts as the main receptor in mediating VEGF signaling in vascular endothelial cells (22, 30, 31) and likewise has been suggested to positively regulate NOTCH/Delta expression (10, 11, 21, 24, 32). Also a reciprocal effect of NOTCH signaling on the expression of VEGFR-2 has been reported, i.e. a reduction in VEGFR-2 and an increase in VEGFR-1 expression with subsequent drop in VEGFA responsiveness (32).

Accordingly, we show that in primary adult HCMvECs, the expression of DLL4 is stimulated by the VEGFR-2 acting ligands VEGF-A165 and VEGF-E, and not by PlGF-2. Intriguingly, no other NOTCH ligands beside DLL4 were affected by VEGF-A-related endothelial signaling (data not shown) suggesting a selectivity of VEGF-A-related signaling in primary adult microvascular ECs. The essential role of VEGFR-2 was confirmed in porcine aortic endothelial cells expressing only one of the receptors and with a specific VEGFR-2 inhibitor. The presented data clearly show that up-regulation of DLL4 by VEGF-A is NOTCH signaling-dependent. VEGF-A failed to induce DLL4 expression when NOTCH signaling was inhibited, indicating the requirement of activated NOTCH for this process. In addition, NOTCH signaling alone was sufficient to induce DLL4 expression as shown by NICD-1 and NICD-4 overexpression. A comparable effect of VEGF-A-induced DLL4 expression was described for embryonic (33) and human bone marrow-derived mesenchymal stem cells (4).

We show that both NICD-1 and NICD-4 were able to transactivate the DLL4 promoter. Previous reports (15, 16) indicated that the FOX2 transcription factor can serve as a mediator for VEGF-A-induced DLL4 expression by interacting with a FBE located in the DLL4 promoter. Besides the FBE, multiple RBP-Jk binding sites are present in the DLL4 promoter sequence, providing the potential molecular cues for NOTCH-induced expression. Indeed, NICD-1 effectively transactivated both the full-length DLL4 promoter and FBE-deleted DLL4 promoter sequences, providing evidence that the FOX transcription factor pathway does not mediate VEGF-induced NOTCH activation in the endothelium. Accordingly, overexpression of a constitutively active FOXC2 transcript did not increase DLL4 expression. The FBE lacking −2616, −1587, and −931 promoter constructs showed a transactivation profile that suggests dependence on the number of RBP-Jk binding sites. As expected, both NICD-1 and -4 were unable to stimulate the −931 DLL4 promoter fragment that lacked any RBP-Jk binding site. NICD-4 had a similar profile of transactivation as NICD-1 with respect to −2616 and −1587 fragments but, in accordance with previous studies, at lower levels (34). This difference likely relates to the presence of a C-terminal autonomous transactivation domain in the NICD-1, which acts independently of RBP-Jk. Such a transactivation domain sequence is absent in the NICD-4, and consequently, it requires RBP-Jk to initiate transcription (35). Therefore, the reduced induction capacity of NICD-4 is likely related to the number of RBP-Jk that are present in the shortened −2616 and −1587 DLL4 promoter fragments. Accordingly, no effect was shown by NICD-4 for the −931 DLL4 promoter...
fragment that contained only one RBP-J binding site, whereas NICD-1 could still effectively transactivate this promoter.

NOTCH is typically activated by one of its ligands, including membrane-bound DLL4. Therefore, similar to VEGF-A, NOTCH activation by surface-bound recombinant DLL4 induced DLL4 expression in HCMvECs. Inhibition of NOTCH signaling, but not of VEGF signaling, was sufficient to prevent this induction. These data indicate that activated NOTCH can induce DLL4 expression and transactivate the DLL4 promoter in absence of FOXC transcription factors and support the observation that NOTCH can induce DLL4 expression independently of FOXC2-mediated VEGF signaling. To our knowledge, these results show for the first time that DLL4 induces its own expression in endothelial cells, most likely through direct NICD-mediated transcriptional regulation. VEGF signaling also induced the up-regulation of HES-1 and RBP-Jk transactivation, indicating that VEGF-A activates NOTCH signaling. This activation is likely not secondary to ligand overexpression, as NOTCH inhibition prevented the VEGF-A-induced expression of DLL4.

The link between VEGF-A and NOTCH signaling most likely relates to VEGFR2 increased ADAM expression and activity. In a previous study, we showed that ADAM expression and protease activity (i.e. sheddase) in HUVECs and VEGFR-2/PAEC was increased upon VEGF-A165 stimulation (36). VEGF-A has also been reported to induce the expression of ADAM-10 and to enhance shedding of VEGFR-2 and its co-receptor NRP1 by activating ADAM-10 and ADAM-17, respectively (14). Although this mechanism has been postulated to modulate VEGF-A signaling (14), VEGF-A increased ADAM sheddase activity might potentiate NOTCH signaling as well. In line with our observations, Hainaud et al. (37)
showed an accumulation of cleaved NOTCH-4 in VEGF-A-treated HUVECs with concomitant up-regulation of the ADAM and presinilin (part of the γ-secretase complex) expression.

The involvement of ADAM-10 and ADAM-17 in NOTCH processing and signaling has been unequivocally established (38–42). ADAM-10 mutant mice phenotypically resemble Notch-1 null mutants and die at embryonic day 9.5, whereas Adam-17 mutants are viable (43). Among others Bozkulak and Weinmaster (41) showed that NOTCH-1 is a substrate for both ADAM-10 and ADAM-17 and, although ADAM-10 was essentially required for ligand induced NOTCH-1 signaling, ADAM-17 mediated ligand independent signaling. The importance of ADAM in NOTCH signaling was also reflected by our experiments showing that inhibition of ADAM activity abrogated the VEGF-A induced expression of DLL4 and the NOTCH transcription factor HES-1. In addition, rDLL4-induced DLL4 expression was depressed by blockade of ADAM activity as well. Altogether, these data indicate that VEGF-A activates ADAM, which is required for NOTCH signaling activation.

The ability of the activated NOTCH receptor to specifically induce the expression of its ligand would provide endothelial cells a mechanism by which NOTCH signaling is distributed and propagated between communicating cells with initially a limited amount of ligand. A comparable positive feed-forward mechanism has recently been described to regulate the interaction between endothelial and mural cells. JAGGED-1 ligand expressing endothelial cells induced NOTCH-3 signaling activation in mural cells (44), that in turn promote JAGGED-1 expression. In this way, mural cells can interact more efficiently with endothelial cells and can activate NOTCH signaling of neighboring cells, maintaining and distributing the signal to other mural cells. In the setting of membrane-bound ligands, the positive feed-forward mechanism is especially attractive to expand the signal to distant cells. The regulation of ligand expression by NOTCH receptors has already been described in Drosophila (45). NOTCH signaling was found to increase the expression of DELTA and SERRATE through a positive feedback loop during the regulation of the dorsoventral boundary of the developing wing (45). Likewise, in vertebrates, DELTA-like ligands are required during somitogenesis for the maintenance of somite borders and accumulate in a NOTCH-dependent manner (46–48).

The expression pattern of DLL4 ligand in arteries and sprouting vessels affects spatial distribution of NOTCH sig-

![Figure 4](image-url)
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During sprouting, tip endothelial cells will express high DLL4 levels and low NOTCH activity, whereas their neighbor stalk cells will have high NOTCH activity but low DLL4 expression. However, a static situation in which a subpopulation of cells presents high level of DLL4 and low NOTCH signaling is unlikely. NOTCH signaling is in fact required for arterial specification, and lack or reduction of NOTCH signaling would cause loss of arterial identity. Here, we show, that in contrast to eGFP-transfected cells, DLL4-eGFP ECs were able to induce DLL4 expression of non-transfected surrounding ECs, through a feed-forward mechanism, that was NOTCH signaling-dependent. Coordinated activation of NOTCH signaling would produce a wave of DLL4 expression that provides periodic NOTCH signaling activation in each cell (9). Such a mechanism could be important to maintain an arterial phenotype along a blood vessel and for regulating spatial patterning of branching as well (9, 49).

In summary, stimulation of VEGFR-2 by VEGF-A165 up-regulates DLL4 expression by activating NOTCH signaling in HCMvECs, independently of FOXC2. The NOTCH involvement in regulating DLL4 expression indicates the existence of a feed-forward mechanism by which NOTCH signaling can be propagated between adjacent endothelial cells.

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