Laminin-Binding Integrins Induce Dll4 Expression and Notch Signaling in Endothelial Cells

Soline Estrach, Laurence Cailleteau, Claudio Areias Franco, Holger Gerhardt, Caroline Stefani, Emmanuel Lemichez, Laurent Gagnoux-Palacios, Guerrino Meneguzzi, Amel Mettouchi

Rationale: Integrins play a crucial role in controlling endothelial cell proliferation and migration during angiogenesis. The Delta-like 4 (Dll4)/Notch pathway establishes an adequate ratio between stalk and tip cell populations by restricting tip cell formation through “lateral inhibition” in response to a vascular endothelial growth factor gradient. Because angiogenesis requires a tight coordination of these cellular processes, we hypothesized that adhesion, vascular endothelial growth factor, and Notch signaling pathways are interconnected.

Objective: This study was aimed at characterizing the cross-talk between integrin and Notch signaling in endothelial cells.

Methods and Results: Adhesion of primary human endothelial cells to laminin-111 triggers Dll4 expression, leading to subsequent Notch pathway activation. SiRNA-mediated knockdown of α2β1 and α6β1 integrins abolishes Dll4 induction, which discloses a selective integrin signaling acting upstream of Notch pathway. The increase in Foxc2 transcription, triggered by α2β1 binding to laminin, is required but not sufficient per se for Dll4 expression. Furthermore, vascular endothelial growth factor stimulates laminin γ1 deposition, which leads to integrin signaling and Dll4 induction. Interestingly, loss of integrins α2 or α6 mimics the effects of Dll4 silencing and induces excessive network branching in an in vitro sprouting angiogenesis assay on three-dimensional matrigel.

Conclusions: We show that, in endothelial cells, ligation of α2β1 and α6β1 integrins induces the Notch pathway, and we disclose a novel role of basement membrane proteins in the processes controlling tip vs stalk cell selection. (Circ Res. 2011;109:172-182.)

Key Words: adhesion molecules ■ angiogenesis ■ signaling pathways

Angiogenesis involves extracellular matrix (ECM) remodeling, sprouting, migration, and proliferation, leading to the shaping of a ramified network. Endothelial cells (EC) are surrounded by a complex ECM microenvironment that constitutes a scaffold for vascular endothelial growth factor (VEGF) gradient patterning and bioavailability. Apart from its structural and reservoir function, ECM also provides positional information to cells for the outgrowth and maturation of a nascent sprout. Cues from ECM proteins are transmitted to cells by integrin adhesion receptors, which are transmembrane α and β heterodimers that convey signals regulating EC migration, proliferation, gene expression, and survival. Once bound to their ECM ligand, integrins form clusters and recruit signaling and structural molecules that couple to the actin cytoskeleton and organize focal adhesion sites. Although the role of ECM in supporting migration, proliferation, and survival is well-established, its specific role in regulating morphogenetic events remains unclear.

The Notch signaling pathway involves interaction between 2 adjacent cells, one expressing a ligand (either Delta or Jagged) and the other expressing Notch receptor. Such cell interaction results in a proteolytic cleavage cascade releasing the Notch intracellular domain, which translocates to the nucleus and activates transcription of target genes such as Hes and Hey. Whereas the ligands Delta-like 1 (Dll1), Delta-like 4 (Dll4), and Jagged 1 are expressed in EC, only Notch 1 and Notch 4 receptors are expressed. In EC, the Notch pathway regulates shaping of a branched vascular network by establishing differential capacity of EC to respond to VEGF stimulation.

VEGF is a key regulator of vasculogenesis and angiogenesis, and it signals via 3 distinct receptors: VEGF receptor (VEGFR)-1, VEGFR-2, and VEGFR-3. VEGF exerts multiple effects on EC, including proliferation, survival, migration, and tip cell sprouting. VEGF induces Dll4 expression downstream of VEGFR-2 activation. Expression of VEGF receptors is in turn regulated by Notch signaling. VEGFR-1 is
induced upon Notch activation,\textsuperscript{13} whereas VEGFR-2 expression is repressed.\textsuperscript{13,14} VEGFR-1 negatively regulates VEGFR-2 signals, and opposite modulation of their expression contributes to reduce or spatially restrict the response of EC to VEGF. VEGF modulates integrin affinity and expression,\textsuperscript{15,16} and a reciprocal activation of VEGFR-2 and $\alpha_v\beta_3$ integrin controls EC migration.\textsuperscript{17} Nevertheless, a role of integrins downstream of VEGF signaling for controlling vessel branching has not been assessed.

Here we show that in EC, laminin-binding integrins specifically induce Dll4 expression. This requires combined $\alpha_2\beta_1$ and $\alpha_6\beta_1$ integrin signaling and involves induction of the Foxc2 transcription factor. Forced expression of Foxc2 in cells cultured on fibronectin was not sufficient to induce high Dll4 levels. Induction of Dll4 expression in EC contacting laminin occurs independently of VEGF signaling and leads to Notch pathway activation. Interestingly, VEGF treatment of EC stimulates laminin-$\gamma_1$ production, leading to integrin signaling and Dll4 induction. Dll4 expression is also detected when EC are cultured on 3-dimensional matrigel (Basement Membrane Matrix), a model for branching morphogenesis. Furthermore, decreasing Dll4 or integrin expression levels

---

**Non-standard Abbreviations and Acronyms**

- **Dll4**: Delta-like 4
- **EC**: endothelial cell
- **ECM**: extracellular matrix
- **Fn**: fibronectin
- **Lm**: laminin-111
- **VEGF**: vascular endothelial growth factor
- **VEGFR**: vascular endothelial growth factor receptor

---

**Figure 1.** Dll4 expression is induced in endothelial cells following adhesion on Laminin-111 or matrigel. A and B, Levels of Delta-like 4 (Dll4) in cells grown on different extracellular matrix (as indicated) were analyzed by quantitative polymerase chain reaction and Western blot after 4 hours of adhesion. C and D, Delta-like 1 (Dll1) and Jagged1 mRNA levels were measured by quantitative polymerase chain reaction. Graphs represent fold induction \pm SD compared with T0 (cells in suspension, n=3; ***P<0.0001). Human umbilical vein endothelial cells were seeded on three-dimensional matrigel lysates and RNA were collected over network formation time course and Dll4 levels were measured by quantitative polymerase chain reaction (E) or Western blot (F). Graph represents fold induction \pm SD compared with T0 (n=3; ***P<0.0001; *P<0.01). G and H, Notch1 and Notch4 mRNA levels were measured by quantitative polymerase chain reaction in cells grown on different extracellular matrix. Graphs represent fold induction \pm SD compared with T0 (n=3).
using SiRNA leads to increased branching of EC network in a situation resembling the in vivo phenotype of Dll4–Notch pathway inhibition.

This study shows that ECM is important for endothelial sprouting by promoting, via selective integrin signaling, the Notch ligand Dll4 expression. Moreover, that VEGF-stimulated expression of Dll4 involves laminin production, and integrins ligation underscores a new mode of action of this angiogenic factor to control sprouting. Overall, we disclose the cross-talk between a cell–ECM-mediated signaling and the cell–cell signaling that governs endothelial tip vs stalk cell selection.

Methods
An expanded Methods section is available in the Online Data Supplement available at http://circres.ahajournals.org, including detailed cell culture conditions, list of material, oligonucleotides and constructs used, reverse-transcription polymerase chain reaction, immunoprecipitation, Flow cytometry, BrdUrd incorporation, and Western blotting protocols.

Results
Cell Adhesion on Laminin-111 Induces Dll4 Expression
To investigate the role of ECM components in signaling events leading to the establishment of endothelial tip and stalk cell subpopulations, we analyzed Dll4 expression in response to adhesion of primary human umbilical vein EC (HUVEC) to individual ECM proteins. We specifically detected Dll4 mRNA induction after adhesion on laminin-111 (Lm), peaking to 6-fold after 6 hours (Figure 1A). No significant induction of Dll4 was observed after cell adhesion on fibronectin (Fn), collagen type I, and collagen type IV.
indicates specificity for Lm-induced signals. Induction of Dll4 by Lm was confirmed by Western blot (Figure 1B). The specific upregulation of Dll4 expression was also observed in aortic EC contacting Lm, indicating that this regulation occurs in arterial cells as well (Online Figure 1A). Interestingly, neither Dll1 nor Jagged1 expression was induced after adhesion on ECM (Figure 1C and 1D). HUVEC cultured on 3-dimensional matrigel, which is mainly composed of laminin and collagen, form a branched network, representing an in vitro model of sprouting angiogenesis. Dll4 expression monitored during network formation showed 2-fold and 5-fold mRNA induction at 4 and 6 hours, respectively. Once the network established, at 8 to 10 hours, mRNA induction declined to 1.5-fold (Figure 1E). Dll4 protein expression was confirmed by Western blot (Figure 1F). Interestingly, a modest variation of Jagged1 and a late induction of Dll1 mRNA expression were measured during network formation, suggesting expressions ensuing from morphogenic-associated signaling events (Online Figure 1B). The expression of Notch1 and Notch4 receptors was not modulated by adhesion on different ECM components (Figure 1G and 1H). In conclusion, specific adhesion of HUVEC on Lm induces the selective expression of the Notch ligand Dll4.

**Notch Pathway Is Activated After Dll4 Induction**

To measure Notch pathway activation after cell adhesion on Lm, we monitored induction of the target genes *Hes* and *Hey*. Our results show that *Hey1* and *Hey2* (Figure 2A) were specifically induced, whereas no significant induction was seen in cells seeded on other substrates. *Hey1* and *Hey2* expression was also detected in the 3-dimensional matrigel assay (Figure 2B), which demonstrated that induction of Dll4 correlates with Notch signaling activation. To confirm that *Hey1* and *Hey2* inductions are attributable to Dll4 activation of Notch pathway, we knocked down Dll4 in EC. In absence of Dll4 expression, *Hey1* and *Hey2* induction was strongly impaired (Figure 2C, Online Figure II A). Furthermore, treating cells with the pharmacological inhibitor of Notch signaling DAPT also impaired *Hey1* and *Hey2* expression in our model (Online Figure II B). These results show that the Notch pathway is activated after Dll4 induction on Lm.

**Dll4 and a2 Integrins Cooperate to Regulate EC Proliferation**

Absence of Notch signaling or decrease in Dll4 expression leads to EC hyperproliferation. Integrin α2β1 serves as an Lm receptor in EC and signals for cell proliferation arrest. We investigated whether Dll4 plays a role downstream of α2 integrin to control EC proliferation. We either overexpressed or knocked down Dll4 and assessed proliferation ability of EC plated on either Fn, which binds α5 integrin, or Lm (Figure 3A and 3B). As previously reported, EC proliferated on Fn but remained quiescent on Lm (Figure 3C and 3D). Interestingly, mimicking the effect of Lm, Dll4 overexpression induced arrest of proliferation in cells plated on Fn (Figure 3C). Conversely, knocking-down Dll4 expression partially rescued cell proliferation on Lm (Figure 3D), whereas no effect was observed in cells on Fn. Because Dll4 knockdown allows regain of proliferation capacity but no gain of function when α2β1 integrin is not bound to its ligand, these results suggest that Dll4 induction contributes to the cell proliferation arrest imposed by Lm and α2β1.

**Lm-Induced Dll4 Expression Requires Integrin Signaling**

In HUVEC, Lm-binding integrins α2, α3, and α6 are expressed but differently contribute to adhesion, with α2 integrin being the main adhesion receptor to Lm. To identify the integrins involved in Lm-induced Dll4 expression, each α subunit was selectively knocked-down. Because α2 is critical for Lm adhesion, α2 knocked-down cells were plated on a mixed matrix (Fn + Lm), which allows cell adhesion and Dll4 expression (Figure 1B). In absence of α2, Dll4 induction was strongly impaired (82% reduction), which demonstrates that Dll4 induction upon adhesion to Lm requires α2β1 integrin (Figure 4A). Interestingly, knocking down α6 reduces from 50% Dll4 induction in cells on Lm, whereas α3 knockdown had no effect (Figure 4B and 4C). We also tested the effect of Notch pathway inhibition on Dll4 levels. Treating cells with DAPT partially impaired Dll4 expression, indicating a positive feedback loop of Dll4 on its own expression via Notch signaling (Figure 4D). We thus establish for the first time to our knowledge a functional link between α2β1/α6β1 ligation to Lm and induction of Dll4 expression in EC.

We next studied localization of α2 and α6 integrins in a three-dimensional embryoid body model in which mouse embryonic stem cells differentiate and form well-established tip cells heading sprouting capillaries. As shown in Figure 4E, α2...
localized in EC but not in pericytes (arrow), whereas α6 was expressed in both cell types. Integrins labeled the overall surface of EC and intense staining was observed for α2 in filopodia projections of tip cells (arrow heads). Such localization is in agreement with a role of these receptors in the sprouting process.

**Lm Induces Dll4 Expression via Foxc2-Dependent Transcription**

To identify the signaling involved in Dll4 induction downstream of integrins, we investigated whether Dll4 mRNA increase requires de novo transcription, as opposed to regulation of mRNA stabilization. Cells were seeded on Fn or mixed matrix (Fn+Lm) in presence of actinomycin D to interfere with transcription initiation. Actinomycin D treatment impaired the induction of Dll4 mRNA after cell adhesion (Figure 5A), thereby implying a transcriptional mechanism. VEGF promotes transient dissociation of the Tel-CtBP transcriptional repressor, resulting in Dll4, sprouty-4, and VE-cadherin expression, together with 16 other genes. We investigated whether signaling by Lm-binding integrins could interfere with Tel-CtBP to derepress Dll4 transcription. The gene profile defined for Tel-CtBP inactivation was analyzed in HUVEC cultured on Lm. Although Dll4 and Hey1 were induced, we did not find a correlation with the genes tested, thereby ruling out a major contribution of this mechanism (Online Figure IIIA). Sequence analysis of the human Dll4 promoter revealed numerous putative transcription factor-binding sites, including transcription factors involved in vascular development such as LEF/TCF, Tal1, Ets, and forkhead family members. It is known that a combinatorial regulation by Ets and forkhead transcription factors tightly controls endothelial gene expression and that Foxc2, a forkhead member, plays a key role in vascular development. Foxc2 directly binds the Dll4
promoter and induces Dll4 expression in arterial EC. To determine whether Foxc2 is involved downstream of integrin signaling, we monitored its expression by quantitative polymerase chain reaction. Foxc2 was transiently and specifically induced by Lm within 2 hours of adhesion (Figure 5B). Importantly, Foxc2 knockdown impaired Dll4 induction on Lm, which demonstrates its requirement for Lm-induced Dll4 expression (Figure 5C, Online Figure IIIB). To determine whether Foxc2 was sufficient to induce Dll4 expression, we analyzed the consequence of its overexpression in cells plated on Fn (Figure 5D, Online Figure IIIC). The transfection of Foxc2 slightly enhanced expression of Dll4 in cells on Fn at early time points but did not allow reaching of the amounts of Dll4 detected in control cells after 4 hours of adhesion on Lm. These data indicate that Lm conveys an additional signal that cooperates with Foxc2 to express optimal levels of Dll4.

To disclose the mechanism of Foxc2 expression, α2 and α6 integrins were selectively knocked-down. In absence of α2 expression, Foxc2 induction by Lm was lost (Figure 5E, Online Figure IIID), whereas α6 depletion had no impact on Foxc2 levels but blocked Dll4 mRNA induction (Figure 5F, Online Figure IIIE and IIIF). Taken together, these results indicate that Foxc2 is required for Dll4 expression and that its induction by Lm is controlled by α2β1 integrin, whereas α6β1 contributes to Dll4 expression by a distinct mechanism. Thus, α2β1 and α6β1 signaling converge to Dll4 expression via nonredundant signaling pathways.

**VEGF Induces Lm Deposition Leading to Subsequent Dll4 Expression**

VEGF signaling induces Dll4 expression during postnatal angiogenesis in the retina. In arterial endothelium, VEGF-A triggers Dll4 expression through PI3K and ERK
signaling in collaboration with forkhead transcription factors.\textsuperscript{12,28,29} VEGFR-2 is mainly responsible for the signaling inducing Dll4 expression.\textsuperscript{10} VEGF-A binds to VEGFR-2 and triggers receptor autophosphorylation.\textsuperscript{11} To assess whether integrin engagement activates VEGFR by coclustering into focal adhesions, HUVEC were plated on either Fn or Lm, and phosphorylation of VEGFR-2 or VEGFR-1, together with induction of Dll4, was monitored. VEGF treatment was the positive control. Neither Fn nor Lm activated VEGFR-2 and VEGFR-1 (Figure 6A, Online Figure IVA) and, accordingly, blocking antibody against VEGFR-2 had no effect on Lm-induced expression of Dll4 (Figure 6B), which demonstrates an induction independent from VEGF signaling.

VEGF-A stimulates tip cell formation in vivo;\textsuperscript{18,21,30} in vitro, it stimulates Dll4 expression within 24 hours.\textsuperscript{12} To investigate the idea that VEGF could trigger a cascade of events leading to ECM remodeling around the future sprout, EC were plated on plastic and stimulated with VEGF-A. After 4 days in culture medium, cells and extracellular matrices were collected and analyzed for ECM protein content. Interestingly, cells treated with VEGF produced and layered-down higher amounts of laminin-\textgamma1 than untreated controls, and induction of Dll4 was detected (Figure 6C). Knocking-down laminin-\textgamma1 in cells submitted to 4-day VEGF stimulation strongly impaired Dll4 induction (Figure 6D). Similar effect was seen after silencing integrins \textalpha2 or \textalpha6, which reveals their involvement in the VEGF signaling cascade leading to Dll4 induction in vitro (Figure 6E and 6F, Online Figure IVB). Interestingly, mice overexpressing Dll4 present increased laminin production and deposition in the dorsal aorta.\textsuperscript{31} Thus, in our model, VEGF could stimulate laminin production in cells via direct VEGFR-2 signaling and as a consequence of induction of Notch signaling.

Tight Regulation of Dll4 Level Influences Endothelial Network Ramification In Vitro

Notch pathway inhibition in EC results in increased sprouting and branching of blood vessels.\textsuperscript{18–21} Moreover, Dll4/Notch signaling restricts tip cell formation in response to a VEGF gradient, thus establishing an adequate ratio between stalk and tip cells.\textsuperscript{18,21,30} In this study, we disclose the interaction between three major signaling pathways involved in angiogenesis: VEGF, Notch, and integrins. To illustrate the role of this interaction, the effect of Dll4 or integrins deletion on in vitro vascular network branching in three-dimensional matrigel was analyzed by scoring branching points during a time course. Interestingly, knocking-down Dll4 expression increased network ramification (Figure 7A and 7B, Online Figure VA), which is in agreement with the increased ramified retinal vasculature observed in Dll4\textsuperscript{-/-} mice.\textsuperscript{18,19,21} Knocking-down either \textalpha2 or \textalpha6 integrin enhanced network formation by raising the number of branching points (Figure 7C, Online Figure VB), which is in agreement with the downregulation of Dll4 expression observed in mice overexpressing Dll4.

Figure 6. Vascular endothelial growth factor (VEGF), integrin, and Notch signaling pathways are connected. Cells were plated on fibronectin or laminin-111 and stimulated with 10 ng/mL VEGF during 4 hours (A) or simultaneously treated with a VEGFR-2 blocking antibody (IgG89106) or mouse IgG1 (B). Lysates were immunoprecipitated using a VEGFR-2 antibody when indicated (Ip) and analyzed by Western blot with indicated antibodies (n=3). C, Human umbilical vein endothelial cells (HUVEC) were kept in culture for 4 days with 10 ng/mL VEGF. Secreted proteins (media), extracellular matrix layered to the plate (matrix), and cell lysates were collected and subjected to Western blot using indicated antibodies (n=3). HUVEC transfected with control siRNA (si Ctrl) or siRNA against laminins (si lam \textgamma1; D), integrin \textalpha2 (si int \textalpha2; E), or integrin \textalpha6 (si int \textalpha6; F) were cultured for 4 days with 10 ng/mL VEGF. Cell lysates were collected and Dll4 level was analyzed by Western blot.
in absence of α2 or α6 integrins. Similar results were obtained in presence of VEGF (Figure 7D), thus reinforcing the link between VEGF, integrins, and Notch pathways that we have unraveled.

Discussion

Angiogenesis requires complex coordination of cell proliferation, migration, and cell–cell signaling. Our study provides molecular insights showing that integrins cross-talk with the pathways controlling tip and stalk cells establishment, the VEGF and Dll4-Notch pathways, and therefore could signal to control the endothelial morphological processes involved during sprouting angiogenesis. Specifically, we provide evidence that the laminin-binding α2β1 and α6β1 integrins act upstream of the Notch pathway and that VEGF stimulation of EC increases laminin production and Dll4 expression. Thus, we hypothesize that, via VEGFR and eventually Notch signaling, VEGF induces laminin production and deposition by EC. In turn, specific production of laminin leads to integrin-dependent signaling, which induces Dll4 expression and Notch pathway activation. Such a regulatory cascade, linking VEGF, integrin, and Notch signaling, participates to regulate tip cell/stalk cell balance (Figure 8).

ECM Specifically Regulates Subsets of Notch Endothelial Ligands

In the vascular system, the Notch pathway is required for induction of arterial cell fate during development and selection of endothelial stalk vs tip cells during sprouting angiogenesis. For both functions, Dll4 ligand is required whereas Dll1 or Jag1 play distinctive roles. We have established that specific stimulation of EC by Lm induces Dll4 expression. Interestingly, none of the two other Notch ligands expressed
by EC, DI1, and Jag1 were induced by Lm. During arterial fate specification, DI1 expression is crucial in maintaining arterial identity marker expression and enhancing arterial cell responsiveness to VEGF by upregulating expression of VEGFR2 and NRP1.32,33 That Lm does not control DI1 expression implies that Lm–integrin control of Notch signaling via DI4 expression is involved in the control of tip–stalk balance. Thus, although DI4 induction in EC cultured on Lm (this study) or on VEGF stimulation for arterial specification36,37 is controlled by a common transcription factor, FoxC2, Lm also signals by specific molecular partners to set-up a selective Notch response in EC. Using endothelial-specific conditional knockout mice for Jag1, Benedito et al34 recently demonstrated that Jag1 antagonizes DI4 effects during sprouting angiogenesis. Jag1 is strongly expressed in stalk cells, whereas DI4 expression is higher in tip cells. The different expression and localization of these two Notch ligands allow the establishment of a regulation loop in which a strong activation of Notch in the stalk cell occurs and, in return, the fringe modification of Jagged1-activated Notch leads to a downregulation of the Notch activity in the leading cell.

Our finding that laminin-binding integrins signal exclusively for DI4 expression makes it tempting to anticipate that a role of this pathway in vivo would be to control relative amounts or spatial localization of different Notch ligands, or both. Laminin-binding integrins could signal to control the temporal extent and the amplitude of DI4 expression to reinforce the competitive advantage of tip cells and contribute to their persistence at the tip position in the sprout.25

### Connections Between Adhesion and Notch Signaling in EC

Lines of evidence involve Notch signaling in the control of cell adhesion because expression of Notch intracellular domain leads to activation of β1 integrins and stimulates cell adhesion.41,42 Also, laminin deposition by tip cells has been reported both in vitro in an embryoid body model36 and in vivo, together with the basement membrane components Nidogen-1 and Nidogen-2.43 Our mechanistic analysis now provides original cues that establish an active role of laminin and its receptors α2β1 and α6β1 integrins in signaling for the control of Delta/Notch pathway. Therefore, besides playing a role in cell migration and in vessel scaffolding and stabilization, basement membrane components appear to be directly involved in the regulation of vessel branching. Moreover, our results unveil the complex and intricate mode of cooperation between Notch and adhesion molecules. In this respect, Brutsch et al44 recently identified ICAP-1 as a novel negative regulator of angiogenesis and showed that this function is mediated by CCM1-mediated activation of Notch signaling. Because ICAP-1 is a protein regulating integrin affinity,45 its action in angiogenesis could also involve integrins.

In conclusion, our study of EC places laminin-binding integrins upstream of the Notch pathway by inducing a transcriptional program leading to the expression of DI4 and activation of the Notch pathway. Our data also suggest that VEGF regulates ECM composition to stimulate these integrins, thereby impacting on the balance between tip and stalk cells selection at an additional level.
Disclosures
None.

References

Novelty and Significance

What Is Known?

- Angiogenesis is a complex process that involves extracellular matrix remodeling, “tip/stalk” population specification, and migration and proliferation of endothelial cells.
- Integrins play a crucial role in controlling proliferation and migration in endothelial cells.
- Delta-like 4 (Dll4)/Notch participate in the establishment of an adequate balance between tip and stalk cells during angiogenic sprouting.

What New Information Does This Article Contribute?

- Adhesion of endothelial cells to laminin induces Dll4 expression and activates Notch pathway.
- The alpha2beta1 and beta6beta1 integrins, receptors of laminin, specifically trigger intracellular signaling pathways leading to Dll4 induction.
- Induction of Dll4 by laminin requires Foxc2-dependent transcription.
- Vascular endothelial growth factor (VEGF) stimulates laminin deposition by endothelial cells, leading to specific integrin signaling and subsequent Dll4 expression.

Because angiogenesis requires a fine regulation of cell proliferation, migration, and tip vs stalk cell balance, we hypothesized that adhesion, VEGF, and Notch pathways are interconnected.

Adhesion to laminin triggers a specific induction of Dll4 expression in endothelial cells, leading to Notch signaling activation. Loss-of-function studies reveal that a selective integrin signaling, involving alpha2beta1 and beta6beta1, is acting upstream of the Dll4/Notch pathway in endothelial cells. Laminin-induced Dll4 expression requires a transcription-dependent mechanism. Specifically, we show that increase in Foxc2 expression and transcriptional activity, triggered by alpha2beta1, is required for Dll4 expression. Furthermore, the proangiogenic factor VEGF stimulates laminin deposition, which leads to integrin signaling and Dll4 expression in cultured endothelial cells.

In conclusion, our work provides the original notion that the extracellular matrix and integrin-mediated signaling modulate Notch signaling and play a central role in determination of the stalk vs tip identity in endothelial cells.

Such novel insights into a matrix-dependent regulation of the endothelial VEGF–Notch signaling during sprouting angiogenesis open perspectives for the development of novel therapeutic approaches.
Laminin-Binding Integrins Induce Dll4 Expression and Notch Signaling in Endothelial Cells

Soline Estrach, Laurence Cailleteau, Claudio Areias Franco, Holger Gerhardt, Caroline Stefani, Emmanuel Lemichez, Laurent Gagnoux-Palacios, Guerrino Meneguzzi and Amel Mettouchi

*Circ Res.* 2011;109:172-182; originally published online April 7, 2011; doi: 10.1161/CIRCRESAHA.111.240622

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/109/2/172

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/04/07/CIRCRESAHA.111.240622.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/
Laminin binding integrins induceDll4 expression and Notch signaling in endothelial cells

Estrach, S. et al.

**Supplemental material:**

**Cell culture and transfection**

Primary Human umbilical vein endothelial cells (HUVEC) were cultured as previously described. All experiments were performed using G₀ synchronized cells in Serum Free Medium (Human endothelial SFM, Gibco) supplemented with EGF 10 ng/ml, heparin 1 µg/ml, FGF-2 20 ng/ml and ITS+1 (sigma-aldrich). When specified, 10 ng/ml VEGF (Peprotech), 10 µM DAPT (Sigma-Aldrich) or 50 ng/ml ActinomycinD (sigma-aldrich) was added. Matrigel™ assays were performed in 35 mm glass bottom culture dishes (MatTek corporation) using matrigel™ at a final concentration of 5 mg/ml. Network formation was monitored by microscopy and quantified by counting branch points (20 fields per condition). Each field represents a surface of 1mm². BAEC were cultured in DMEM supplemented with 10% fetal serum, experiments were performed using G₀ synchronized cells in serum free conditions.

**Material and constructs:**

Human Dll4 cDNA (IMAGE clone 40034887) was subcloned using PCR amplification into pcDNA3 expressing vector. pcDNA3.1-Hygro-FoxC2-3XFLAG was kindly provided by Pr. S. Enerbäck (Gotenburg Univ.). For RNAi experiments, ON-TARGETplus SMARTpool Si RNA L-010490; L-008987; L-004571; L-007214; L-011714 (Dharmacon), directed respectively against DLL4, Foxc2, α3 integrin, α6 integrin, lamin and α2 integrin (GAA ACG CCC TTG ATA CTA ATT) and luciferase as a control (si Ctrl) (CGU ACG CGG AAU ACU UCG A) were used at 100 nM. Sequences Dll4 #1(TCA CAA GGC TGC GCT ACT C); #2(CAG AATGGC TAC TGC AGC A).

Antibodies against the following proteins were used: human integrin α2 (AB1936, Chemicon), mouse integrin α2 (Emfret analytics), integrin α3 (AB2056, Chemicon), Integrin α6 (G0H3, R&D systems) Dll4 (Cell Signaling), VEGFR-2 (Cell Signaling), P-VEGFR2 (cell Signaling), VEGFR-1 (sc-316, Santa Cruz Biotechnology), Phosphotyrosine P-Tyr-100 (Cell Signaling), GAPDH (SC-25778; Santa Cruz Biotechnology), βTubulin (TUB2.1, Sigma-Aldrich), laminin γ1 (B4, Santa Cruz Biotechnology), FLAG-tag (Sigma), BrdU (Roche), blocking anti-human VEGFR2 clone 89106 (R&D systems).

Human plasma Fibronectin, Matrigel™, rat collagen-I, mouse EHS and Collagen IV were purchased from BD-Bioscience or Upstate and used at 15 µg/ml. When Fn and Lm were mixed, each protein was used at 15 µg/ml.

**EC differentiation and sprouting assay**

Culture of ES cells, generation of embryoid bodies and wholemount immunostaining were performed as in. Briefly, embryoid bodies derived from a hybrid ES cell line (DsRed-MST; 129S6B6-F1) were transferred to a polymerized collagen I gel and cultured in ES cell medium supplemented with 30 ng/ml VEGF-A. Samples were analysed on a Zeiss LSM710 confocal microscope, images were treated using ImageJ and Photoshop CS3 software.

**RT-PCR and Q-PCR**

RNAs were extracted from cultured HUVECs using Trizol reagent (Gibco) and RNeasy kit (QUIAGEN). Reverse transcription was performed on 1 µg of total RNA using Superscript II reverse transcriptase (invitrogen) and anchored oligo(dT)₂₀ primers (invitrogen). Sets of specific primers (see Online Table1) were used for amplification using 7900HT Real Time PCR System (Applied Biosystems, Foster USA). Samples were normalized to a ribosomal protein SB34 using the ΔCt method. Statistical significance was determined with Student’s t-test.
**Protein extraction, Immunoprecipitation**
Proteins were extracted using ice-cold RIPA buffer (20 mM Tris pH 8, 150 mM NaCl, 1%NP40, 0.1% SDS, 0.5% Deoxycholic acid) containing Na<sub>3</sub>VO<sub>4</sub> 1 mM, βglycerophosphate 10 mM, NaF 10 mM, AEBSF 1 mM, and protease inhibitors (Roche). Lysates were analyzed by immunoblotting either on total proteins or following immunoprecipitation using 3x10<sup>6</sup> cells. For matrix production analysis, HUVECs were maintained in serum-containing medium in presence of VEGF (10 ng/ml) for 4 days. The day before collection, cells were placed in serum free medium overnight, medium was collected and proteins were precipitated with acetone. Cells were detached using 5 mM EDTA and extracted in RIPA buffer. Matrices layered down to the culture dishes were collected after extraction for 20 min at room temperature in Laemmli buffer.

**BrdU incorporation**
BrdU incorporation was performed on G0-synchronized cells plated for 24h, 10μM BrdU was added in the last 5h and detected as previously described 24. 300 cells per condition were scored for positive staining. Results are expressed as % incorporation compared to controls normalized as 100 %. Graphs represent means of 3 independent experiments. A student t-test was performed.

**Dll4 promoter analysis**
We used TESS software to analyze the human promoter sequence of Dll4. This software is freely available online: [http://www.cbil.upenn.edu/cgi-bin/tess/tess](http://www.cbil.upenn.edu/cgi-bin/tess/tess)

**FACS analysis of surface-protein expression**
Cells were detached and incubated with antibodies against α2 (AK-7), α3 (ASC-1) or α6 (GOH3) integrins, followed by secondary FITC-conjugated antibody and fixation in 1% formaldehyde. Mean fluorescence intensities were determined on a BD FACSCalibur flow cytometer.
Supplemental References


### Online Table 1: Oligonucleotides sequences used for Q-PCR analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dll4 F</td>
<td>TGGGTCAGAACTGGTTATTGGA</td>
</tr>
<tr>
<td>Dll4 R</td>
<td>GTCATTGCGCTTTCTTGACAG</td>
</tr>
<tr>
<td>Jag1 F</td>
<td>CAGTTGAGTTGGAGATCCTGT</td>
</tr>
<tr>
<td>Jag1 R</td>
<td>CCTTGAGGCACACTTTGGAAGTA</td>
</tr>
<tr>
<td>Dll1 F</td>
<td>ACTGCAGCTCTTCCACCCTG</td>
</tr>
<tr>
<td>Dll1 R</td>
<td>CAGGTGCCAGGAGAAGTCGTT</td>
</tr>
<tr>
<td>Notch1 F</td>
<td>CACTGTGGGCGGGTCC</td>
</tr>
<tr>
<td>Notch1 R</td>
<td>GTTGATTTGCTGGCCACCAT</td>
</tr>
<tr>
<td>Notch4 F</td>
<td>TAGGGCTCCCCACGCTCCT</td>
</tr>
<tr>
<td>Notch4 R</td>
<td>GGCAGGTGCCCCATT</td>
</tr>
<tr>
<td>Hey1 F</td>
<td>GAGAAGCAGGGGATCTGCTAA</td>
</tr>
<tr>
<td>Hey1 R</td>
<td>CCCAAACTCCGATAGTCCAT</td>
</tr>
<tr>
<td>Hey2 F</td>
<td>GTCGCTCTCCACAAACTTCAG</td>
</tr>
<tr>
<td>Hey2 R</td>
<td>GAATCCGCAATGGCCAAAC</td>
</tr>
<tr>
<td>Foxc2 F</td>
<td>CAG TCC ATC ATG GAC CGC TCC</td>
</tr>
<tr>
<td>Foxc2 R</td>
<td>CCA GCC CTG CTT GTT CTC C</td>
</tr>
<tr>
<td>Foxc2 F_2</td>
<td>ACC AGT TCA TCA TGG ACC GC</td>
</tr>
<tr>
<td>Foxc2 R_2</td>
<td>CAG CCC TGC TTG TTC TCC C</td>
</tr>
<tr>
<td>VE-Cadherin F</td>
<td>ATCGGTTGTCAATGCGTCC</td>
</tr>
<tr>
<td>VE-Cadherin R</td>
<td>CCTCCAGATTTGATCATGACA</td>
</tr>
<tr>
<td>IL8 F</td>
<td>TGCCAAGGAGTGCTAAAG</td>
</tr>
<tr>
<td>IL8 R</td>
<td>CTCACAACACCCTGCAAC</td>
</tr>
<tr>
<td>IL6 F</td>
<td>TCTCCACAAGCGCTTCG</td>
</tr>
<tr>
<td>IL6 R</td>
<td>CTCAGGGCTGAGATGCCG</td>
</tr>
<tr>
<td>DUSP1 F</td>
<td>CAGTACCACCTCTACGATCA</td>
</tr>
<tr>
<td>DUSP1 R</td>
<td>ACCCTCAAATGGTGGGACA</td>
</tr>
<tr>
<td>DUSP5 F</td>
<td>GGCCCTCAGATTACATCAAGCA</td>
</tr>
<tr>
<td>DUSP5 R</td>
<td>TGCCCATGAAAGCATAAGA</td>
</tr>
<tr>
<td>SPRY4 F1</td>
<td>CTGGGTCTGCAACCCAGGA</td>
</tr>
<tr>
<td>SPRY4 R1</td>
<td>GACCAGTTGATACCGTGCA</td>
</tr>
<tr>
<td>SPRY4 F2</td>
<td>AGACCAGCCATGTCGAGATG</td>
</tr>
<tr>
<td>SPRY4 R2</td>
<td>CCCCGGGTGTCGCTT</td>
</tr>
<tr>
<td>SPRY4 F3</td>
<td>CTCAGCCCAGACTCTGGTGCA</td>
</tr>
<tr>
<td>SPRY4 R3</td>
<td>CCTCATCGCTCCATCGT</td>
</tr>
</tbody>
</table>
**Online figure legends:**

**Online figure I: Expression of Notch ligands in arterial cells and in HUVECs during network formation on matrigel™.**

A) Dll4 mRNA levels were analysed by QPCR in BAECs cells following 4h adhesion on Fn or Lm. Values represent fold induction compared to T0 ±SD (**p ≤0.0001). B) HUVECS were seeded on 3D matrigel™, mRNA were collected over network formation time course, and Dll1 and Jagged1 levels were measured by QPCR. Graph represents fold induction±SD compared to T0 (n=3, *p ≤0.01).

**Online figure II: Control of Dll4 knockdown efficiencies and DAPT effect on Notch pathway target genes.**

A) mRNA levels of Dll4 in cells transfected with either si Ctrl or si Dll4#1, #2 after 4h adhesion on different matrices were analysed by QPCR. Values represent fold induction compared to T0 ±SD (n=3). B) mRNA levels of Hey1 and Hey2 were analysed by QPCR in cells treated as indicated with 10µM of DAPT after 4h of adhesion on Fn or Lm. Values represent fold induction compared to T0 ±SD.

**Online figure III: Analysis of Tel/CtBP-controlled gene profile in HUVECs on Lm and control of FoxC2, integrin α2 and integrin α6 levels for figure 5.**

A) mRNA levels of Tel/CtBP-responsive genes in HUVECs following 4h of adhesion on Fn or Lm. Values represent fold induction compared to T0 ±SD. B) mRNA levels of FoxC2 in cells transfected with either si Ctrl or si FoxC2 following an adhesion time course on different matrices were analysed by QPCR. Values represent fold induction compared to T0 ±SD (**p ≤0.0001). C) FoxC2 overexpression in HUVECs transfected with empty vector (-) or a vector encoding Flag-tagged human FoxC2 (+). D) Cells transfected with either si Ctrl or si int α2 were plated on different extracellular matrices. Lysates were collected and analysed by Western blot to control knockdown efficiency. E) RNAi mediated knockdown of integrin α6 was verified by surface staining and FACS analysis. F) Dll4 mRNA level was analyzed by QPCR in cells transfected with either si Ctrl or si int α6 after 4h adhesion on Fn or Lm. Values represent fold induction compared to T0 ±SD (n=3).

**Online figure IV: laminin-induced Dll4 expression is independent of VEGF signaling.**

A) Cells were plated on Fn or Lm and stimulated or not with 10ng/ml VEGF during 4h. Lysates were collected and immunoprecipitated using VEGFR1 antibody. Total lysates and Immunoprecipitation were analysed by Western Blot with different antibodies as indicated (n=3). B) RNAi mediated knockdown of integrin α6 was verified by surface staining and FACS analysis.

**Online figure V: Control of Dll4, integrin α2 and integrin α6 knockdown efficiencies for figure 7**

A) Cells transfected with si Ctrl, si Dll4#1, si Dll4#2 were plated on Fn or Lm for 4h in parallel of the matrigel™ network formation assay. Lysates were collected and analysed by Western blot. B) RNAi mediated knockdowns of integrin α2 and α6 were verified by surface staining and FACS analysis.
A

![Graph A: Dll4 expression over time]

B

![Graph B: Dll1 and Jagged1 expression over time]