Angiopoietin-1/Tie2 signal augments basal Notch signal controlling vascular quiescence by inducing delta-like 4 expression through AKT-mediated activation of β-catenin

Jianghui Zhang‡, Shigetomo Fukuhara‡, Keisuke Sako‡, Takato Takenouchi§, ¶, Hiroshi Kitani§, Tsutomu Kumé¶, Gou Young Koh***, Naoki Mochizuki‡

‡Department of Structural Analysis, National Cardiovascular Center Research Institute, Fujishirodai 5-7-1, Suita, Osaka 565-8565, Japan. §Transgenic Animal Research Center, National Institute of Agrobiological Sciences, Ibaraki, Japan. ¶Laboratory for Chemistry and Metabolism, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan. **Feinberg Cardiovascular Research Institute, Northwestern University, Chicago, IL 60611. ***Biomedical Research Center and Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Guseong-dong, Daejeon, 305-701, Korea

Running title: Ang1 upregulates Dll4/Notch signal through β-catenin
Correspondence should be addressed to Shigetomo Fukuhara (fuku@ri.ncvc.go.jp) and Naoki Mochizuki (nmochizu@ri.ncvc.go.jp).
Address: Department of Cell Biology, National Cerebral and Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan
Tel.: +81-6-6833-5012; Fax: +81-6-6835-5461

Angiopoietin-1 (Ang1) regulates both vascular quiescence and angiogenesis through the receptor tyrosine kinase Tie2. We and another group previously showed that Ang1 and Tie2 form distinct signaling complexes at cell-cell and cell-matrix contacts. We further demonstrated that the former upregulates Notch ligand delta-like 4 (Dll4) only in the presence of cell-cell contacts. Since Dll4/Notch signal restricts sprouting angiogenesis and promotes vascular stabilization, we investigated the mechanism how Ang1/Tie2 signal induces Dll4 expression to clarify the role of Dll4/Notch signal in β-catenin-dependent Dll4 expression. Under the confluent endothelial cells, basal Notch signal was observed. Ang1, moreover, induced Dll4 expression and production of Notch intracellular domain (NICD). Ang1 stimulated transcriptional activity of β-catenin through phosphoinositide 3-kinase (PI3K)/Akt-mediated phosphorylation of glycogen synthase kinase 3β (GSK3β). Correspondingly, GSK3β inhibitor upregulated Dll4, while depletion of β-catenin by siRNA blocked Ang1-induced Dll4 expression, indicating the indispensability of β-catenin in Ang1-mediated upregulation of Dll4. In addition, Dll4 expression by the GSK3β inhibitor was only observed in the confluent cells, and impeded by DAPT, a γ-secretase inhibitor, implying the requirement of Notch signal in β-catenin-dependent Dll4 expression. Consistently, we found that either Ang1 or NICD upregulates Dll4 through the RBP-J binding site within the intron 3 of DLL4 gene and that β-catenin forms a complex with NICD/RBP-J to enhance Dll4 expression. Ang1 induced the deposition of extracellular matrix that is preferable for basement membrane formation through the Dll4/Notch signaling. Collectively, Ang1/Tie2 signal potentiates basal Notch signal controlling vascular quiescence by upregulating Dll4 through AKT-mediated activation of β-catenin.

Angiopoietin-1 (Ang1) is a ligand for endothelium-specific receptor tyrosine kinase Tie2. Ang1/Tie2 signaling is essential for developmental vascular formation, as evidenced by the gene-targeting analyses of either Ang1 or Tie2 in mice(1-3). In quiescent adult vasculature, Ang1 secreted from mural cells induces Tie2 activation in endothelial cells to maintain mature blood vessels by enhancing vascular integrity and endothelial survival(4-6). Ang1/Tie2 signaling also plays an important role in physiological and pathological angiogenesis,
as opposed to its function in quiescent vasculature(7-9). As to the dual functions of Ang1/Tie2 signaling, we and Alitalo’s group have previously reported that Ang1 assembles distinct Tie2 signaling complexes in the presence or absence of endothelial cell-cell junctions, thereby regulating both vascular quiescence and angiogenesis(10;11). Ang1 induces \textit{trans}-association of Tie2 in the presence of cell-cell contacts, whereas Tie2 is anchored to the cell-substratum contacts through extracellular-matrix (ECM)-bound Ang1 in the isolated endothelial cells. \textit{Trans}-associated Tie2 and ECM-anchored Tie2 stimulate AKT and extracellular signal-regulated kinase 1/2 pathways preferable for vascular quiescence and angiogenesis, respectively.

By performing DNA microarray analyses, we have revealed that \textit{trans}-associated Tie2, but not ECM-anchored Tie2, regulates the expression of genes involved in vascular quiescence which include Krüppel-like factor 2 (KLF2), delta-like 4 (Dll4), TIS11d and connexin-40(10). We have extended the studies on Ang1-mediated vascular quiescence and have shown that Ang1-induced KLF2 expression occurs through phosphoinositide 3-kinase (PI3K)/AKT pathway-mediated activation of myocyte enhancer factor 2, and counteracts vascular endothelial growth factor (VEGF)-mediated inflammatory responses (12).

Dll4 is a type 1 membrane protein belonging to the Delta/Serrate/Lag2 (DSL) family of Notch ligands. Notch signaling is an evolutionally conserved pathway involved in cell fate specification during embryonic and postnatal development, and plays crucial roles in multiple aspects of vascular development such as arterial-venous cell fate determination and tip/stalk cell specification during sprouting angiogenesis(13-15). DSL ligands bind to Notch family receptors in a cell-cell contact-dependent manner, leading to cleavage of the Notch intracellular domain (NICD). NICD cleaved from Notch enters into the nucleus, associates with transcription factor RBP-J, and regulates the expression of Hes (Hairy/Enhancer of Slit) and Hey (Hes related with YRPW, also known as HesR, HRT and HERP) family of transcriptional repressors(16).

During the tip-stalk cell communication during sprouting angiogenesis, Dll4/Notch signal is well characterized(17-21). VEGF upregulates Dll4 expression in endothelial tip cells, which in turn leads to Notch activation in adjacent stalk cells. The stalk cells subsequently lose their responsiveness to VEGF through down-regulation of VEGF receptors such as VEGFR2 and Neuropilin-1(22), thereby maintaining a quiescent and stabilized phenotype. Similarly, Dll4/Notch signaling is reported to be involved in tumor angiogenesis. In tumor vasculature, tumor-derived VEGF induces Dll4 expression in endothelial cells, which acts as a negative regulator of tumor angiogenesis, but is required for formation of functional vascular network(23-25). Indeed, blockade of Dll4/Notch signaling in tumor vasculature inhibits tumor growth by promoting non-productive angiogenesis associated with excessive sprouting from tumor vessels. The effect of Dll4/Notch signaling on tumor vasculature is reminiscent of that of Ang1/Tie2 signaling. Ang1/Tie2 signaling is also capable of inducing normalization of tumor vasculature by reducing excessive endothelial sprouting and promoting pericyte coverage(26-29).

The Notch signaling not only restricts angiogenesis but also maintains the vascular quiescence(15;30). It has been reported that conditional deletion of RBP-J, the key transcription factor downstream of Notch receptor, induces spontaneous angiogenesis in quiescent adult vasculature(31). Similarly, Tie2 is activated in the endothelium of quiescent adult vasculature, and is believed to be involved in the maintenance of vascular quiescence(6;32). Furthermore, both Ang1/Tie2 and Dll4/Notch signaling promote recruitment of mural cells to vessel wall and induce deposition of basement membrane proteins around the vessels, both of which are important for vascular stabilization(26;33-37).

Besides the role for Dll4/Notch signal in the tip-stalk communication, Dll4/Notch signal appears to function in the mature blood vessels with tight interendothelial cell-cell contacts. Functional similarity between Ang1/Tie2 signal and Dll4/Notch signal and our previous data that Ang1 induced Dll4 expression prompted us to test our hypothesis
that Ang1/Tie2 signal may promote the vascular stabilization through DLL4/Notch signal and to investigate how DLL4 is induced by Ang1/Tie2 signaling. In this study, we found that Ang1 induces activation of β-catenin through PI3K/AKT pathway-mediated inhibition of glycogen synthase kinase 3β (GSK3β) and that the stabilized β-catenin subsequently enhances Notch signal-induced DLL4 expression by forming a complex with NICD/RBP-J on the DLL4 intron3 enhancer, thereby potentiating DLL4/Notch signal leading to vascular stabilization.

EXPERIMENTAL PROCEDURES
Reagents, antibodies and siRNAs
Ang1, Angiopoietin-2 (Ang2) and cartilage oligomeric matrix protein (COMP)-Ang1 were prepared as described before(38). Other reagents were purchased as follows: wortmannin, AKT inhibitor IV, N-(3,5-difluorophenacetyl)-L-alanyl-S-p henylglycine t-butyl ester (DAPT) from Calbiochem; SB216763 from Sigma-Adrich; lithium chloride (LiCl) from Wako Pure Chemical Industries; basic fibroblast growth factor (bFGF) from PeproTech. Antibodies were purchased as follows: anti-DLL4, anti-Cleaved Notch1 (Val1744) (anti-NICD), anti-AKT, anti-phospho-AKT, anti-GSK3β, anti-phospho-GSK3β (Ser9) from Cell Signaling Technology; anti-tubulin from Sigma-Aldrich; lithium chloride (LiCl) from Wako Pure Chemical Industries; basic fibroblast growth factor (bFGF) from PeproTech. Antibodies were purchased as follows: anti-DLL4, anti-Cleaved Notch1 (Val1744) (anti-NICD), anti-AKT, anti-phospho-AKT, anti-GSK3β, anti-phospho-GSK3β (Ser9) from Cell Signaling Technology; anti-tubulin from Sigma-Aldrich; anti-β-catenin from BD Biosciences; anti-RBP-J (K0043) from Tokusyu-meneki Laboratory; anti-collagen type IV from Millipore; rhodamine-phalloidin from Invitrogen Corp.; horseradish peroxidase-coupled sheep anti-mouse and anti-rabbit IgG from GE Healthcare Life Science. Stealth small interfering RNAs (siRNAs) targeting the genes indicated below were purchased from Invitrogen Corp.: human DLL4 (HSS123068, HSS182569), human β-catenin (VHS50819, VHS50822).

Plasmids and adenoviruses
The DNA including the intron 3 of human DLL4 gene was amplified by PCR using the genomic DNA extracted from HUVECs as a template and the following primer set (5’-gtgagtagctcgctccgc-3’ and 5’-ctgagggggcagagggtc-3’). The amplified DNA was cloned into pGL3 Promoter vector (Promega Corporation) to construct the DLL4-Int3-Luc reporter plasmid. To generate the DLL4-Int3mut-Luc reporter plasmid, the RBP-J binding site was mutated using QuickChange Site-directed Mutagenesis kit (Stratagene) using the DLL4-Int3-Luc plasmid as a template. To construct the p3xFLAG-NICD plasmid encoding FLAG-tagged NICD, a DNA fragment encoding Notch1 intracellular domain was excised from pcDNA-FLAG-Notch1-ICD vector, a gift from M. Kurabayashi (Gunma University), and subcloned into p3xFLAG-CMV10 vector (Sigma-Aldrich). A cDNA encoding human Foxc2 was amplified by PCR using human heart cDNAs as a template, and cloned into pERE/NLS vector, a gift from M. Matsuda(40), namely...
pERed-NLS-Foxc2 plasmid. A 3.7 kb fragment of the mouse Dll4 promoter (-3631/+76) cloned in pGL3 Basic vector (Promega Corporation) has already been reported(41). An expression plasmid encoding constitutively active form of β-catenin (CA-βCat) in which Ser37 is replaced with Ala, was kindly provided by J. S. Gutkind (National Institute of Health). Other vectors are purchased as follows: pRL-SV40 and pRL-TK from Promega Corporation; TOPflash reporter plasmid from Millipore Corporation. Recombinant adenovirus vectors encoding LacZ and constitutively active form of AKT (CA-AKT) were kindly provided by M. Matsuda (Kyoto University) and Y. Fujio (Osaka University), respectively.

**Real-time reverse transcription-PCR**

Endothelial cells placed on collagen-coated plates under either sparse or confluent culture condition were starved in medium 199 containing 1% BSA for 12 h, and stimulated with either 400 ng/ml COMP-Ang1 or 10 μM SB216763 as described in the figures. After the stimulation, total RNA was purified using Trizol (Invitrogen Corp.). Quantitative real-time reverse transcription (RT)-PCR was carried out using QuantiFast SYBR Green RT-PCR kit (Qiagen) as described before(12). For each reaction, 100 ng of total RNA was transcribed for 10 min at 50 °C, followed by a denaturing step at 95 °C for 5 min and 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Fluorescence data were collected and analyzed using Mastercycler ep realplex (Eppendorf). The primers used for amplification were as follows: human Dll4, 5'-tcaaatgcctcatctttac-3' and 5'-ttttgatggcttttgtct-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-atggaaggcttgctg-3' and 5'-ggccttcttggtcagca-3'. For normalization, expression of human GAPDH was determined in parallel as an endogenous control.

**Immunoprecipitation and Western blot analysis**

Confluent and sparse HUVECs plated on collagen-coated dish were starved in medium 199 containing 1% BSA for 12 h, and stimulated as described in the figures. After the stimulation, the cells were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl at PH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 20 mM sodium fluoride, 1 mM sodium vanadate and 1 x protease inhibitor cocktail (Roche Applied Science), and centrifuged at 15,000g for 20 min at 4 °C. The supernatant was used as preclreated cell lysate. To detect the Dll4 protein expression and the NICD production, the cell lysates were subjected to SDS-PAGE and Western blot analysis with anti-Dll4 and anti-NICD antibodies. To evaluate the phosphorylation of AKT and GSK3β, aliquots of cell lysate were subjected to Western blot analysis with anti-phospho-AKT and anti-phospho-GSK3β antibodies, respectively. The total contents of AKT and GSK3β in each cell lysate were also assayed in a parallel run using corresponding antibodies. To detect the interaction between NICD and β-catenin, NICD was immunoprecipitated with anti-NICD antibody from the precleared lysates. Immunoprecipitated NICD and aliquots of cell lysate were subjected to SDS-PAGE and Western blot analysis with anti-β-catenin and anti-NICD antibodies, respectively.

**Luciferase reporter assay**

Luciferase reporter assay was performed as described previously(42). Briefly, HUVECs plated on collagen-coated dish were transfected with different expression vectors, together with reporter plasmids as described in the figures. Total amount of plasmid DNA was adjusted with empty vector. The cells were harvested, and re-plated on the collagen-coated 24-well plate in confluent culture condition. Luciferase activity was assayed 48 h after the transfection. To examine the effect of COMP-Ang1, the cells were starved and stimulated as described in the figures. The cells were lysed using passive lysis buffer (Promega Corporation), and luciferase activities in cell extract were determined using a dual luciferase assay system (Promega Corporation).

**Chromatin immunoprecipitation assay**
Chromatin immunoprecipitation (ChIP) assay was performed using EZ ChIP™ kit (Millipore Corporation) according to the manufacturer’s instruction. Confluent HUVECs plated on collagen-coated dish were starved in medium 199 containing 1% BSA for 12 h, and stimulated with 400 ng/ml COMP-Ang1 for 30 min. After the stimulation, genomic DNA and protein were cross-linked by addition of formaldehyde (1% final concentration) directly to culture medium, and incubated for 10 min at room temperature (RT). The cells were then harvested, lysed and sonicated to generate 0.3-1.0 kb DNA fragments. After centrifugation, the cleared supernatant was incubated with anti-RBP-J, anti-NICD, anti-β-catenin, and control antibodies for immunoprecipitation. Co-immunoprecipitated and input DNA were used as a template for PCR amplification. PCR amplifications were carried out using the primers specific for the intron 3 of human Dll4 gene (5'-gacgcttagcttggcctggagctg-3' and 5'-tgtaaaatacaggaaggggcccgtcag-3'). The sensitivity of the PCR reactions was evaluated with serial dilutions of input DNA collected after sonication. Amplified DNA was separated on 2% agarose gels and visualized with ethidium bromide.

Endothelial cell tube formation assay
Endothelial cell tube formation assay was performed according to Davis’s method(43). HUVECs were suspended in 2.5 mg/ml collagen type I matrices (Nitta Gelatin) at density of 2 x 10⁶ cells/ml, and incubated at 37 °C for 48 h in medium 199 containing reduced serum supplement, bFGF at 40 ng/ml, and ascorbic acid at 50 μg/ml. During the incubation, the cells were stimulated with or without COMP-Ang1 in the presence or absence of 20 μM DAPT. The cultures were fixed in PBS containing 2% paraformaldehyde for 2 h at RT, and blocked with PBS containing 1% BSA for 12 h at 4 °C. To detect extracellular deposition of collagen type IV, the cultures were stained with anti-collagen type IV antibody for 12 h at 4 °C, and visualized with Alexa 488-labeled donkey anti-goat IgG. To visualize filamentous actin, the cultures were subsequently permeabilized with 0.1% Triton X-100 for 1 h at RT, and stained with rhodamine-phalloidin for 12 h at 4 °C. Fluorescence images of Alexa 488 and rhodamine were recorded with a FV1000 confocal microscope (Olympus Corporation) with a 20x water immersion objective lens. To quantify the extracellular deposition of collagen type IV, fluorescence intensity of Alexa 488 within the areas of rhodamine-marked tube structures was determined using FluoView software (Olympus Corporation). Data were expressed as average pixel intensity in the areas of tube structures.

Statistical analysis
The values are expressed as means ± standard deviation (s.d.). Statistical significance was determined using one-way ANOVA or unpaired t-test. P values < 0.05 were considered statistically significant.

RESULTS
Ang1 induces Notch signaling by upregulating Dll4 under confluent, but not sparse cultures of HUVECs.
We previously found that Dll4 expression was upregulated in the confluent HUVECs stimulated with Ang1 by the microarray analyses(10). To first confirm whether the Dll4 expression is increased by Ang1 in the HUVECs with cell-cell contacts, HUVECs were stimulated with COMP-Ang1, a potent Ang1 variant, under either confluent or sparse culture condition. Before the stimulation, Dll4 mRNA was approximately 4 times higher in the confluent HUVECs than in the sparse cells, indicating the basal Notch signal is present in the HUVECs with cell-cell contacts (Fig. 1A). COMP-Ang1 significantly increased Dll4 mRNA in the confluent HUVECs, which peaked at 1 h after the stimulation and immediately declined to the basal level by 2 h (Fig. 1, A & B). Similarly, Dll4 mRNA levels were increased by stimulation with COMP-Ang1 in HAECs and HMVECs under confluent culture condition (Supplementary Fig. S1). However, in the sparse HUVECs, Dll4 mRNA was not affected by the stimulation with COMP-Ang1 (Fig. 1A). Consistently, Dll4 in the confluent HUVECs was higher than that in the sparse cells, and was increased in response to COMP-Ang1 (Fig. 1, C & D). In contrast, COMP-Ang1 did not induce Dll4 in
the sparse cells (Fig. 1, C & D). Dll4 was induced by native Ang1 as well as COMP-Ang1, but not by Ang2, an antagonist for Tie2 (Fig. 1, E & F). These results suggest that Dll4 upregulation by Ang1 depends on the cell-cell contacts that allow the activation of Notch signaling, and is dependent on the specific signal downstream of trans-associated Tie2 by Ang1.

To further investigate whether Dll4 expression by trans-associated Tie2 leads to the activation of Notch signaling, we examined the amount of NICD. COMP-Ang1 increased NICD in parallel with Dll4 upregulation under confluent culture conditions, which peaked at 1-2 h after the stimulation and declined to the basal level by 4 h, although NICD was not induced by COMP-Ang1 in the sparse cells (Fig. 1G). In addition, depletion of Dll4 by siRNA blocked increase in NICD by COMP-Ang1 (Fig. 1H). Collectively, these results indicate that Tie2 activation in the presence of cell-cell contacts results in the activation of Notch signaling by upregulating Dll4 expression.

A PI3K/AKT pathway is involved in Ang1-induced Dll4 expression. To understand the molecular mechanism underlying Dll4 expression by Ang1/Tie2 in the confluent cells, we focused on the downstream signaling of Tie2 in the presence of cell-cell contacts. We previously demonstrated that PI3K/AKT signal is preferentially activated by trans-associated Tie2(10). Thus, we next investigated the involvement of PI3K/AKT pathway in Ang1-induced Dll4 expression by using specific inhibitors for PI3K (wortmannin) and AKT (AKT inhibitor). Either inhibitor prevented not only COMP-Ang1-induced AKT activation but also COMP-Ang1-induced Dll4 expression (Fig. 2, A-D, Supplementary Fig. S2, A & B), indicating the requirement of PI3K/AKT pathway for Ang1-induced Dll4 expression. We further tested whether the activation of PI3K/AKT pathway is sufficient to induce Dll4 expression by infecting HUVECs with adenovirus-encoding CA-AKT, an active mutant of AKT. Over-expression of CA-AKT led to the increase in both Dll4 and NICD (Fig. 2E). These findings indicate that Ang1 activates Notch signaling through PI3K/AKT pathway-mediated Dll4 expression.

β-catenin is required for Ang1-induced Dll4 expression. β-catenin is one of the major substrates of GSK3β, and undergoes proteasomal degradation through GSK3β-mediated phosphorylation(45). Recently, Corada et al. have reported that Wnt/β-catenin pathway upregulates Dll4 transcription through the TCF-binding site located 706 bp upstream from the transcription start site of mouse Dll4 gene(46). Considering these evidences, we hypothesized that the stabilization of β-catenin through AKT-mediated inactivation of GSK3β is involved in Ang1-induced Dll4 expression. To address this possibility, HUVECs were transfected with a β-catenin -responsive luciferase reporter construct.
containing four native TCF binding sites (TOPflash). COMP-Ang1 significantly induced luciferase activity driven by TOPflash reporter (Fig. 4A), indicating the ability of Ang1 to induce β-catenin-dependent transcription. We further clarified the requirement of β-catenin in Ang1-induced Dll4 expression by transfecting HUVECs with two independent siRNAs targeting β-catenin. Depletion of β-catenin by siRNAs completely abolished COMP-Ang1-induced Dll4 expression (Fig. 4B). Similarly, Dll4 expression induced by SB216763 did not occur in the absence of β-catenin (Fig. 4C). These results suggest that Ang1 stimulates β-catenin—dependent transcriptional activity through AKT-mediated inhibition of GSK3β, thereby inducing Dll4 expression.

We next investigated whether Ang1 stimulates Dll4 transcription through the TCF-binding site located 706 bp upstream from the transcription initiation site of mouse Dll4 gene. For that, HUVECs were transfected with luciferase reporter plasmid in which the reporter is driven by the 3.7 kb mouse Dll4 promoter (Dll4-3.7k-Luc). COMP-Ang1 did not activate 3.7 kb mouse Dll4 promoter, although Foxc2 significantly stimulated the Dll4-3.7k-Luc reporter activity as previously reported (Supplementary Fig. S4, A & B)(41). However, CA-βCat, an active mutant of β-catenin, did not induce luciferase expression driven by Dll4-3.7k-Luc reporter gene, although the TOPflash reporter activity was significantly enhanced by CA-βCat (Supplementary Fig. S4, B & C). These results indicate that Ang1 stimulates Dll4 transcription independently of TCF-binding element located in the proximal Dll4 promoter.

Cell-cell contact-dependent Notch signaling is required for Ang1-induced Dll4 expression. Expression of Dll4 is higher in the confluent endothelial cells than in the sparse cells (Fig. 1, C, D & G). In addition, either COMP-Ang1 or GSK3β inhibitor induced Dll4 upregulation only in the confluent but not sparse endothelial cells (Fig. 1, A & C, Fig. 3, E & F). These results imply that cell-cell contact-dependent signal induces Dll4 expression and is required for β-catenin-mediated Dll4 upregulation.

Recently, Yamamizu et al. have reported that β-catenin forms a complex with NICD on the RBP-J binding sites of genes that determine the arterial fate of endothelial cells(47). Importantly, they also identified the RBP-J binding site within the intron 3 of both mouse Dll4 gene and human DLL4 gene by performing in silico analysis of the cis-acting elements (Fig. 5A). These findings prompted us to hypothesize that Notch signal is cell-cell contact-dependent signal responsible for Ang1-induced Dll4 expression. To address this possibility, we examined the effect of depletion of NICD by DAPT, a γ-secretase inhibitor, on Ang1-induced Dll4 expression. Treatment of confluent HUVECs with DAPT not only depleted NICD but also reduced basal Dll4 expression. In addition, DAPT prevented COMP-Ang1-induced Dll4 expression and subsequent NICD production (Fig. 5, B & C). Dll4 upregulation induced by SB216763 was also inhibited by treatment with DAPT (Fig. 5, D & E). These results indicate that cell-cell contact-dependent Notch signaling contributes to basal Dll4 expression and is indispensable for Ang1-induced Dll4 upregulation through β-catenin.

To further investigate whether intron 3 of DLL4 gene containing the RBP-J binding site acts as an Ang1-responsive enhancer element, HUVECs were transfected with either a plasmid expressing luciferase reporter gene under the control of human DLL4 intron 3 (Dll4-Int3-Luc) or its mutant plasmid in which the RBP-J binding site is mutated (Dll4-Int3mut-Luc) (Fig. 5A). COMP-Ang1 significantly stimulated Dll4-Int3-Luc reporter activity, which was inhibited by wortmannin (Fig. 5, F & G). In contrast, Dll4-Int3mut-Luc reporter was not activated by COMP-Ang1 (Fig. 5F). In addition, inhibition of Notch signaling by DAPT abolished COMP-Ang1-induced activation of Dll4-Int3-Luc reporter (Fig. 5H). These results indicate that Ang1 stimulates the enhancer activity of DLL4 intron 3 in a Notch signal-dependent manner.

Since β-catenin was essential for Ang1-induced Dll4 expression (Fig. 4B), we assumed that β-catenin and NICD might cooperatively stimulate the enhancer activity of DLL4 intron 3. To address this possibility, HUVECs were transfected with either
Dll4-Int3-Luc or Dll4-Int3mut-Luc reporter together with the plasmid encoding CA-βCat and/or that expressing NICD. NICD stimulated Dll4-Int3-Luc but not Dll4-Int3mut-Luc reporter activity (Fig. 5I). Although CA-βCat did not stimulate both reporter genes, it potently augmented NICD-stimulated Dll4-Int3-Luc reporter activity (Fig. 5I). However, Dll4-Int3mut-Luc reporter activity did not increase even if CA-βCat and NICD were co-expressed (Fig. 5I). Collectively, these results indicate that NICD stimulates the enhancer activity of DLL4 intron 3 via the RBP-J binding site and that β-catenin potentiates the NICD-induced stimulation of the enhancer activity.

Ang1 recruits β-catenin to the NICD/RBP-J complexes on the Dll4 intron 3. To understand how β-catenin potentiates Notch signal-mediated Dll4 expression, we examined the complex formation of β-catenin, NICD and RBP-J on the Dll4 intron 3 enhancer region by performing ChIP assay. Binding of NICD and RBP-J to the DLL4 intron 3 was detected in the confluent HUVECs irrespective of the presence and absence of COMP-Ang1 (Fig. 6A). Although β-catenin did not exist in the DLL4 intron 3 in the unstimulated confluent cells, COMP-Ang1 potently induced binding of β-catenin to the DLL4 intron 3 (Fig. 6A). Together with the results of Dll4-Int3-Luc reporter assays, these findings suggest that Ang1/Tie2 signal recruits β-catenin to the NICD/RBP-J complexes on the Dll4 intron 3. To confirm it, we carried out co-immunoprecipitation assay using anti-NICD antibody. Only a small fraction of β-catenin interacted with NICD in the confluent HUVECs (Fig. 6B). However, stimulation with COMP-Ang1 enhanced the association between β-catenin and NICD without affecting the expression of Dll4 and NICD (Fig. 6B). Collectively, these findings indicate that Ang1/Tie2 signal recruits β-catenin to the NICD/RBP-J complexes on the enhancer region of Dll4 intron 3, thereby inducing Dll4 upregulation.

Ang1 induces extracellular deposition of collagen type IV through Dll4/Notch signaling. Both Ang1/Tie2 and Dll4/Notch signaling are known to induce formation of vascular basement membrane(33;36;37), which is a hallmark of vascular stabilization. Therefore, we investigated whether Ang1 induces deposition of collagen type IV, a major basement membrane component, during endothelial cell tube formation in 3D collagen matrices. Extracellular deposition of collagen type IV was markedly increased by the stimulation with COMP-Ang1 (Fig. 7, A & B). However, inhibition of Notch signaling by treatment with DAPT inhibited COMP-Ang1-induced deposition of collagen type IV (Fig. 7, A & B). Consistently, collagen type IV depletion was not induced by COMP-Ang1 in Dll4-depleted cells (Fig. 7C, Supplementary Fig. S5). These findings suggest that Ang1 induces basement membrane formation through the Dll4/Notch signaling.

**DISCUSSION**

We here explored how Ang1 induces Dll4 expression and suggested its contribution to Ang1-regulated vascular quiescence. Ang1 assembles distinct Tie2 signaling complexes in the present or absence of cell-cell junctions, thereby regulating both vascular quiescence and angiogenesis. In the presence of cell-cell junctions, Ang1 induces formation of trans-associated Tie2, which induces expression of the genes involved in vascular stabilization which include Notch ligand Dll4. Since Dll4/Notch signal is known to restrict sprouting angiogenesis and promote vascular stabilization(15;17-21;30;31), we hypothesized that Dll4/Notch signal is involved in Ang1/Tie2 signal-mediated vascular quiescence. To address this possibility, we decided to delineate the signaling pathways underlying Ang1-induced Dll4 expression. We found that Ang1/Tie2 signal induces activation of β-catenin through AKT-mediated inhibition of GSK3β and that β-catenin resistant to the degradation enhances Notch signal-mediated Dll4 expression by forming a complex with NICD/RBP-J on the RBP-J binding site in the Dll4 intron 3, thereby potentiating Dll4/Notch signal leading to vascular quiescence (Fig. 7).

Basal Dll4 expression and NICD is higher in the confluent cells than in the sparse cells (Fig. 1G), consistent with the
previous report that cell-cell contact-dependent Notch signaling induces Dll4 expression(48). Importantly, either Ang1- or GSK3β inhibitor-induced Dll4 expression requires endothelial cell-cell contacts, and is sensitive to DAPT, suggesting that the Notch signaling is prerequisite for β-catenin-mediated Dll4 expression.

Augmentation of Dll4 expression by Ang1 is dependent on β-catenin. We have previously shown that Ang1/Tie2 signal preferentially activates PI3K/AKT signaling(10). Although phosphorylation of β-catenin by GSK3β leads to its degradation, β-catenin is stabilized by the inhibition of GSK3β by AKT. Dll4 expression by Ang1 was inhibited by depletion of β-catenin and by inhibition of either PI3K or AKT (Fig. 2, A-D, Fig. 4B), indicating the essential role of β-catenin for Ang1-induced Dll4 expression. Thus, we further extend the study on the transcriptional regulation of Dll4 by β-catenin.

We first analyzed -3.7 kb promoter region of mouse Dll4 gene, because this region contains the transcription factor binding sites for forkhead transcription factors and TCF. The forkhead transcription factors, Foxc1 and Foxc2, are the first transcription factors identified to regulate Dll4 expression during vascular development(41). Mouse embryos deficient in both Foxc1 and Foxc2 exhibit arteriovenous malformation and lack of expression of arterial genes such as Dll4 and ephrinB2. Consistently, Foxc1 and Foxc2 directly activate Dll4 promoter via the forkhead binding element (FBE) located approximately 3.7 kb upstream from the transcription initiation site(41). Thus, Dll4 induction responsible for arterial-venous cell fate determination appears to be mediated by Foxc genes. However, Ang1 did not stimulate the -3.7 kb Dll4 promoter containing the FBE, suggesting that Foxc1 and Foxc2 are not involved in Ang1-induced Dll4 expression. In addition, Corada et al. have recently reported that β-catenin upregulates Dll4 transcription through the TCF-binding site located 706 bp upstream from the transcription initiation site of mouse Dll4 gene(46). However, in our experiments, neither Ang1 nor CA-βCat activated the -3.7 kb mouse Dll4 promoter containing the corresponding TCF binding site. Instead, our luciferase reporter assays and ChIP experiments performed in this study revealed that the Dll4 intron 3 is an enhancer element responsible for Ang1-induced Dll4 transcription through β-catenin. Currently, the reason for this discrepancy remains unclear, but it may be due to the different cell types used for the experiments. We performed the experiments with HUVECs, while they used endothelial cells isolated from mouse embryos(46). Consistent with this idea, VEGF-induced Dll4 expression occurs only in arterial endothelial cell, but not in venous cells(49). Thus, the signaling pathways leading to Dll4 expression may vary in different endothelial cell types and in different upstream mediators.

Ang1 induces recruitment of β-catenin to NICD/RBP-J complexes on the RBP-J binding site in the Dll4 intron 3, which enhances NICD-mediated Dll4 expression. Thus, Ang1/Tie2 and Notch signaling converges into β-catenin/NICD/RBP-J complexes on the Dll4 intron 3 enhancer to cooperatively induce Dll4 expression. Consistently, functional interaction between NICD and β-catenin has recently been reported(47;50). In arterial, but not venous, endothelial cells, β-catenin/NICD/RBP-J complexes are formed on the RBP-J binding sites of arterial genes, thereby regulating their expression leading to arterial fate specification(47). In addition, it has also been shown that β-catenin/NICD/RBP-J complexes on the Hes1 promoter induce Hes1 expression to suppress the differentiation of neural precursor cells(50). Thus, the functional interaction between Notch and β-catenin signaling may be involved in a variety of biological processes.

Both Ang1/Tie2 and Notch signal are known to regulate vascular quiescence. Functional similarity between them and our present evidence that Ang1 induces Dll4 expression leading to Notch activation imply the role of Dll4/Notch signal in Ang1/Tie2-mediated vascular quiescence. We further revealed that Ang1 induces extracellular deposition of collagen type IV, a major component of basement membrane, during endothelial cell tube formation. This Ang1-mediated deposition of collagen type IV is dependent of Dll4/Notch signal, as
demonstrated by the evidence that inhibition of Notch signal by DAPT and deletion of Dll4 by siRNA prevented this effect (Figure 7). Since basement membrane matrix assembly is a crucial step for vascular maturation and stabilization(51), these findings suggest that Ang1/Tie2 signal might promote vascular stabilization through activation of Dll4/Notch signal.

Dll4/Notch signal is also involved in tip/stalk cell specification(15). Activation of Notch signal in the stalk cells restricts their angiogenic behavior, thereby maintaining a quiescent and stabilized phenotype of stalk cells. Interestingly, Yana et al. have found by using ex vivo angiogenesis system that Tie2 is specifically expressed in stalk cells and is involved in vessel maturation(37). Thus, Ang1/Tie2 signal may also regulate Dll4 expression in the stalk cells, leading to the maturation of neovessels. However, in vivo study must be required to clarify the role of cross-talk between Ang1/Tie2 and Dll4/Notch signal in vascular stabilization.

In conclusion, we found that Ang1/Tie2 signal induces activation of β-catenin through PI3K/AKT pathway-mediated inhibition of GSK3β in the presence of cell-cell contacts, and that the undegraded β-catenin subsequently potentiates Notch signal-mediated Dll4 expression by forming a complex with NICD/RBP-J on the RBP-J binding site in the Dll4 intron 3, which in turn upregulates Dll4/Notch signal. In addition, we also revealed that Dll4/Notch signal augmented by Ang1/Tie2 signal promotes formation of vascular basement membrane leading to vascular stabilization (Fig. 8).

ACKNOWLEDGMENTS
We are grateful to J. S. Gutkind (National Institute of Health) for the CA-βCat plasmid, to M. Kurabayash (Gunma University) for the Notch1 ICD plasmid, to M. Matsuda (Kyoto University) and Y. Fujio (Osaka University) for the adenovirus encoding LacZ and CA-AKT, respectively. We also thank K. Hiratomi, M. Sone, M. Minamimoto, and Y. Matsuura for technical assistance, N. Takakura (Osaka University), J. K. Yamashita (Kyoto University), K. Yamamizu (Kyoto University), and M. Masuda for helpful advice.

REFERENCES
1. Dumont, D. J., Gradwohl, G., Fong, G. H., Puri, M. C., Gertsenstein, M., Auerbach, A., and Breitman, M. L. (1994) Genes Dev. 8, 1897-1909
2. Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995) Nature 376, 70-74
3. Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996) Cell 87, 1171-1180
4. Brindle, N. P., Saharinen, P., and Alitalo, K. (2006) Circ. Res. 98, 1014-1023
5. Peters, K. G., Kontos, C. D., Lin, P. C., Wong, A. L., Rao, P., Huang, L., Dewhirst, M. W., and Sankar, S. (2004) Recent Prog. Horm. Res. 59, 51-71
6. Wong, A. L., Haroon, Z. A., Werner, S., Dewhirst, M. W., Greenberg, C. S., and Peters, K. G. (1997) Circ. Res. 81, 567-574
7. Asahara, T., Chen, D., Takahashi, T., Fujikawa, K., Kearney, M., Magner, M., Yancopoulos, G. D., and Isner, J. M. (1998) Circ. Res. 83, 233-240
8. Eklund, L. and Olsen, B. R. (2006) Exp. Cell Res. 312, 630-641
9. Lin, P., Polverini, P., Dewhirst, M., Shan, S., Rao, P. S., and Peters, K. (1997) J. Clin. Invest. 100, 2072-2078
10. Fukuhara, S., Sako, K., Minami, T., Noda, K., Kim, H. Z., Kodama, T., Shibuya, M., Takakura, N., Koh, G. Y., and Mochizuki, N. (2008) Nat. Cell Biol. 10, 513-526
11. Saharinen, P., Eklund, L., Miettinen, J., Wirkkala, R., Anisimov, A., Winderlich, M., Nottebaum, A., Vestweber, D., Deutsch, U., Koh, G. Y., Olsen, B. R., and Alitalo, K. (2008) Nat. Cell Biol. 10, 527-537
12. Sako, K., Fukuhara, S., Minami, T., Hamakubo, T., Song, H., Kodama, T., Fukamizu, A., Gutkind, J. S., Koh, G. Y., and Mochizuki, N. (2009) J. Biol. Chem. 284, 5592-5601
13. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) *Science* **284**, 770-776
14. Gridley, T. (1997) *Mol. Cell. Neurosci.* **9**, 103-108
15. Phng, L. K. and Gerhardt, H. (2009) *Dev. Cell* **16**, 196-208
16. Iso, T., Kedes, L., and Hamamori, Y. (2003) *J. Cell. Physiol.* **194**, 237-255
17. Hellstrom, M., Phng, L. K., Hofmann, J. J., Wallgard, E., Coutlas, L., Lindblom, P., Alva, J., Nilsson, A. K., Karlsson, L., Gaiano, N., Yoon, K., Rossant, J., Iruela-Arispe, M. L., Kalen, M., Gerhardt, H., and Betsholtz, C. (2007) *Nature* **445**, 776-780
18. Leslie, J. D., Ariza-McNaughton, L., Bermange, A. L., McDow, R., Johnson, S. L., and Lewis, J. (2007) *Development* **134**, 839-844
19. Lobov, I. B., Renard, R. A., Papadopoulos, N., Gale, N. W., Thurston, G., Yancopoulos, G. D., and Wiegand, S. J. (2007) *Proc. Natl. Acad. Sci. U.S.A* **104**, 3219-3224
20. Siekmann, A. F. and Lawson, N. D. (2007) *Nature* **445**, 781-784
21. Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A., and Eichmann, A. (2007) *Proc. Natl. Acad. Sci. U.S.A* **104**, 3225-3230
22. Williams, C. K., Li, J. L., Murga, M., Harris, A. L., and Tosato, G. (2006) *Blood* **107**, 931-939
23. Li, J. L., Sainson, R. C. A., Shi, W., Leek, R., Harrington, L. S., Preussner, M., Biswas, S., Turley, H., Heikamp, E., Hainfellner, J. A., and Harris, A. L. (2007) *Cancer Res.* **67**, 11244-11253
24. Noguer-Trisse, I., Daly, C., Papadopoulos, N. J., Coetzee, S., Boland, P., Gale, N. W., Lin, H. C., Yancopoulos, G. D., and Thurston, G. (2006) *Nature* **444**, 1032-1037
25. Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W. C., Chantery, Y., Kowalski, J., Watts, R. J., Callahan, C., Kasman, I., Singh, M., Chien, M., Tan, C., Hongo, J. A., de Sauvage, F., Plowman, G., and Yan, M. (2006) *Nature* **444**, 1083-1087
26. Augustin, H. G., Young, K. G., Thurston, G., and Alitalo, K. (2009) *Nat. Rev. Mol Cell Biol.* **10**, 165-177
27. Falcon, B. L., Hashizume, H., Koumoutsakos, P., Chou, J., Bready, J. V., Coxon, A., Oliner, J. D., and McDonald, D. M. (2009) *Am. J. Pathol.* **175**, 2159-2170
28. Hawighorst, T., Skobe, M., Steit, M., Hong, Y. K., Velasco, P., Brown, L. F., Riccardi, L., Lange-Asschenfeldt, B., and Detmar, M. (2002) *Am. J. Pathol.* **160**, 1381-1392
29. Macchein, M. R., Knedla, A., Knoth, R., Wagner, S., Neuschl, E., and Plate, K. H. (2004) *Am. J. Pathol.* **165**, 1557-1570
30. Phng, L. K., Potente, M., Leslie, J. D., Babbage, J., Nyqvist, D., Lobov, I., Ondr, J. K., Rao, S., Lang, R. A., Thurston, G., and Gerhardt, H. (2009) *Dev. Cell* **16**, 70-82
31. Dou, G. R., Wang, Y. C., Hu, X. B., Hou, L. H., Wang, C. M., Xu, J. F., Wang, Y. S., Liang, Y. M., Yao, L. B., Yang, A. G., and Han, H. (2008) *FASEB J.* **22**, 1606-1617
32. Fukushima, S., Sako, K., Noda, K., Zhang, J., Minami, M., and Mochizuki, N. (2010) *Histol. Histopathol.* **25**, 387-396
33. Benedito, R., Trindade, A., Hirashima, M., Henrique, D., da Costa, L. L., Rossant, J., Gill, P. S., and Duarte, A. (2008) *BMC Dev. Biol.* **8**, 117
34. Iivanainen, E., Nelimarkka, L., Elenius, V., Heikkinen, S. M., Juntila, T. T., Sihombing, L., Sundvall, M., Maatta, J. A., Laine, V. J., Yla-Herttuala, S., Higashiyama, S., Alitalo, K., and Elenius, K. (2003) *FASEB J.* **17**, 1609-1621
35. Kobayashi, H., DeBusk, L. M., Babichev, Y. O., Dumont, D. J., and Lin, P. C. (2006) *Blood* **108**, 1260-1266
36. Trindade, A., Ram Kumar, S., Schenget, J. S., Lopes-da-Costa, L., Becker, J., Ji, W., Liu, R., Gill, P. S., and Duarte, A. (2008) *Blood* **112**, 1720-1729
37. Yana, I., Sagara, H., Takaki, S., Takatsu, K., Nakamura, K., Nakao, K., Katsuki, M., Taniguchi, S. i., Aoki, T., Sato, H., Weiss, S. J., and Seiki, M. (2007) *J. Cell Sci.* **120**, 1607-1614
38. Cho, C. H., Kammerer, R. A., Lee, H. J., Steinmetz, M. O., Ryu, Y. S., Lee, S. H., Yasunaga, K., Kim, K. T., Kim, I., Choi, H. H., Kim, W., Kim, S. H., Park, S. K., Lee, G. M., and Koh, G. Y. (2004) *Proc. Natl. Acad. Sci. U.S.A* **101**, 5547-5552
39. Fukushima, S., Sacramento, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y.,
Kangawa, K., and Mochizuki, N. (2005) Mol. Cell. Biol. 25, 136-146
40. Aoki, K., Nakamura, T., Fujikawa, K., and Matsuda, M. (2005) Mol. Biol. Cell 16, 2207-2217
41. Seo, S., Fujita, H., Nakano, A., Kang, M., Duarte, A., and Kume, T. (2006) Dev. Biol. 294, 458-470
42. Fukuhara, S., Marinissen, M. J., Chiariello, M., and Gutkind, J. S. (2000) J. Biol. Chem. 275, 21730-21736
43. Koh, W., Stratman, A. N., Sacharidou, A., and Davis, G. E. (2008) Methods Enzymol. 443, 83-101
44. Frame, S. and Cohen, P. (2001) Biochem. J. 359, 1-16
45. Wu, D. and Pan, W. (2010) Trends Biochem. Sci. 35, 161-168
46. Corada, M., Nyqvist, D., Orsenigo, F., Caprini, A., Giampietro, C., Taketo, M. M., Iruela-Arispe, M. L., Adams, R. H., and Dejana, E. (2010) Dev. Cell 18, 938-949
47. Yamamizu, K., Matsunaga, T., Uosaki, H., Fukushima, H., Katayama, S., Hiraoka-Kanie, M., Mitani, K., and Yamashita, J. K. (2010) J. Cell Biol. 189, 325-338
48. Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M., and Adams, R. H. (2009) Cell 137, 1124-1135
49. Liu, Z. J., Shirakawa, T., Li, Y., Soma, A., Oka, M., Dotto, G. P., Fairman, R. M., Velazquez, O. C., and Herlyn, M. (2003) Mol. Cell. Biol. 23, 14-25
50. Shimizu, T., Kagawa, T., Inoue, T., Nonaka, A., Takada, S., Aburatani, H., and Taga, T. (2008) Mol. Cell. Biol. 28, 7427-7441
51. Davis, G. E. and Senger, D. R. (2005) Circ. Res. 97, 1093-1107

FOOTNOTES
This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan (to S.F., N.M.); the Ministry of Health, Labour, and Welfare of Japan (to N.M.); and the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (to S.F., N.M.); the Naito Foundation (to S.F.); Takeda Science Foundation (to S.F., N.M.); the Sagawa Foundation for Promotion of Cancer Research (to S.F.), Mochida Memorial Foundation for Medical and Pharmaceutical Research (to S.F.), Kowa Life Science Foundation (to S.F.), Kanoe Foundation for the Promotion of Medical Science (to S.F.), The Novartis Foundation (Japan) for the Promotion of Science (to S.F.), Senri Life Science Foundation (to S.F.), the Mitsubishi Foundation (to N.M.), and AstraZeneca Research Grant (to N.M.).

The abbreviations used are: Ang1, angiopoietin-1; ECM, extracellular matrix; KLF2, Krüppel-like factor 2; Dll4, delta-like 4; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; DSL, Delta/Serrate/Lag2; NICD, Notch intracellular domain; GSK3β, glycogen synthase kinase 3β; Ang2, angiopoietin-2; COMP, cartilage oligomeric matrix protein; DAPT, N-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester; LiCl, lithium chloride; siRNA, small interfering RNA; HUVECs, human umbilical vein endothelial cells; CA-βCat, constitutively active mutant of β-catenin; CA-AKT, constitutively active mutant of AKT; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation.

FIGURE LEGENDS
Figure 1. Ang1 induces Dll4 expression leading to activation of Notch signaling in confluent endothelial cells. (A) Sparse and confluent HUVECs were starved in medium 199 containing 1% BSA for 12 h, and stimulated with vehicle (-) or COMP-Ang1 at 400 ng/ml (C-Ang1) for 1 h. (COMP-Ang1 was used at the concentration of 400 ng/ml throughout the following experiments.) After the stimulation, total RNA was extracted and subjected to real-time RT-PCR analysis to determine the expression of Dll4 mRNA as described under “Experimental Procedures”. Bar graphs show relative mRNA levels of Dll4 mRNA normalized
to that of GAPDH. Data are expressed as fold induction relative to that in the vehicle-treated sparse cells, and shown as means ± s.d. of three independent experiments. (B) Confluent HUVECs starved for 12 h were stimulated with COMP-Ang1 for the periods indicated at the bottom (h). Dll4 mRNA levels were analyzed by real-time RT-PCR as described in A. Values are expressed as fold induction relative to that in the unstimulated cells, and shown as means ± s.d. of five independent experiments. (C) Confluent and sparse HUVECs were starved in medium 199 containing 1% BSA for 12 h, and stimulated with COMP-Ang1 for the periods indicated at the top (h). Cell lysates were subjected to Western blot analysis with anti-Dll4 (Dll4) and anti-tubulin (tubulin) antibodies. (D) The relative expression of Dll4 observed in C are quantified by normalizing the expression of Dll4 by that of tubulin. Values are expressed as fold induction relative to that observed in the confluent unstimulated cells, and shown as means ± s.d. of three independent experiments. (E) Confluent HUVECs starved for 12 h were stimulated with vehicle (control), 600 ng/ml Ang1 (Ang1), 600 ng/ml Ang2 (Ang2) and COMP-Ang1 (C-Ang1) for 1h. Cell lysates were subjected to Western blot analysis with anti-Dll4 (Dll4) and anti-tubulin (tubulin) antibodies. (F) Expression of Dll4 protein observed in E are quantified as described in D. Values are expressed as fold induction relative to that observed in the control cells, and shown as means ± s.d. of four independent experiments. (G) Confluent and sparse HUVECs were starved in medium 199 containing 1% BSA for 12 h, and stimulated with COMP-Ang1 for the periods indicated at the top (h). Cell lysates were subjected to Western blot analysis with anti-NICD (NICD), anti-Dll4 (Dll4) and anti-tubulin (tubulin) antibodies. Significant differences between two groups (A) or from the control (B, D, F) are indicated as *, p<0.05, **, p<0.01, or ***, p<0.001. n.s. indicates no significance between two groups or from the control.

Figure 2. Ang1 induces Dll4 expression through a PI3K/AKT pathway. (A) Confluent HUVECs starved for 12 h were pretreated with vehicle (control) or 60 nM wortmannin for 30 min, and subsequently stimulated with vehicle (-) or COMP-Ang1 (+) for 1h. Dll4 protein expression was examined by Western blot analysis as described in the legend of Fig. 1C. (B) Expression of Dll4 protein observed in A are quantified as described in legend of Fig. 1D. Values are expressed as fold induction relative to that in the wortmannin-untreated cells stimulated with vehicle, and shown as means ± s.d. of five independent experiments. (C) Confluent HUVECs starved for 12 h were pretreated with vehicle (control) or 8 μM AKT inhibitor for 10 min and subsequently stimulated with vehicle (-) or COMP-Ang1 (+) for 1h. Dll4 protein expression was analyzed as described in A. (D) Expression of Dll4 protein observed in C are quantified as described in legend of Fig. 1D. Values are expressed as fold induction relative to that in the Akt inhibitor-untreated cells stimulated with vehicle, and shown as means ± s.d. of four independent experiments. (E) Confluent HUVECs were infected with adenoviruses encoding LacZ or with two different titers of adenoviruses encoding AKT-CA for 48 h. Cell lysates were subjected Western blot analysis with anti-Dll4 (Dll4), anti-NICD (NICD), anti-phospho-AKT (P-AKT), anti-AKT (AKT) and anti-tubulin (tubulin) antibodies. Significant differences between two groups (B, D) are indicated as **, p<0.01. n.s. indicates no significance between two groups.

Figure 3. Ang1 induces Dll4 expression through AKT-mediated inhibition of GSK3β. (A) Confluent HUVECs starved for 12 h were stimulated with COMP-Ang1 for the periods indicated at the top (h). Cell lysates were subjected to Western blot analysis with anti-phospho-GSK3β (P-GSK3β), anti-GSK3β (GSK3β), and anti-tubulin (tubulin) antibodies. (B) Confluent HUVECs starved for 12 h were pretreated with vehicle (control) or 60 nM wortmannin for 30 min, and subsequently stimulated with vehicle (-) or COMP-Ang1 (+) for 30 min. Cell lysates were subjected to Western blot analysis with anti-phospho-GSK3β (P-GSK3β), anti-GSK3β (GSK3β), anti-phospho-AKT (P-AKT), and anti-AKT (AKT)
antibodies. (C) Phosphorylated GSK3β levels observed in B are quantified by normalizing the expression of phosphorylated GSK3β by that of total GSK3β. Values are expressed as fold induction relative to that in the wortmannin-untreated cells stimulated with vehicle, and shown as means ± s.d. of three independent experiments. (D) Confluent HUVECs were infected with adenoviruses encoding either LacZ or CA-AKT. Cell lysates were subjected to Western blot analysis as described in B. (E) Confluent and sparse HUVECs were starved in medium 199 containing 1% BSA for 12 h, and treated with 10 μM SB216763 for the periods indicated at the top (h). Cell lysates were subjected to Western blot analysis with anti-Dll4 (Dll4), anti-NICD (NICD) and anti-tubulin (tubulin) antibodies. (F) The effect of 20 mM LiCl on the expression of Dll4 and NICD was analyzed as described in E. In C, significant difference between two groups is indicated as *, p<0.05. n.s. indicates no significance between two groups.

Figure 4. Ang1 induces Dll4 expression through activation of β-catenin. (A) Confluent HUVECs were transfected with TOPflash reporter plasmid together with pRL-TK vector. After the transfection, the cells were starved in medium 199 containing 1% BSA for 4 h, and stimulated with vehicle (control) or COMP-Ang1 (C-Ang1) for 4h. After the stimulation, the cells were collected, and the lysates were assayed for firefly and renilla luciferase activities as described under “Experimental Procedures”. The data represent firefly luciferase activity normalized by the renilla luciferase activity present in each cellular lysate. Values are expressed relative to that observed in the cells treated with vehicle, and shown as mean ± s.d of four independent experiments. (B) Confluent HUVECs were transfected without (-) or with either control siRNA (control) or two independent siRNAs targeting β-catenin (βCat#1 and βCat#2). Then, the cells were starved and stimulated with vehicle (-) or COMP-Ang1 (+) for 1 h. Cell lysates were subjected to Western blot analysis with anti-Dll4 (Dll4), anti-β-catenin (βCat) and anti-tubulin (tubulin) antibodies. (C) Confluent HUVECs transfected with siRNAs as described in B were starved, and treated with vehicle (-) or 10 μM SB216763 (+) for 2 h. Cell lysates were subjected to Western blot analysis as described in B. In A, significant difference between two groups is indicated as ***, p<0.001.

Figure 5. Ang1 stimulates the enhancer activity of the inton 3 of Dll4 gene in a Notch signal-dependent manner. (A) The exon-intron organization of the human Dll4 gene and the structures of luciferase reporter constructs. Note that the human Dll4 intron 3 contains the RBP-J binding site. Dll4-Int3-Luc reporter plasmid expresses the firefly luciferase reporter gene under the control of human Dll4 intron 3. In Dll4-Int3mut-Luc reporter construct, the RBP-J binding site is disrupted. (B) Confluent HUVECs starved for 12 h were pretreated with vehicle (control) or 10 μM DAPT for 8 h, and subsequently stimulated with vehicle (-) or COMP-Ang1 (+) for 1h. Western blot analysis was performed as described in the legend of Fig. 1G. (C) The expression of Dll4 observed in B are quantified as described in legend of Fig. 1D. Values are expressed as fold induction relative to that in the DAPT-untreated cells stimulated with vehicle, and shown as means ± s.d. of 5 independent experiments. (D) Confluent HUVECs pretreated with DAPT as described in B were stimulated with vehicle (-) or SB216763 (+) for 2 h. Western blot analysis was performed as described in B. (E) The expression of Dll4 observed in D are quantified as described in legend of Fig. 1D. Values are expressed as described in C, and shown as means ± s.d of 4 independent experiments. (F) Confluent HUVECs transfected with either Dll4-Int3-Luc or Dll4-Int3mut-Luc reporter construct together with pRL-SV40 vector were starved in medium 199 containing 1% BSA for 12 h, and stimulated with vehicle (control) or COMP-Ang1 (+) for 3 h. After the stimulation, the cells were collected, and the lysates were assayed for firefly and renilla luciferase activities as described in the legend of Fig. 4A. Values are expressed relative to that observed in the control cells expressing Dll4-Int3-Luc plasmid, and shown as mean ± s.d of three independent experiments. (G) Confluent HUVECs co-transfected with Dll4-Int3-Luc reporter plasmid and pRL-SV40 vector were starved in medium 199 containing 1% BSA for 12 h, pretreated with vehicle (control) or 60 nM wortmannin for 30 min, and stimulated with vehicle (-) or COMP-Ang1 (+) for 3 h. After the stimulation, the cells were collected, and the lysates were assayed for firefly and renilla
luciferase activities as described in the legend of Fig. 4A. Values are expressed relative to that observed in the wortmannin-untreated cells stimulated with vehicle, and shown as mean ± s.d of four independent experiments. (H) Confluent HUVECs co-expressing both Dll4-Int3-Luc plasmid and pRL-SV40 vector were starved for 12 h, pretreated with vehicle (control) or 10 μM DAPT for 1 h, and stimulated with vehicle (-) or COMP-Ang1 (+) for 3 h. Firefly and renilla luciferase activities were assayed as described in the legend of Fig. 4A. Values are expressed relative to that in the DAPT-untreated cells stimulated with vehicle, and shown as means ± s.d. of three independent experiments. (I) Confluent HUVECs were transfected with either Dll4-Int3-Luc or Dll4-Int3mut-Luc reporter construct together with pRL-SV40 vector and the empty vector (control) or the plasmid encoding either CA-βCat or NICD. Cell lyses were assayed for firefly and renilla luciferase activities as described in the legend of Fig. 4A. Values are expressed relative to that observed in the control cells expressing Dll4-Int3-Luc reporter plasmid, and shown as mean ± s.d of five independent experiments. Significant differences between two groups (C, E, F, G, H, I) are indicated as *, p<0.05, **, p<0.01, ***, p<0.001. n.s. indicates no significance between two groups.

Figure 6. β-catenin is recruited to the NICD/RBP-J complexes on the Dll4 intron 3 in response to Ang1. (A) Confluent HUVECs starved for 12 h were stimulated with vehicle (control) or COMP-Ang1 (C-Ang1) for 30 min. After the stimulation, the cells were fixed with formaldehyde, and the cross-linked chromatin was immunoprecipitated with anti-β-catenin (βCat), anti-NICD (NICD), anti-RBP-J (RBP-J) and control (negative) antibodies. Input (input) and co-immunoprecipitated DNA were used as a template for PCR amplification. PCR amplification was performed using the primers specifically targeting the Dll4 intron 3. (B) Confluent HUVECs starved for 12 h were stimulated with COMP-Ang1 as described in A. Cell lyses were immunoprecipitated with anti-NICD antibody. Immunoprecipitates (IP; NICD) and aliquots of cell lyses (total cell lystate) were subjected to Western blot analysis with anti-β-catenin (βCat), anti-Dll4 (Dll4), anti-NICD (NICD), and anti-tubulin (tubulin) antibodies as indicated at the left.

Figure 7. Extracellular deposition of collagen type IV is increased by Ang1 during endothelial cell tube formation. (A) HUVECs were cultured to form tube structures in 3D collagen matrices for 48 h. During this period, the cells were stimulated with vehicle (-) or COMP-Ang1 (+) in the presence (DAPT) or absence (control) of 20 μM DAPT as indicated at the top. To detect the extracellular deposition of collagen type IV, the cultures were fixed, immunostained with anti-collagen type IV antibody, and visualized with Alexa 488-conjugated secondary antibody. After permeabilization, the cells were further stained with rhodamine-phalloidin to visualize filamentous actin. Alexa 488 and rhodamine images were obtained through a confocal microscope. Alexa 488 (collagen IV) and rhodamine (F-actin) images and the merged images (merge) are shown as indicated at the left. Scale bar. 100 μm. (B) Extracellular deposition of collagen type IV was quantified as described under “Experimental Procedures”. Values are expressed as fold induction relative to that observed in DAPT-untreated cells stimulated with vehicle, and shown as mean ± s.d of five different fields. Similar results were obtained in four independent experiments. (C) HUVECs transfected with either control siRNA (control) or two independent siRNAs targeting Dll4 (Dll4#1 and Dll4#2) were cultured to form tube structures in 3D collagen matrices for 48 h. During this period, the cells were stimulated with vehicle (-) or COMP-Ang1 (+) as indicated at the bottom. The extracellular deposition of collagen type IV was detected and quantified as described in A and B. Values are expressed as fold induction relative to that observed in control siRNA-transfected cells stimulated with vehicle, and shown as mean ± s.d of five different fields. In B and C, significant differences between two groups are indicated as ***P<0.001. n.s. indicates no significance between two groups.

Figure 8. Schematic representation of a proposed model for how Ang1/Tie2 signal induces Dll4 expression to potentiate Notch signal. (A) In the confluent endothelial cells, cell-cell
contact-dependent Notch signaling induces production of NICD, which subsequently binds to the RBP-J binding site in the Dll4 intron 3, leading to Dll4 expression. (B) In the confluent cells, Ang1/Tie2 signal stimulates the transcriptional activity of β-catenin through PI3K/AKT pathway-mediated inhibition of GSK3β. The stabilized β-catenin enhances NICD-mediated Dll4 expression by forming a complex with NICD and RBP-J on the Dll4 intron 3, which augments the Notch signal. Dll4/Notch signal augmented by Ang1/Tie2 signal promotes formation of vascular basement membrane leading to vascular quiescence. In the absence of cell-cell contacts, Dll4 expression is very low due to the lack of Notch signaling. Even if the cells are stimulated with Ang1 under this condition, Dll4 upregulation does not occur, since Ang1/Tie2 signal is unable to induce Dll4 expression in the absence of Notch signaling (not described in this figure).
Figure 1 Zhang et al.
Figure 2 Zhang et al.
Figure 3 Zhang et al.
Figure 4 Zhang et al.
Figure 5 Zhang et al.
Figure 6 Zhang et al.
Figure 7 Zhang et al.
Figure 8 Zhang et al.
Angiopoietin-1/Tie2 signal augments basal Notch signal controlling vascular quiescence by inducing delta-like 4 expression through AKT-mediated activation of \( \beta \)-catenin

Jianghui Zhang, Shigetomo Fukuhara, Keisuke Sako, Takato Takenouchi, Hiroshi Kitani, Tsutomu Kume, Gou Young Koh and Naoki Mochizuki

*J. Biol. Chem.* published online January 6, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.192641

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/01/06/M110.192641.DC1