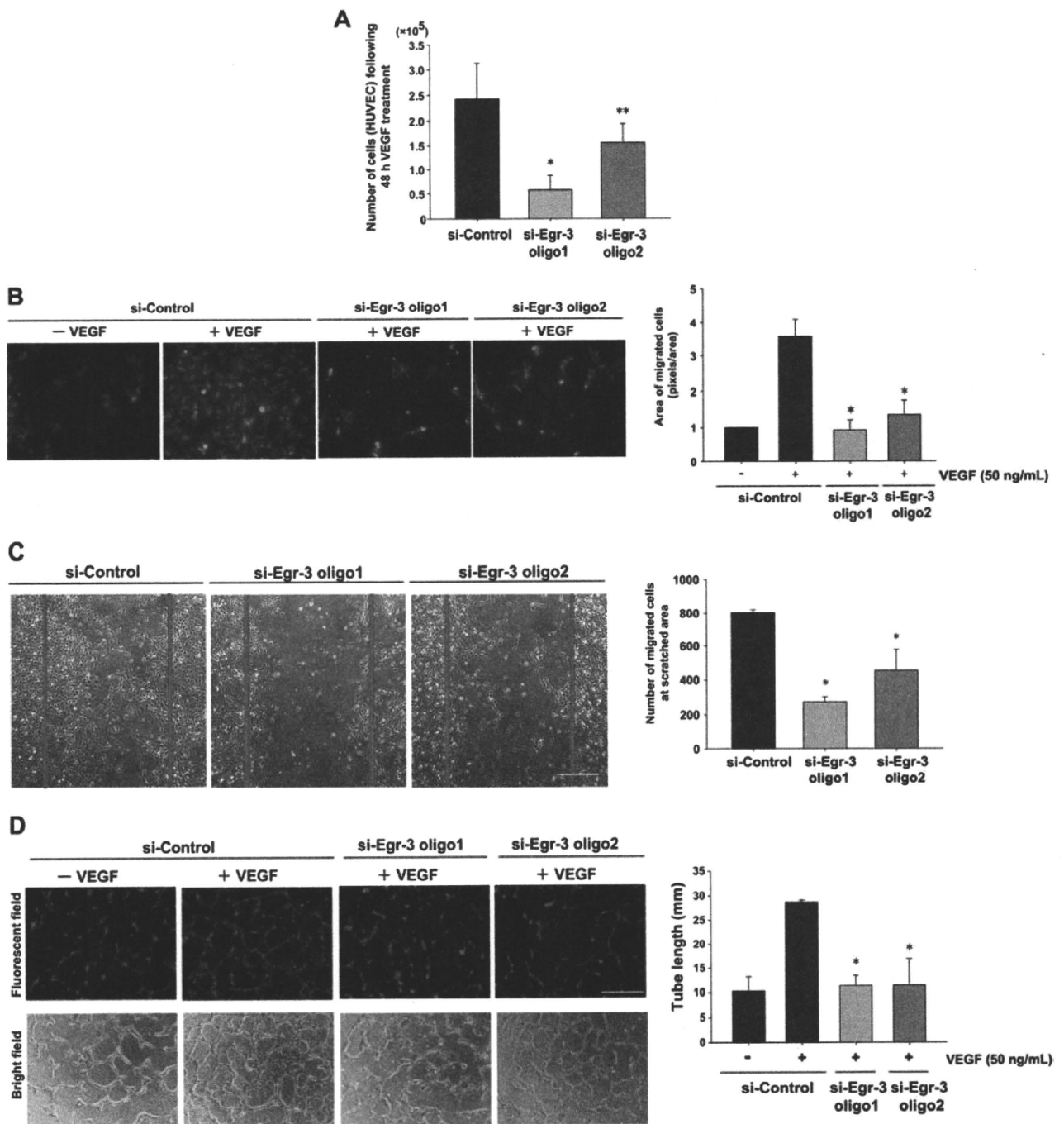**Figure 3. Egr-3 is required for VEGF-mediated induction of proangiogenic and proinflammatory genes in primary human endothelial cells.** (A) Quantitative real-time PCR analysis of Egr-3 mRNA expression in HUVECs transfected with control siRNA (si-Control) or 2 independent siRNAs against Egr-3 (si-Egr-3 oligo1 and -2) and then treated in the presence of 50 ng/mL VEGF for 1 hour. Egr-3 expression is normalized to Cyclophilin A mRNA levels. The results show the mean ± SD of expression relatives to si-Control obtained from at least 5 independent experiments. *$P < .001$ compared with si-Control. (B) Western blot analysis of Egr-3 protein in HUVECs transfected with control siRNA or 2 independent siRNAs against Egr-3 and treated in the absence of presence of 50 ng/mL VEGF for 1 hour. Nucleoporin was used as a loading control. The data are representative of 4 independent experiments. (C) DNA microarrays of HUVECs transfected with si-Control, si-Egr-3 oligo1, or si-Egr-3 oligo2 and treated with 50 ng/mL VEGF for 1 hour. Shown are heat maps of genes whose VEGF response was most profoundly inhibited by si-Egr-3. Transcriptome data were derived from triplicate arrays of VEGF-treated si-Control–transfected cells and duplicate arrays of each of the VEGF-treated si-Egr-3–transfected cells. (D) DNA microarrays of HUVECs transduced with Ad-control or Ad-expressing Egr-1 (Ad-Egr-1) or Egr-3 (Ad-Egr-3; multiplicity of infection = 5). Cells were harvested for RNA at 24 hours after infection. Shown is heat map of siEgr-3–mediated down-regulated genes in Ad-Egr vs Ad-control–transduced cells.

To further address the functional role of Egr-3 in mediating angiogenesis, we used an in vivo Matrigel plug assay. In these experiments, Matrigel impregnated with or without VEGF, Ad-miControl, or Ad-miEgr-3 was implanted subcutaneously into the flank of C57BL/6 mice. Fourteen days later, the Matrigel plugs were harvested, processed for tissues sectioning, and stained for

Figure 4. Egr-3 is required for VEGF-mediated induction of proliferation, migration, and tube formation of primary human endothelial cells. (A) Proliferation assay. HUVECs (10^5) were transfected with si-Control, si-Egr-3 oligo1, or si-Egr-3 oligo2. Cells were serum-starved for 18 hours, incubated in the presence of 50 ng/mL VEGF for 48 hours, and subsequently enumerated. The results show the mean ± SD derived from 6 independent experiments. *$P < .01$; **$P < .04$ compared with si-Control. (B) Migration assay. HUVECs were transfected with si-Control, si-Egr-3 oligo1, or si-Egr-3 oligo2; labeled with PKH2; serum-starved; and plated in upper layer of a Transwell. A total of 50 ng of VEGF (or vehicle) was added to the lower chamber. After 24 hours' incubation, migrated cells were detected by the use of a fluorescent microscope. The number of migrated cells (green) was quantified with image analysis software. Means ± SD were derived from 3 independent experiments, each performed in triplicate. *$P < .01$ compared with si-Control plus VEGF. (C) Scratch wound assay. Confluent HUVECs transfected with si-Control, si-Egr-3 oligo1, or si-Egr-3 oligo2 were scratched with the use of a 1-mm fine tip. After 24 hours of VEGF treatment, the number of cells migrating into the scratched area was counted. Red lines correspond to the borders of the scratched area. The graph shows mean ± SD of migrated cells derived from 4 independent experiments, each performed in triplicate. *$P < .01$, compared with si-Control. (D) Tube-formation assay. HUVECs were transfected with si-Control si-Egr-3 oligo1 or si-Egr-3 oligo2, labeled with PKH26, serum-starved, and grown on a collagen gel in the presence or absence of 50 ng/mL VEGF. Cells were observed under fluorescence (top) or bright field (bottom). White bar indicates 100 μm. The mean ± SD of total tube length was calculated with image analyzer from 3 independent experiments performed in triplicate (bottom bar graph). *$P < .02$ compared the activity from si-Control plus VEGF.

endothelial-restricted PECAM-1 as a marker for blood vessels. In the presence of Ad-miControl, VEGF induced significant new blood vessel formation (Figure 6C middle, shown with dark brown). In contrast, Ad-miEgr-3 treatment markedly attenuated VEGF-mediated neovascularization (Figure 6C left). Evans blue

was used to quantify the extent of neoangiogenesis. Matrigel that contained no virus or Ad-miControl yielded comparable levels of Evans blue (data not shown), whereas Matrigel containing Ad-miEgr-3 demonstrated a marked (56%) reduction in Evans blue content (Figure 6C bottom bar graph).

A
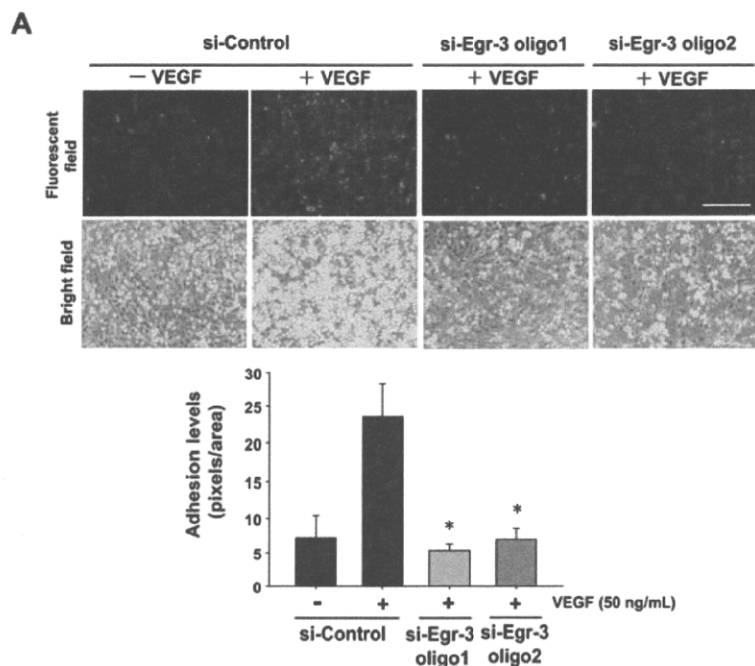
Figure 5. Egr-3 is required for VEGF-mediated induction of leukocyte adhesion to primary human endothelial cells. HUVECs were transfected with si-Control, si-Egr-3 oligo1, or si-Egr-3 oligo2 (A) or preincubated with control IgG or antibodies against E-selectin, VCAM-1 (B) and treated with or without 50 ng/mL VEGF for 6 hours. PKH26-stained U937 monocytes were plated on top of HUVEC monolayers and incubated for 90 minutes. After washing, adhered U937 cells were observed under fluorescent and phase-contrast microscopy. Adhesion levels were quantified on the basis of fluorescent intensity by the use of image analysis software. The mean ± SD was derived from 3 arbitral optical images with 5 independent experiments (bar graph). *$P < .01$ compared with si-Control plus VEGF.

B
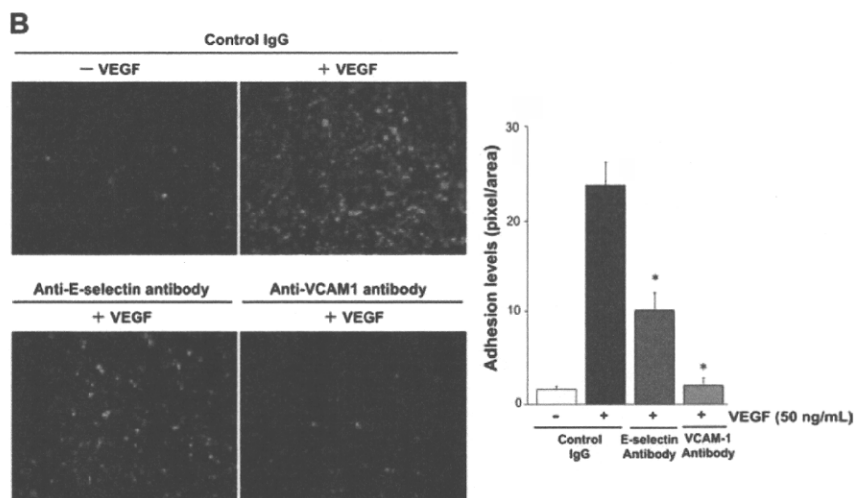


Egr-3 knockdown impairs tumor growth in mice

On the basis of the findings of Egr-3–regulated endothelial cell activation, we hypothesized that Egr-3–dependent angiogenesis may be important for tumor growth. Indeed, immunohistochemical studies of B16-F10 melanoma and Lewis lung carcinoma xenografts revealed colocalization of Egr-3 and PECAM-1 in tumor microvascular endothelium (Figure 7A arrows). B16-F10 melanoma xenografts (10 mm³ in volume) were injected with Ad-miControl or Ad-miEgr-3 (n = 6, each condition), and measured daily for volume. Compared with control, Ad-miEgr-3 treatment resulted in greater than 50% reduction of tumor size beginning 3 days after the injection and lasting until the end of the experiment (day 14; Figure 7B). Moreover, Egr-3 was associated with significantly reduced (88%) blood vessel density (Figure 7C).

To determine whether the attenuation of Egr-3 would suppress infiltration of tumors with inflammatory leukocytes, the xenografts were digested, sectioned, and stained with anti-CD45 antibody. As shown in Figure 7D, Ad-miControl–treated xenografts demonstrated large numbers of CD45-positive cells. Ad-miEgr-3 treatment resulted in greater than 85% reduction of CD45-positive cells per area. The latter effects were accompanied by a parallel reduction in VCAM-1 and E-selectin

levels (supplemental Figure 8 shows VCAM-1). There was no difference in body weight between the 2 groups (data not shown). Together, these findings suggest that Egr-3 attenuates inflammation, neovascularization, and secondary tumor growth in mice.

Discussion

Activation of endothelial cells by extracellular stimuli is a key mechanism underlying the development of vascular disease. In the present study, we used DNA microarrays to analyze the global gene expression profiles of agonist-treated primary human endothelial cells. We have shown that VEGF, thrombin, and TNF-α result in rapid, high-level induction of the early-immediate transcription factors Egr-1 and Egr-3. VEGF-mediated up-regulation of Egr-3 was greater and more sustained compared with Egr-1. Egr-3, in turn was shown to mediate many downstream functions of VEGF, including proinflammatory activity, cell growth and migration in vitro, and neovascularization of Matrigel and tumor growth in vivo.
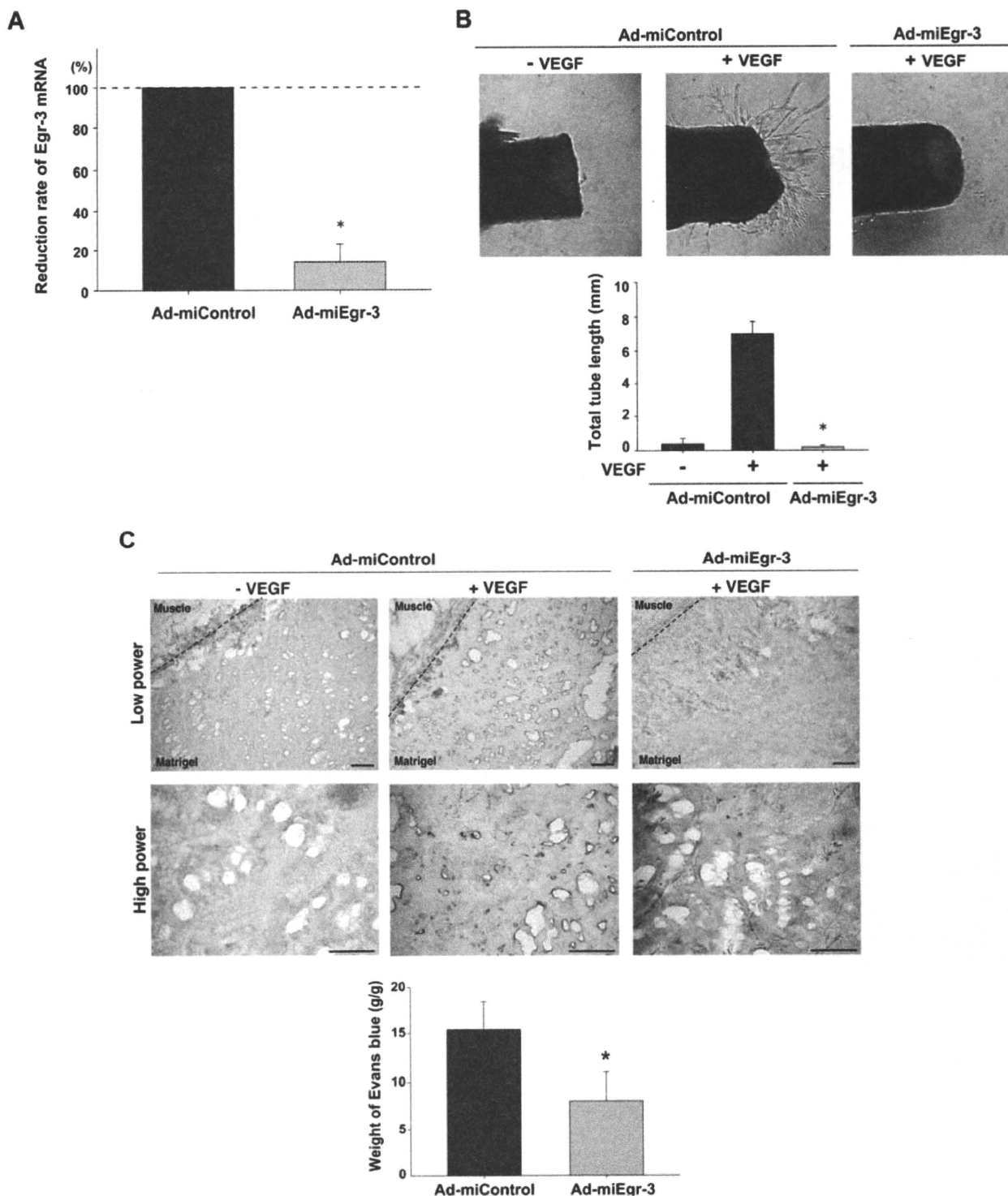
Figure 6. Egr-3 plays a role in VEGF-mediated neovascularization of ex vivo aortic explants and in vivo Matrigel plugs in mice. (A) Quantitative real-time PCR analysis of Egr-3 mRNA expression in VEGF-treated murine MS-1 cells transduced with adenovirus expressing miControl (Ad-miControl) or miRNA against Egr-3 (Ad-miEgr-3). Egr-3 expression is normalized to Cyclophilin A mRNA levels. The results show the mean ± SD of expression levels relative to miControl obtained from 3 independent experiments. *$P < .001$ compared with Ad-miControl. (B) C57/BL6 mice were injected intravenously with 10^9 pfu Ad-miControl or Ad-miEgr-3. At 3 days later, a short segment of the aorta was removed, embedded in Matrigel, and incubated with MCDB131 medium containing 10^9 pfu of Ad-miControl or Ad-miEgr-3. Outgrowth of neovessels from the aorta was observed under phase-contrast microscopy, and tube length was calculated by the use of a cell image analyzer. The mean ± SD were derived from 4 independent experiments. *$P < .001$ compared with Ad-miControl plus VEGF. (C) Matrigel containing 10^9 pfu Ad-miControl or Ad-miEgr-3 was injected subcutaneously into the flank of C57/BL6 mice. Fourteen days later, Matrigel plugs were removed, and sections were immunostained with anti–PECAM-1 antibody. Broken line indicates the boundary between flank muscle and explanted Matrigel. Bar indicates 50 μm. The data are representative of 6 independent experiments. To quantify neoangiogenesis, 1% Evans blue was injected intravenously into mice. At 10 minutes later, Matrigel plugs were removed and incubated in formamide. The amount of Evans blue dye was quantified by OD_{620} and normalized to weight of Matrigel. The mean ± SD were derived from 6 Matrigel plugs in each condition. *$P < .01$ compared with Ad-miControl.
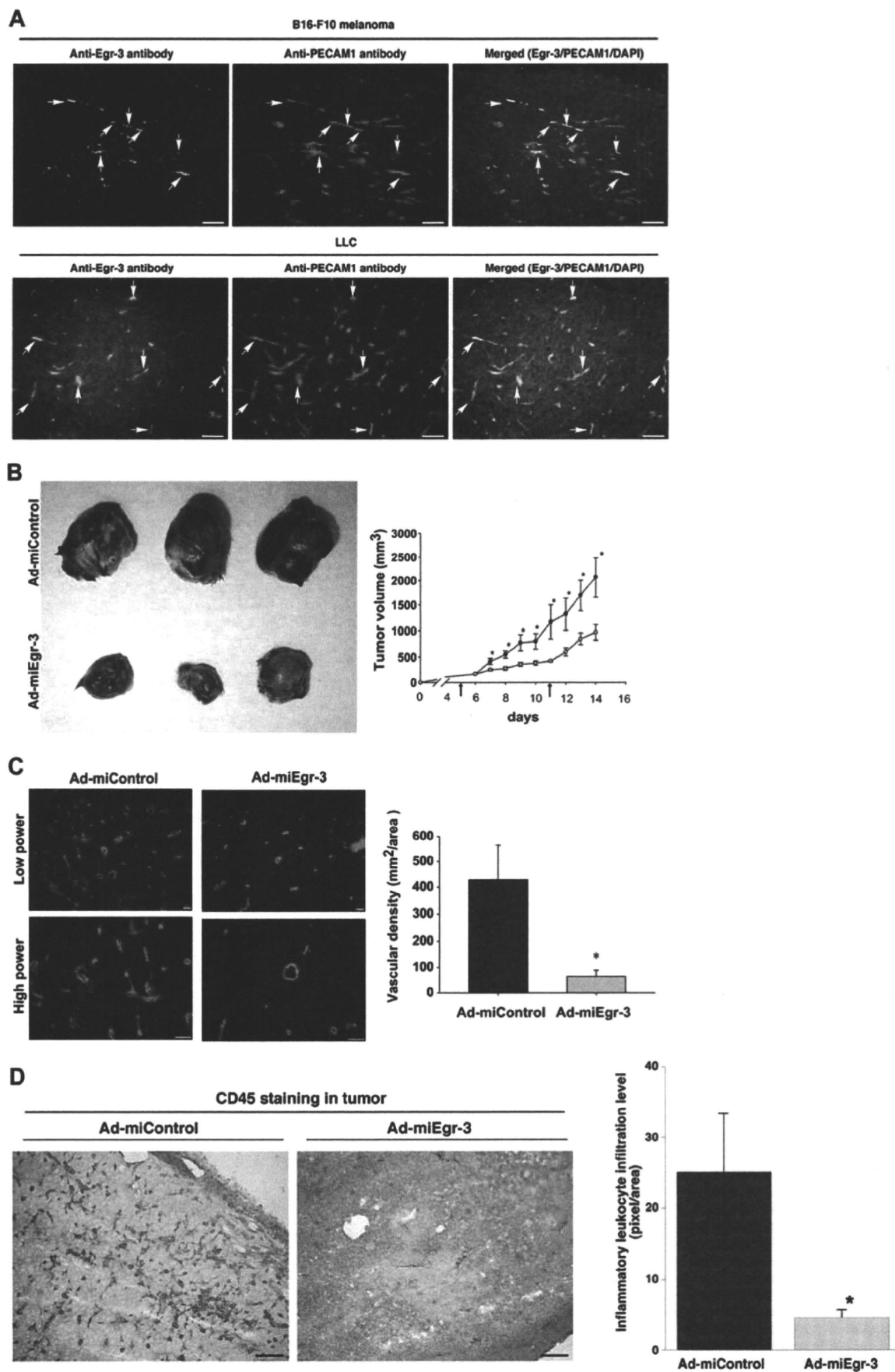
Figure 7.

Previous studies of the Egr-1 promoter have implicated an important functional role for 2 CRE motifs and 5 SRE sites in mediating inducible gene expression.[27-29] In contrast to Egr-1, remarkably little is known about the transcriptional regulation of Egr-3. In T cells, cyclosporine was shown to inhibit Egr-3 induction, suggesting a positive effect of NFAT transcription factors.[7,30] However, cyclosporine A did not influence the induction of Egr-3 in primary human fibroblasts.[30] In endothelial cells, our data implicate a role for NFAT in mediating the positive effect of VEGF on Egr-3 but not Egr-1 expression. Specifically, we have shown that 2 NFAT/NF-κB–like elements in the upstream promoter of Egr-3 are necessary for transcriptional activation, that NFATc1 and NFATc2 each bind to this region, and that NFATc-mediated transactivation of the promoter is dependent on intact NFAT/NF-κB–like elements.

Our data are the first to demonstrate a role for SRF and CREB in mediating inducible expression of Egr-3. Unlike Egr-1, Egr-3 contains only a single SRE consensus motif. We have shown that VEGF induces SRF binding to this element. Moreover, mutation of the SRE abrogated SRF-mediated transactivation of Egr-3. Similar to Egr-1, Egr-3 has 2 CRE motifs. VEGF promoted the binding of phospho-CREB to the CRE motif and mutation of CRE abrogated CREB-mediated Egr-3 promoter activation. Thus, although certain transcriptional control mechanisms are shared between Egr-1 and Egr-3 (eg, they both have functional CRE and SRE sites), others are fundamentally different (eg, the presence or absence of NFAT, and the number of SRE motifs). Further studies are required to determine how these convergent and divergent pathways ultimately govern Egr-dependent endothelial activation.

There is increasing evidence that negative feedback pathways play a key role in modulating the temporal course of endothelial cell activation. For example, we have previously demonstrated that VEGF activation of the calcineurin-NFATc pathway induces expression of DSCR-1s, which in turn negatively feeds back to inhibit NFATc activity.[21,24] Our findings suggest that Egr-3 contributes to the up-regulation of DSCR-1 expression in VEGF-treated endothelial cells and that DSCR-1 in turn inhibits NFAT-dependent activation of Egr-3 (Figure 3C-D).

Previous studies have shown that the corepressors NAB-1 and NAB-2 inhibit Egr-1 activity.[11,12,31] Consistent with these findings, we have demonstrated that NAB-1 and NAB-2 inhibit Egr-1 activity in endothelial cells. In contrast, NAB-2 alone inhibited Egr-3 activity. Given that HUVECs constitutively express NAB-1≫NAB-2, it seems likely that NAB serves as an immediate negative feedback inhibitor of Egr-1. In contrast, overexpression of Egr-3 was shown to induce NAB-2 (and NAB-1) levels, suggesting that NAB-2 may result in delayed feedback inhibition of Egr-3. These observations may explain why VEGF induction of Egr-3 is more profound and sustained compared with Egr-1. Collectively,

our data point to dual feedback control of Egr-3 whereby VEGF induces Egr-3 whereas at the same time triggering expression of NAB-2 and DSCR-1. NAB-2 and DSCR-1, in turn, feed back to inhibit Egr-3 activity and expression, respectively.

In a previous study, DNAzyme-mediated inhibition of Egr-1 blocked serum-mediated proliferation, migration, and tube formation of endothelial cells in vitro and repressed neovascularization in corneas and tumors.[19] Here, we have shown that Egr-3 plays a critical role in VEGF-mediated endothelial cell growth, migration, and tube formation in vitro and angiogenesis in vivo. These findings suggest that both Egr-1 and Egr-3 may contribute to new blood vessel formation. Mice that are null for Egr-1 or Egr-3 develop normally.[6,8,9] Thus, it is possible that these transcription factors compensate for one another during development of the vascular system. Future studies are required to determine whether Egr-3 is required for pathologic angiogenesis in adult mice.

In addition to promoting angiogenesis, VEGF induces a proinflammatory phenotype. For example, VEGF increases permeability, promotes a procoagulant state, and induces the expression of cell adhesion molecules (eg, VCAM-1, E-selectin) and chemokines (eg, CXCL1, interleukin-8).[21] Elevated levels of VEGF play a pathogenic role in sepsis.[32] Our present findings suggest that Egr-3 mediates many of the effects of VEGF on inflammation. siRNA knockdown of Egr-3 significantly inhibited the effect of VEGF on the expression of inflammatory mediators. Moreover, Egr-3 knockdown abolished VEGF-mediated monocyte adhesion. We recently reported that DSCR-1 inhibits VEGF-calcineurin-NFATc induction of inflammatory gene expression and monocyte adhesion,[21,24] in essence phenocopying the effect of Egr-3 knockdown. These data suggest that many of the proinflammatory effects of VEGF-calcineurin-NFATc signaling are mediated by Egr-3.

During the course of our studies, Liu et al[20,22] reported that VEGF induces Egr-3 expression and transcriptional activity in HUVECs. In the latter study, Egr-3 siRNA inhibited VEGF- and FGF-2–mediated cell proliferation and tube formation in cultured endothelial cells.[22] Our data add to these findings in several important ways. First, we compare the dynamics of Egr-3 expression with that of Egr-1. Second, we address the role of Egr-3 multiple types of endothelial cells, including those from veins, arteries, and capillaries. Third, we delineate for the first time the transcriptional mechanisms underlying agonist induction of Egr-3 in endothelial cells. Fourth, we have used DNA microarrays to systematically identify Egr-3–dependent VEGF target genes in endothelial cells. Finally, and most importantly, we provide the first evidence for a role of Egr-3 in mediating angiogenesis in vivo.

An important goal in vascular biology is to understand the molecular mechanisms by which the microenvironment regulates vascular function in space and time. In this study, we have uncovered a key role for Egr-3 in VEGF-mediated signaling, gene transcription, and cell phenotype. On the basis of this knowledge, Egr-3 may represent a novel target in vascular diseases.

Figure 7. Egr-3 contributes to B16-melanoma tumor progression in mice. (A) B16-F10 melanoma cells and Lewis lung carcinoma (LLC) cells were injected subcutaneously into C57/BL6j mice. After 14 days, solid tumor xenografts (1 mm³) were removed, cryosectioned, and immunostained with anti-Egr-3 antibody (left) or anti–PECAM-1 antibody (middle). Merged images with DAPI are shown on the right. White bar indicates 50 μm. (B) Gross pathology of B16-F10 melanoma from Ad-miControl– or Ad-miEgr-3–injected groups immediately after resection (left). Graph shows tumor volume in Ad-miControl–injected (●) or Ad-miEgr-3–injected (○) mice. Arrow indicates the day of adenovirus injection. Data represent mean ± SEM n = 6. *P < .01 compared with Ad-control and Ad-miEgr3. (C) Representative cryosections of B16-F10 melanoma stained with anti–PECAM-1 antibody (left). Vascular density was calculated on the basis of the number of PECAM-1–positive cells. The mean ± SD were derived from 3 optical images of 3 separate xenografts in each condition (right). *P < .001 compared with Ad-miControl. (D) Representative cryosections of B16-F10 melanoma stained with anti-CD45 antibody. Inflammatory leukocytes infiltration levels were calculated on the basis of numbers of CD45-positive cells per optical field (×200, Leica [DMLB]). The mean ± SD were derived from 6 optical images of 6 independent xenografts in each condition (bottom). *P < .001 compared with Ad-miControl.

Acknowledgments

Authorship

Contribution: J.-i.S. performed research and analyzed data; T.H. and T.K. contributed vital new reagents; W.C.A. designed research and wrote the paper; and T.M. designed research, analyzed data, and wrote the paper.

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Review

Angiopoietin-1/Tie2 receptor signaling in vascular quiescence and angiogenesis

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Summary. Angiopoietin (Ang) 1 is a ligand for endothelium-specific receptor tyrosine kinase Tie-2. In adult vasculature, Ang1/Tie2 signaling is thought to regulate both maintenance of vascular quiescence and promotion of angiogenesis. However, it has been unknown how Tie2 signal regulates these distinct biological functions. Recently, we and Alitalo's group have clarified that Ang1 assembles distinct Tie2 signaling complexes in either presence or absence of endothelial cell-cell adhesions. Ang1 induces *trans*-association of Tie2 at cell-cell contacts, whereas Tie2 is anchored to the extracellular matrix (ECM) by Ang1 at the cell-substratum interface. *Trans*-associated Tie2 and ECM-anchored Tie2 activate distinct signaling pathways. In this review, we discuss how Ang1/Tie2 signal regulates both maintenance of vascular quiescence and promotion of angiogenesis, especially focusing on the roles of *trans*-associated Tie2 and ECM-anchored Tie2.

Key words: Angiopoetin-1, Tie2, Quiescence, Angiogenesis, KLF2

Introduction

During embryogenesis, vascular blood vessels form through both vasculogenesis and angiogenesis (Yancopoulos et al., 2000). After birth, most blood vessels in the body become quiescent. However, angiogenesis occurs in some specific tissues, such as the cycling ovary and the placenta during pregnancy, and is promoted during wound healing and repair (Peters et al.,

2004; Adams and Alitalo, 2007). Therefore, angiogenesis is implicated in the pathogenesis of various diseases, such as cancer, arthritis, diabetic retinopathy and macular degeneration (Folkman, 2001; Bergers and Benjamin, 2003; Peters et al., 2004). In the quiescent vessels, the endothelial cells lining vessels tightly contact with each other and do not proliferate and migrate (Dejana, 2004; Dejana et al., 2008, 2009). On the other hand, during angiogenesis, the cells that lose cell-cell contacts are allowed to proliferate and migrate, thereby resulting in sprouting and branching of new capillaries from the pre-existing vessels (Shibuya and Claesson-Welsh, 2006; Wallez et al., 2006; Adams and Alitalo, 2007; Lamalice et al., 2007).

Whether endothelial cells remain quiescent or undergo angiogenesis is determined by the balance between angiostatic and angiogenic signals. Angiogenic signals are mainly induced by endothelial-specific receptor tyrosine kinases (RTKs) and their ligands; vascular endothelial growth factor (VEGF)-VEGF-receptor (VEGFR), ephrin-Eph receptor, and fibroblast growth factor (FGF)-FGF receptor (Yancopoulos et al., 2000; Jones et al., 2001; Shibuya and Claesson-Welsh, 2006; Adams and Alitalo, 2007; Kuijper et al., 2007). On the other hand, angiogenesis is negatively regulated by the signals evoked by angiostatic factors such as thrombospondin, angiostatin, endostatin and vasohibin (Dawson et al., 1999; Rege et al., 2005; Sato and Sonoda, 2007; Tabruyn and Griffioen, 2007). Interestingly, angiopoietin (Ang) 1/Tie2 receptor signal is known to regulate both vascular quiescence and angiogenesis (Jones et al., 2001; Peters et al., 2004; Brindle et al., 2006; Eklund et al., 2006; Fiedler and Augustin, 2006; Augustin et al., 2009; Fukuhara et al., 2009). However, it was unknown how Ang1/Tie2 signal regulates these paradoxical actions. Recently, we and Alitalo's group have clarified that Ang1 and Tie2 form distinct signaling complexes at cell-cell and cell-

extracellular matrix (ECM) contacts in the presence or absence of inter-endothelial cell adhesions, respectively (Fukuhara et al., 2008; Saharinen et al., 2008). In this review, we further discuss the significance of these distinct signaling complexes in vascular quiescence and angiogenesis by referring to the new findings thereafter.

Ang-Tie receptor system

Tie1 and Tie2 belong to a family of Tie RTKs found to be expressed in endothelial cells. The Ang family consists of Ang1, Ang2, Ang3 and Ang4. Of them, Ang1 and Ang2 are well characterized. Ang1 binds to Tie2 and stimulates its kinase activity, whereas Ang2 is known to act as a context-dependent agonist or antagonist for Tie2 (Augustin et al., 2009). Mice lacking either *Ang1* or *Tie2* exhibit embryonic lethality due to defective vascular development (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). The vessels of both mutant animals show fewer number of endothelial cells and a defect in the adhesions between endothelial cells and the underlying mural cells. Therefore, Ang1/Tie2 signal is thought to play a crucial role in the maturation and stabilization of the embryonic vasculature. Tie1-deficient embryos also exhibit compromised vascular maintenance owing to impaired endothelial integrity (Puri et al., 1995, 1999; Sato et al., 1995). Although none of the Ang family members directly bind to Tie1, it has been reported that Tie1 can interact with Tie2 and signal in a heterodimeric complex in vitro (Saharinen et al., 2005). However, it has been also reported that Tie1 negatively regulates Tie2-driven signaling and endothelial cell survival (Marron et al., 2007; Yuan et al., 2007). Thus, how Tie1 functionally interacts with Tie2 and is involved in developmental vascular formation remains unclear.

Role of Ang1/Tie2 signal in vasculature quiescence

Tie2 is expressed and tyrosine-phosphorylated in endothelium of most adult tissues (Schlaeger et al., 1997; Wong et al., 1997). Ang1 is produced primarily by peri-endothelial support cells such as pericytes and vascular smooth muscle cells (Davis et al., 1996; Stratmann et al., 1998; Sugimachi et al., 2003). Therefore, it is believed that activation of Tie2 by Ang1 secreted from peri-endothelial cells contributes to the maintenance of vascular quiescence.

The most important cellular function of Ang1/Tie2 signal is its ability to promote cell survival, which is essential for the maintenance of vascular quiescence. Consistently, cartilage oligometric matrix protein (COMP)-Ang1, a potent and stable Ang1 variant, protects against radiation-induced endothelial cell apoptosis in vivo (Cho et al., 2004). Ang1 stimulates phosphatidylinositol 3-kinase (PI3K) activity through recruitment of its regulatory p85 subunit into the phosphorylated tyrosine residue 1102 in the cytoplasmic domain of Tie2, which subsequently leads to activation of cell survival kinase AKT (Kontos et al., 1998; Jones et al., 1999; Kim et al., 2000b; Papapetropoulos et al., 2000). Ang1/Tie2 signal-induced AKT activation results in phosphorylation and inhibition of the forkhead transcription factor Foxo1 (Daly et al., 2004). Since Foxo1 up-regulates expression of genes associated with vascular destabilization/remodeling and endothelial cell apoptosis, inhibition of Foxo1 by Ang1 may contribute to endothelial cell survival and blood vessel stability. In addition, Ang1-induced PI3K/AKT pathway induces up-regulation of the apoptosis inhibitor, survivin, in endothelial cells (Papapetropoulos et al., 2000). Furthermore, Tadros et al. have also suggested the role of A20 binding inhibitor of nuclear factor-κB (NF-κB) activation-2 (ABIN-2) in Ang1-induced cell survival (Tadros et al., 2003).

Ang1 potentiates endothelial barrier function by strengthening inter-endothelial cell adhesions. Mice over-expressing Ang1 develop vessels resistant to inflammatory agent-induced vascular leakage (Thurston et al., 1999, 2000). Consistently, Ang1 augments inter-endothelial cell adhesions regulated by vascular endothelial (VE)-cadherin, leading to the decreased permeability of cultured endothelial cell monolayer (Gamble et al., 2000). VEGF weakens VE-cadherin-dependent cell adhesions through the Src tyrosine kinase pathway (Paul et al., 2001; Weis et al., 2004; Gavard and Gutkind, 2006). Recently, Gavard et al. reported that Ang1/Tie2 signal counteracts Src activation by VEGF, thereby inhibiting VEGF-induced endothelial barrier dysfunction (Gavard et al., 2008). Moreover, it has been reported that Ang1 inhibits VEGF-induced Ca^{2+} influx through the transient receptor potential canonical-1 to preserve endothelial barrier function (Jho et al., 2005). Two members of Rho family small GTPases, Rac and Rho, have opposite effects on endothelial barrier function; the former increasing barrier function and the latter reducing barrier function. Mammoto et al. have reported that Rac-mediated inhibition of Rho through p190RhoGAP is critical for the protective effect of Ang1 against endotoxin-induced vascular leakage (Mammoto et al., 2007). In addition, it has been suggested that sphingosine kinase-1, a key enzyme catalyzing the formation of sphingosine-1-phosphate, is responsible for the vessel-stabilizing effect of Ang1 (Li et al., 2008).

Ang1/Tie2 signal has been shown to exert potent anti-inflammatory effects. Ang1 attenuates VEGF-induced adhesion of leukocytes to endothelial cells by inhibiting expression of cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Kim et al., 2001). In addition, tumor necrosis factor-α (TNF-α)- and VEGF-induced expression of tissue factor is also reduced by Ang1 stimulation in human umbilical vein endothelial cells (Kim et al., 2002). It has been suggested that these anti-inflammatory effects of Ang1 are mediated by the PI3K/AKT signaling pathway (Kim et al., 2001, 2002). In addition, Ang1 induces recruitment of ABIN-2 to Tie2, which in turn inhibits

NF-κB, a transcription factor involved in inflammatory gene expression (Hughes et al., 2003).

Role of Ang1/Tie2 signal in angiogenesis

In addition to maintenance of vascular quiescence, Tie2 signaling is involved in physiological and pathological angiogenesis in adult vasculature. Tie2 is highly expressed in the endothelium of adult tissues undergoing angiogenesis such as ovary and uterus, and up-regulated in the neovessels during wound healing (Wong et al., 1997; Peters et al., 2004). In addition, Tie2 expression is elevated in the endothelium of angiogenic tumor vessels, suggesting the role of Tie2 in tumor angiogenesis (Peters et al., 2004). Consistently, it has been reported that soluble Tie2 receptor that is capable of blocking Tie2 activation by Ang1 reduced tumor growth and metastasis by inhibiting tumor angiogenesis in vivo (Lin et al., 1997, 1998).

COMP-Ang1 is shown to promote angiogenesis in the mouse corneal micropocket assay (Cho et al., 2004). Another report also indicates that VEGF-induced angiogenesis is potentiated by Ang1 in vivo (Asahara et al., 1998). However, Nambu et al. reported that increased expression of Ang1 in the retina significantly suppresses ischemia- or VEGF-induced renal neovascularization (Nambu et al., 2004, 2005). These results imply that Ang1/Tie2 signal may exert both angiogenic and angiostatic functions in a context-dependent manner.

Angiogenesis requires proliferation and migration of vascular endothelial cells. Although Ang1 induces activation of extracellular signal-regulated kinase (ERK) 1/2, a key regulator of cell proliferation (Harfouche et al., 2003; Brindle et al., 2006), the mitogenic effect of Ang1/Tie2 signal is still controversial (Witzenbichler et al., 1998; Kanda et al., 2005; Brindle et al., 2006). In contrast, Ang1/Tie2 signal potently promotes endothelial cell migration (Witzenbichler et al., 1998; Fujikawa et al., 1999; Cascone et al., 2003; Chen et al., 2004). Activated Tie2 associates with the adaptor protein, Dok-R (also known as Dok-2), which in turn establishes binding sites for Nck and p21-activated kinase (PAK). The Dok-R/Nck/PAK pathway is involved in Ang1-induced endothelial cell migration (Jones and Dumont, 1998; Master et al., 2001; Jones et al., 2003). Adaptor protein ShcA is also recruited to the activated Tie2 to enhance endothelial cell migration (Audero et al., 2004). Activation of PI3K by Ang1/Tie2 signal is involved not only in cell survival, but also in cell motility. Endothelial cell migration induced by Ang1 may be mediated by PI3K-dependent activation of focal adhesion kinase (FAK) and Rho family small GTPases Rho and Rac (Kim et al., 2000a; Cascone et al., 2003). In addition, the production of nitric oxide and reactive oxygen species is also suggested to be involved in Ang1-induced cell migration (Babaei et al., 2003; Chen et al., 2004, 2006; Kim et al., 2006).

Distinct signaling pathways induced by *trans*-associated Tie2 and extracellular matrix-anchored Tie2

Endothelial cell-cell adhesions are essential for maintenance of vascular quiescence. On the other hand, the endothelial cells undergoing angiogenesis weaken or lose the cell-cell contacts. Recently, we and Alitalo's group have clarified that Ang1-activated Tie2 forms distinct signaling complexes at cell-cell and cell-ECM contacts in the presence or absence of inter-endothelial cell adhesions, respectively (Fukuhara et al., 2008; Saharinen et al., 2008). In confluent endothelial cells, Tie2 is recruited to cell-cell contacts in response to Ang1. At the cell-cell contacts, oligomerized Ang1 bridges Tie2 expressed in adjoining cells, resulting in the formation of homophilic Tie2 *trans*-association. In clear contrast, in the absence of cell-cell adhesions, Tie2 is anchored to the cell-ECM interface through Ang1 binding to the ECM.

To further investigate the biological significance of distinct Tie2 localizations, we examined the downstream signaling pathways induced by Ang1 in the presence or absence of cell-cell contacts. Among Tie2-mediated signaling molecules, AKT and ERK1/2 are important for cell survival and cell migration/proliferation, respectively. AKT is preferentially activated by Ang1 in the presence of cell-cell contacts (Fukuhara et al., 2008). Consistently, Alitalo's group has also reported that phosphorylation of endothelial nitric oxide synthase (eNOS), a substrate for AKT, is induced only in cell-cell junctions, but not in motile cells without cell-cell contacts (Saharinen et al., 2008). In contrast, Ang1 preferentially activates ERK1/2 signaling pathway in the absence of cell-cell contacts (Fukuhara et al., 2008). In the cell lacking cell-cell adhesions, Ang1 induces integrin-dependent focal complex formation, leading to activation of FAK. FAK is involved in integrin-mediated ERK1/2 activation. Thus, the preferential activation of ERK1/2 by Ang1 occurs partly through integrin-dependent FAK activation. These findings imply that *trans*-associated Tie2 at cell-cell contacts and ECM-anchored Tie2 at cell-substratum contacts induce preferential activation of AKT and ERK1/2 pathways, respectively.

Vascular quiescence regulated by *trans*-associated Tie2

The preferential activation of AKT by *trans*-associated Tie2 may contribute to the vessel-stabilizing effect of Ang1 (Fig. 1). AKT phosphorylates the forkhead transcription factor Foxo1 and eNOS, which play important roles in endothelial functions (Daly et al., 2004; Chen et al., 2005). Previous work has suggested that AKT-Foxo1 pathway is involved in Ang1-induced endothelial cell survival and blood vessel stability (Daly et al., 2004). In addition, it has been reported that AKT-

eNOS pathway is required for vascular maturation (Chen et al., 2005). Since Ang1 preferentially induces phosphorylation of Foxo1 and eNOS in the presence of cell-cell contacts, *trans*-associated Tie2 may mediate vessel stabilization through the AKT-Foxo1 and AKT-eNOS signaling pathways.

Tie2 is known to interact with vascular endothelial protein tyrosine phosphatase (VE-PTP), which plays an important role in maintenance of vascular stability (Fachinger et al., 1999). VE-PTP also associates with VE-cadherin and reduces the tyrosine phosphorylation of VE-cadherin, thereby restricting vascular permeability (Nawroth et al., 2002; Nottebaum et al., 2008). Analysis of VE-PTP-deficient mice also reveals the requirement of VE-PTP for developmental vascular formation (Dominguez et al., 2007). Alitalo's group has reported that VE-PTP co-localizes with Tie2 at cell-cell contacts, but not at cell-ECM contacts (Fig. 1) (Saharinen et al., 2008). Furthermore, it has been suggested that Tie2 and VE-PTP cooperatively reduce the permeability of endothelial cell monolayer (Saharinen et al., 2008). Thus, trans-associated Tie2 may form a complex with VE-PTP at cell-cell contacts, thereby maintaining vascular quiescence.

Angiogenesis regulated by extracellular matrix-anchored Tie2

In contrast to the effect of *trans*-associated Tie2 on vascular quiescence, ECM-anchored Tie2 may promote angiogenesis (Fig. 2). Alitalo's group noticed that in migrating endothelial cells lacking the cell-cell adhesions, Dok-R is phosphorylated by Ang1 and colocalized with Tie2 at the cell-ECM contacts in the rear of the motile cells, suggesting the involvement of Dok-R in endothelial cell migration upon Ang1 stimulation (Saharinen et al., 2008). We also found that Ang1-induced endothelial cell migration is prevented by inhibition of the ERK1/2 pathway, suggesting that ECM-anchored Tie2 enhances endothelial motility through the ERK1/2 signaling pathway (Fukuhara et al., 2008). Our results are consistent with the previous report that the ERK 1/2 pathway is involved in endothelial cell migration and angiogenesis (Eliceiri et al., 1998).

Differential gene regulation by *trans*-associated Tie2 and extracellular matrix-anchored Tie2

We employed DNA microarray analyses to identify the genes regulated by Ang1/Tie2 signal in the presence or absence of cell-cell contacts. Ang1 regulates distinct sets of genes in the presence or absence of cell-cell contacts (Fukuhara et al., 2008). Interestingly, in the presence of cell-cell contacts, Ang1 up-regulates the genes involved in vascular stability, which include *Krüppel-like factor 2* (KLF2), *zinc finger protein 36, C3H type-like 2 (TIS11d), connexin 40 (Cx40), delta-like1 (Dll1)* and *delta-like 4 (Dll4)*. KLF2 is known to maintain vascular quiescence by negatively regulating inflammation and angiogenesis (SenBanerjee et al., 2004; Bhattacharya et al., 2005; Dekker et al., 2006; Lin et al., 2006). (We discuss KLF2 later in this manuscript). TIS11d interacts with 3'-untranslated region of VEGF mRNA and decreases its stability, leading to down-
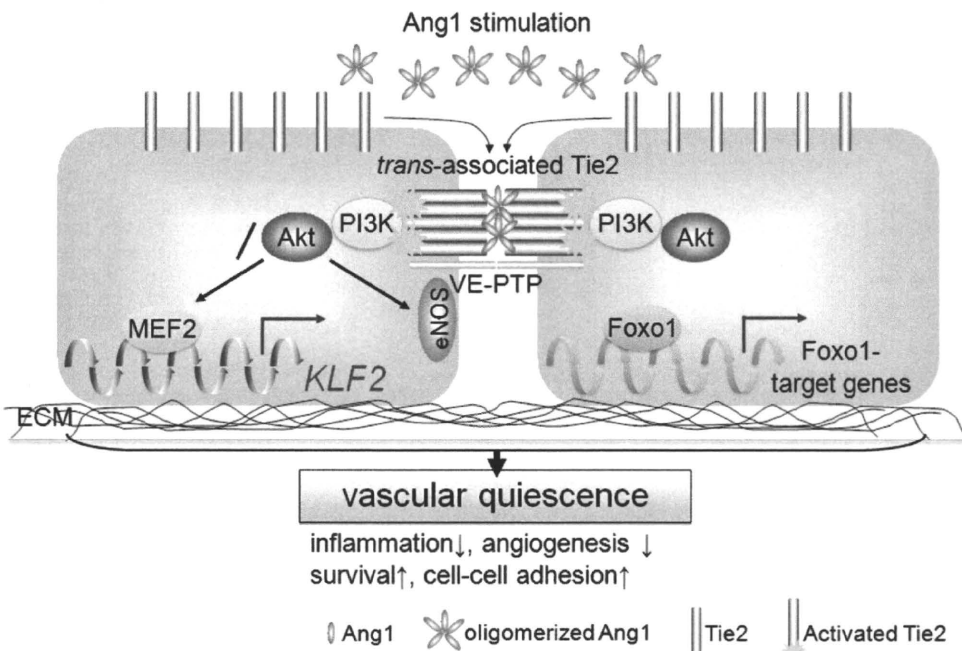
Fig. 1. Angiopoietin-1/Tie2-mediated signaling pathways involved in maintenance of vascular quiescence? Angiopoietin (Ang) 1 induces *trans*-association of Tie2 at cell-cell contacts, which leads to preferential activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Activated AKT stimulates transcriptional activity of myocyte enhancer factor 2 (MEF2) to induce expression of Krüppel-like factor 2 (KLF2), which in turn inhibits inflammation and angiogenesis. At the same time, the activated AKT phosphorylates and inhibits forkhead transcription factor Foxo1, thereby down-regulating the expression of Foxo1 target genes involved in endothelial cell apoptosis and vascular remodeling. AKT also activates eNOS, contributing to vascular maturation. *Trans*-associated Tie2 associates with vascular endothelial protein tyrosine phosphatase (VE-PTP), thereby enhancing endothelial cell-cell adhesions. ECM, extracellular matrix.

regulation of VEGF protein expression (Ciais et al., 2004). Since recent work suggests the role of endothelium-expressed VEGF in vascular homeostasis (Lee et al., 2007), the Ang1/Tie2 signal may regulate a cell-autonomous VEGF signaling pathway in endothelial cells through TIS11d. Cx40 is the predominant gap junction protein expressed in vascular endothelium and is required for inter-endothelial cell communication (Sohl and Willecke, 2004). Dll1 and Dll4, ligands for Notch receptor, have been reported to be involved in angiogenic sprouting (Noguera-Troise et al., 2006; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007; Phng and Gerhardt, 2009). Within angiogenic sprout, VEGF induces Dll4 expression in endothelial tip cells, which leads to Notch activation in adjacent stalk cells and the suppression of VEGF receptor expression, thereby establishing the adequate ratio between tip and stalk cells required for correct sprouting and branching patterns. Yana et al. previously found that Tie2 is expressed in endothelial stalk cells, but not in tip cells (Yana et al., 2007). Thus, Dll1 and Dll4 expression induced by trans-associated Tie2 may lead to inhibition of angiogenic sprouting in stalk cells through the Notch signaling pathway.

KLF2 induction by *trans*-associated Tie2

As described above, DNA microarray analyses revealed that Ang1 selectively induces KLF2 expression in the presence of cell-cell contacts. KLF2 belongs to a zinc-finger family of transcription factors and plays an important role in vascular stability (Atkins and Jain, 2007). In adult endothelium, KLF2 is induced by laminar shear stress and exerts atheroprotective effects (Dekker et al., 2002, 2005; Huddleson et al., 2004). KLF2 inhibits cytokine-induced expression of pro-inflammatory target genes such as VCAM-1 and E-selectin (SenBanerjee et al., 2004; Lin et al., 2006).

Furthermore, KLF2 down-regulates expression of VEGFR2, thereby inhibiting VEGF-induced angiogenesis and hyperpermeability (Bhattacharya et al., 2005; Dekker et al., 2006). Interestingly, Ang1 also acts as an anti-inflammatory and anti-permeability factor (Gamble et al., 2000; Kim et al., 2001; Brindle et al., 2006). Therefore, these findings prompted us to examine the involvement of KLF2 in Ang1/Tie2 signal-dependent vascular stabilization. Consistently, we found that induction of KLF2 by Ang1 is essential for inhibition of VEGF-mediated inflammation (Sako et al., 2009). Furthermore, we investigated the intracellular signaling pathways involved in Ang1-induced KLF2 expression (Sako et al., 2009). It has been previously reported that activation of myocyte enhancer factor 2 (MEF2) transcription factor by ERK5 MAP kinase is involved in shear stress-induced KLF2 expression (Kumar et al., 2005; Parmar et al., 2006). Similarly, our analysis showed that MEF2 is responsible for Ang1-induced KLF2 expression. However, siRNA knockdown experiments revealed the dispensability of ERK5 for Ang1-induced KLF2 expression. Instead, we found that PI3K/AKT signaling pathway is involved in transcriptional activation of MEF2 by Ang1 (Sako et al., 2009). Collectively, *trans*-associated Tie2 stimulates transcriptional activity of MEF2 through a PI3K/AKT pathway, thereby inducing KLF2 expression and contributing to the maintenance of vascular quiescence (Fig. 1).

How is the transition between distinct Tie2 signaling complexes regulated?

Ang1 maintains the vascular quiescence through *trans*-associated Tie2, whereas ECM-anchored Tie2 by Ang1 promotes angiogenesis (Fukuhara et al., 2008; Saharinen et al., 2008). However, it remains unknown how the transition between *trans*-associated Tie2 and
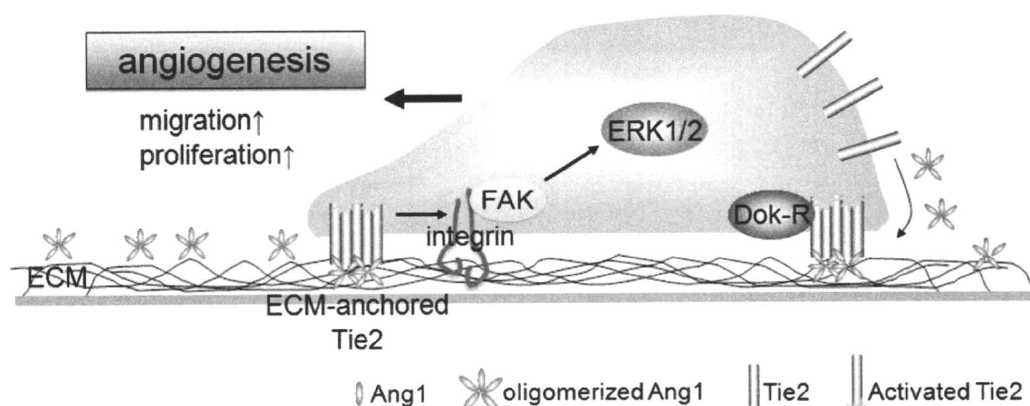
Fig. 2. Angiopoietin-1/Tie2-mediated signaling pathways involved in angiogenesis Angiopoietin (Ang) 1 is capable of binding to extracellular matrix (ECM). Therefore, Tie2 is anchored by ECM-bound Ang1 to cell-substratum interface in the absence of cell-cell contacts. ECM-anchored Tie2 induces integrin-dependent focal complex formation, leading to activation of focal adhesion kinase (FAK). The activated FAK subsequently induces the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway involved in endothelial cell migration and proliferation, thereby promoting angiogenesis. ECM-anchored Tie2 also regulates endothelial cell motility through an adaptor protein, Dok-R.

ECM-anchored Tie2 is regulated. Downstream signaling of Ang1/Tie2 is dependent on the presence or absence of cell-cell contacts. Therefore, disruption of endothelial cell-cell adhesions may trigger the formation of ECM-anchored Tie2 and the subsequent induction of angiogenic signaling. VEGF not only promotes angiogenesis, but also induces hyperpermeability by disrupting the cell-cell adhesions (Weis and Cheresh, 2005; Gavard and Gutkind, 2006; Shibuya and Claesson-Welsh, 2006). Thus, it is possible that, when cell-cell adhesions are disrupted by VEGF, Ang1 induces formation of ECM-anchored Tie2 and accelerates angiogenesis cooperatively with VEGF (Fig. 3). Consistently, it has been reported that VEGF-induced angiogenesis is potentiated by Ang1 *in vivo* (Asahara et al., 1998). However, since Ang1 also counteracts VEGF-mediated disruption of cell-cell contacts, the synergistic or counteractive effect of VEGF and Ang1 may be determined by a balance between Ang1 and VEGF levels and the timing of cell-cell destruction (Fig. 3).

Ang2 may also play an important role in transition between *trans*-associated Tie2 and ECM-anchored Tie2. Ang2 is produced by endothelial cells under hypoxic conditions and antagonizes Ang1-induced Tie2 activation, thereby destabilizing the blood vessels (Fiedler and Augustin, 2006; Augustin et al., 2009). In the destabilized vessels where endothelial cells lose the cell-cell contacts, activation of ECM-anchored Tie2 by Ang1 may promote angiogenesis cooperatively with VEGF produced from hypoxic tissues.

Conclusion

We summarized the recent findings on the mechanisms by which Ang1/Tie2 signal regulates vascular quiescence and angiogenesis. We and Alitalo's group have clearly demonstrated that the dual functions of Ang1/Tie2 signal are mediated through Ang1-assembled Tie2 signaling complexes at cell-cell and cell-ECM contacts (Fukuhara et al., 2008; Saharinen et al., 2008). These studies have, however, also raised a number of new questions. For instance, how does *trans*-associated Tie2 specifically activate the AKT signaling pathway responsible for vascular maintenance? Does *trans*-association of Tie2 physically enhance vascular integrity by acting as a cell-adhesion molecule? How does ECM-anchored Tie2 promote assembly of integrin-mediated cell adhesions and regulate angiogenesis? In addition, it has to be addressed whether Ang1/Tie2 signal utilizes these molecular mechanisms to regulate vascular quiescence and angiogenesis in vivo. Solving these questions will be useful for the development of
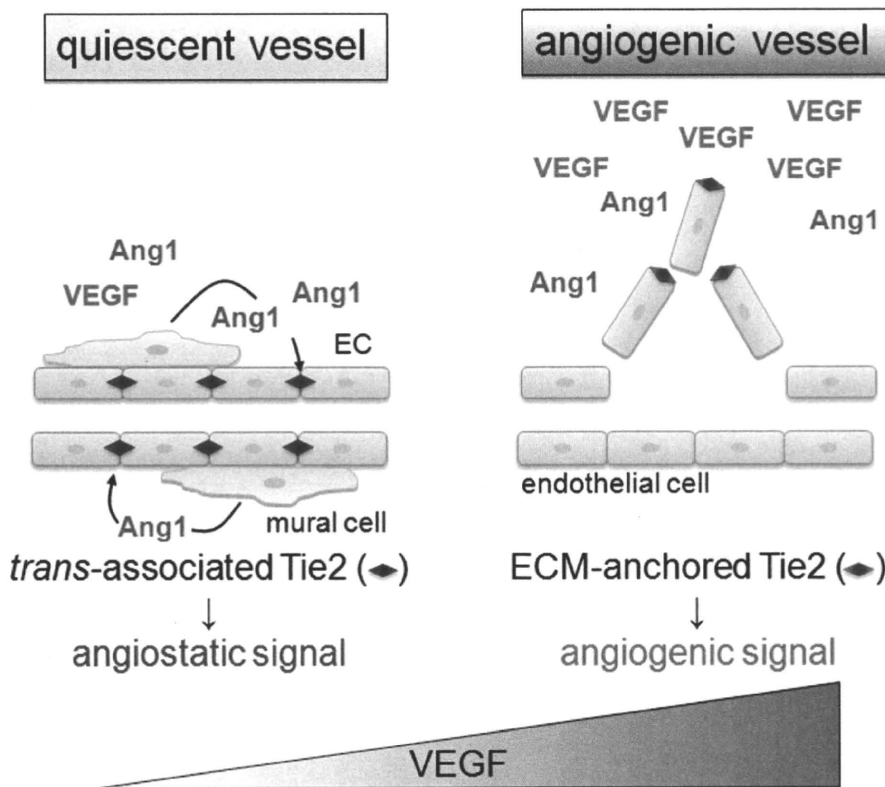
Fig. 3. Proposed model for the dual effects of angiopoietin-1/Tie2 signaling on vascular quiescence and angiogenesis (Left) In quiescent vessels, angiopoietin (Ang) 1 released from mural cells induces trans-association of Tie2 at endothelial cell-cell contacts, which activates the angiostatic signaling pathway to maintain vascular quiescence. Since only a limited amount of vascular endothelial growth factor (VEGF) exists in this condition, VEGF-induced angiogenesis is suppressed by the Ang1/Tie2 signal. (Right) However, once VEGF is released from ischemic tissues, detachment of mural cells from endothelial cells and disruption of inter-endothelial cell adhesions occur. In this situation, Tie2 is anchored to extracellular matrix-bound Ang1 and activates the angiogenic signaling pathway, thereby promoting angiogenesis cooperatively with VEGF.

novel pro- and anti-angiogenic therapies.

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Angiopoietin-1/Tie2 Signal Augments Basal Notch Signal Controlling Vascular Quiescence by Inducing Delta-Like 4 Expression through AKT-mediated Activation of β-Catenin*[S]

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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 up-regulates Notch ligand delta-like 4 (Dll4) only in the presence of cell-cell contacts. Because Dll4/Notch signal restricts sprouting angiogenesis and promotes vascular stabilization, we investigated the mechanism of how the Ang1/Tie2 signal induces Dll4 expression to clarify the role of the Dll4/Notch signal in Ang1/Tie2 signal-mediated vascular quiescence. Under confluent endothelial cells, the basal Notch signal was observed. Ang1, moreover, induced Dll4 expression and production of the 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, the GSK3β inhibitor up-regulated Dll4, whereas depletion of β-catenin by siRNA blocked Ang1-induced Dll4 expression, indicating the indispensability of β-catenin in Ang1-mediated up-regulation of Dll4. In addition, Dll4 expression by the GSK3β inhibitor was only observed in confluent cells, and was impeded by DAPT, a γ-secretase inhibitor, implying requirement of the Notch signal in β-catenin-dependent Dll4 expression. Consistently, we found that either Ang1 or NICD up-regulates Dll4 through the RBP-J binding site within intron 3 of the 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 Dll4/Notch signaling. Collectively, the Ang1/Tie2 signal potentiates basal Notch signal controlling vascular quiescence by up-regulating Dll4 through AKT-mediated activation of β-catenin.

Angiopoietin-1 (Ang1)[3] 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 Saharinen et al. (11) 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). Ang1 induces trans-association of Tie2 in the presence of cell-cell contacts, whereas Tie2 is anchored to the cell-substratum contacts through extracellular matrix-bound Ang1 in the isolated endothelial cells. Trans-associated Tie2 and extracellular matrix-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 trans-associated Tie2, but not extracellular matrix-anchored Tie2, regulates the expression of genes involved in vascular quiescence, which include Krüppel-like factor 2, delta-

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like 4 (Dll4), TIS11d, and connexin-40 (10). We have extended the studies on Ang1-mediated vascular quiescence and have shown that Ang1-induced Krüppel-like factor 2 expression occurs through the 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 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). Delta/Serrate/Lag2 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, the Dll4/Notch signal is well characterized (17–21). VEGF up-regulates 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 vascular quiescence (15, 30). It has been reported that conditional deletion of RBP-J, the key transcription factor downstream of the 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 the 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 the Dll4/Notch signal in the tip-stalk communication, the Dll4/Notch signal appears to function in mature blood vessels with tight interendothelial cell-cell contacts. Functional similarity between the Ang1/Tie2 signal and

Dll4/Notch signal and our previous data that Ang1 induced Dll4 expression prompted us to test our hypothesis that the Ang1/Tie2 signal may promote vascular stabilization through the 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 the 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 (38). Other reagents were purchased as follows: wortmannin, AKT inhibitor IV, and *N*-(*N*-(3,5-difluorophenacetyl)-L-alanyl)-*S*-phenylglycine *t*-butyl ester (DAPT) from Calbiochem; SB216763 from Sigma; lithium chloride (LiCl) from Wako Pure Chemical Industries; and basic fibroblast growth factor (bFGF) from PeproTech. Antibodies were purchased as follows: anti-Dll4, anti-cleaved Notch1 (Val[1744]) (anti-NICD), anti-AKT, anti-phospho-AKT, anti-GSK3β, and anti-phospho-GSK3β (Ser[9]) from Cell Signaling Technology; anti-tubulin from Sigma; 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.; and horseradish peroxidase-coupled sheep anti-mouse and anti-rabbit IgG from GE Healthcare. Stealth small interfering RNAs (siRNAs) targeting the genes indicated were purchased from Invitrogen Corp.: human Dll4 (HSS123068, HSS182569) and human β-catenin (VHS50819, VHS50822).

Cell Culture, Transfection, siRNA-mediated Protein Knockdown, and Adenovirus Infection—Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells were purchased from Kurabo, maintained as described previously (10, 39), and used for the experiments before passages 8 and 10, respectively. Human dermal microvascular endothelial cells were purchased from Kurabo, and maintained in HuMedia-MvG with a growth additive set. The cells were placed on collagen-coated plates at densities of 2,000 and 40,000 cells/cm^2, and cultured overnight to obtain sparse and confluent cell cultures, respectively. HUVECs were transfected using Lipofectamine 2000 (Invitrogen) and Lipofectamine Plus reagents (Invitrogen) according to the manufacturer's instructions. For siRNA-mediated gene silencing, HUVECs were transfected with 20 nM siRNA duplexes using Lipofectamine RNAi MAX reagent (Invitrogen), and cultured for 36–48 h. As a control, siRNA duplexes with irrelevant sequences were used. Then, the cells were harvested, re-plated on collagen-coated plates, and cultured for an additional 24 h before experiments. HUVECs were infected with adenovirus vectors at the appropriate multiplicity of infection. Forty-eight h after infection, the cells were used for experiments.

Plasmids and Adenoviruses—The DNA, including intron 3 of the human *DLL4* gene, was amplified by PCR using the genomic DNA extracted from HUVECs as a template and the following

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primer set: 5'-gtgagtagctcgctccgc-3' and 5'-ctgaggggca-gagggtc-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 the QuikChange Site-directed Mutagenesis kit (Stratagene) with the Dll4-Int3-Luc plasmid as a template. To construct the p3xFLAG-NICD plasmid encoding FLAG-tagged NICD, a DNA fragment encoding the Notch1 intracellular domain was excised from the pcDNA-FLAG-Notch1-ICD vector, a gift from M. Kurabayashi (Gunma University), and subcloned into p3xFLAG-CMV10 vector (Sigma). A cDNA encoding human Foxc2 was amplified by PCR using human heart cDNAs as a template, and cloned into pERed-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 the pGL3 Basic vector (Promega Corporation) has already been reported (41). An expression plasmid encoding the constitutively active form of β-catenin (CA-βCat) in which Ser^{37} 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 and TOPflash reporter plasmid from Millipore Corporation. Recombinant adenovirus vectors encoding LacZ and the 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 conditions were starved in medium 199 containing 1% BSA for 12 h, and stimulated with either 400 ng/ml of COMP-Ang1 or 10 μM SB216763 as described in the figure legends. After stimulation, total RNA was purified using TRIzol (Invitrogen). Quantitative real time reverse transcription (RT)-PCR was carried out using the QuantiFast SYBR Green RT-PCR kit (Qiagen) as described (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'-tccaactgc-ccttcaatttcac-3' and 5'-ctggatggcgatcttgctga-3'; for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-atggg-gaaggtgaaggtcg-3' and 5'-ggggtcattgatggcaacaata-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 a collagen-coated dish were starved in medium 199 containing 1% BSA for 12 h, and stimulated as described in the figure legends. After stimulation, the cells were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM sodium fluoride, 1 mM sodium vanadate, and 1× protease inhibitor mixture (Roche Applied Science), and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was used as precleared 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 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 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 a collagen-coated dish were transfected with different expression vectors, together with reporter plasmids as described in the figure legends. The 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 conditions. 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 figure legends. 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 the EZ ChIP™ kit (Millipore Corporation) according to the manufacturer's instructions. Confluent HUVECs plated on collagen-coated dishes were starved in medium 199 containing 1% BSA for 12 h, and stimulated with 400 ng/ml of COMP-Ang1 for 30 min. After 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 intron 3 of the human *Dll4* gene (5'-gacgcttagcttggcctg-gagctg-3' and 5'-tgtaaaatacaggaaggggcccgtcag-3'). PCR sensitivity 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 the method of Davis and co-workers (43). HUVECs were suspended in 2.5 mg/ml of collagen type I matrices (Nitta Gelatin) at a density of $2 × 10^6$ 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

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