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Tie1 deletion inhibits tumor growth and improves angiopoietin antagonist therapy

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The endothelial Tie1 receptor is ligand-less, but interacts with the Tie2 receptor for angiopoietins (Angpt). Angpt2 is expressed in tumor blood vessels, and its blockade inhibits tumor angiogenesis. Here we found that Tie1 deletion from the endothelium of adult mice inhibits tumor angiogenesis and growth by decreasing endothelial cell survival in tumor vessels, without affecting normal vasculature. Treatment with VEGF or VEGFR-2 blocking antibodies similarly reduced tumor angiogenesis and growth; however, no additive inhibition was obtained by targeting both Tie1 and VEGF/VEGFR-2. In contrast, treatment of Tie1-deficient mice with a soluble form of the extracellular domain of Tie2, which blocks Angpt activity, resulted in additive inhibition of tumor growth. Notably, Tie1 deletion decreased sprouting angiogenesis and increased Notch pathway activity in the postnatal retinal vasculature, while pharmacological Notch suppression in the absence of Tie1 promoted retinal hypervasularization. Moreover, substantial additive inhibition of the retinal vascular front migration was observed when Angpt2 blocking antibodies were administered to Tie1-deficient pups. Thus, Tie1 regulates tumor angiogenesis, postnatal sprouting angiogenesis, and endothelial cell survival, which are controlled by VEGF, Angpt, and Notch signals. Our results suggest that targeting Tie1 in combination with Angpt/Tie2 has the potential to improve antiangiogenic therapy.

Introduction

Angiogenesis, the sprouting of new blood vessels from preexisting ones, is required for a variety of physiological processes, such as embryonic development, reproduction, wound healing, and organ regeneration in adults. Furthermore, angiogenesis is involved in several pathological processes, including age-related macular degeneration and cancer (1, 2), and compounds that inhibit the VEGF/VEGFR-2 pathway are in clinical use for the treatment of these diseases. However, targeting the VEGF/VEGFR-2 pathway is often insufficient to control tumor growth. Thus, novel approaches to further develop antiangiogenic therapies for cancer are needed (3).

The angiopoietin (Angpt) growth factors Angpt1 and Angpt2 and the Tie receptors Tie1 and Tie2 form another endothelial receptor tyrosine kinase signaling system with a pivotal role in embryonic vessel morphogenesis and vascular homeostasis (4). Constitutive deletion of Tie1, Tie2, or Angpt1 results in embryonic lethality (5–8). In adults, Angpt1-mediated signals are important for stabilization of the vascular endothelium after angiogenic processes (8). Angpt2 is required for lymphatic development, and postnatally, both Angpt1 and Angpt2 contribute to the development of the retinal vasculature (9, 10).

The Angpt-Tie pathway also regulates tumor angiogenesis (4, 11). Angpt2 levels are increased in many human cancers, and its blockage inhibits tumor growth and angiogenesis (12–14). Blocking Angpt2 also inhibits tumor metastasis via hematogenous and lymphatic routes (13, 15).

All angiopoietins bind to Tie2, while Tie1 is as an orphan receptor (4, 12, 14). However, Tie1 interacts with Tie2, and both translocate to endothelial cell-cell contacts upon Angpt stimulation (16–18). During embryonic development, Tie1 is required for the integrity of the vascular endothelium, particularly in regions undergoing angiogenic capillary growth (5). Tie1 expression is increased in adults during wound healing, ovarian follicle maturation, and tumor angiogenesis (11, 19). According to Woo et al., postnatal loss of 40%–80% of Tie1 did not result in obvious pathology, but instead conferred an atheroprotective effect, in a murine model (20).

In the present study, we showed that endothelial-specific deletion of Tie1 inhibited tumor angiogenesis and growth and delayed developmental angiogenesis occurring postnatally in the retina. Interestingly, angiopoietin inhibitors increased antiangiogenic effects on both tumor and retinal angiogenesis in Tie1-deleted mice.

Results

Validation of a conditional Tie1 loss-of-function mouse model. To study the importance of Tie1 during postnatal angiogenesis, given that homozygous constitutive Tie1 deletion (*Tie1$^{-/-}$*) is embryonic lethal, we generated conditionally Tie1-targeted mice (*Tie1$^{flox/flox}$*) using the *Cre/loxP* system to delete the first coding exon of *Tie1* (5, 21, 22). We validated the mouse model by deleting Tie1 ubiquitously, using the *Tie1$^{flox/flox}$* allele and the *PGK-Cre* transgenic strain, in which the expression of the Cre recombinase starts during the diploid phase of oogenesis (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI68897DS1; and refs. 22, 23). Southern, Northern, and Western blotting demonstrated virtually complete Tie1 gene ablation at the
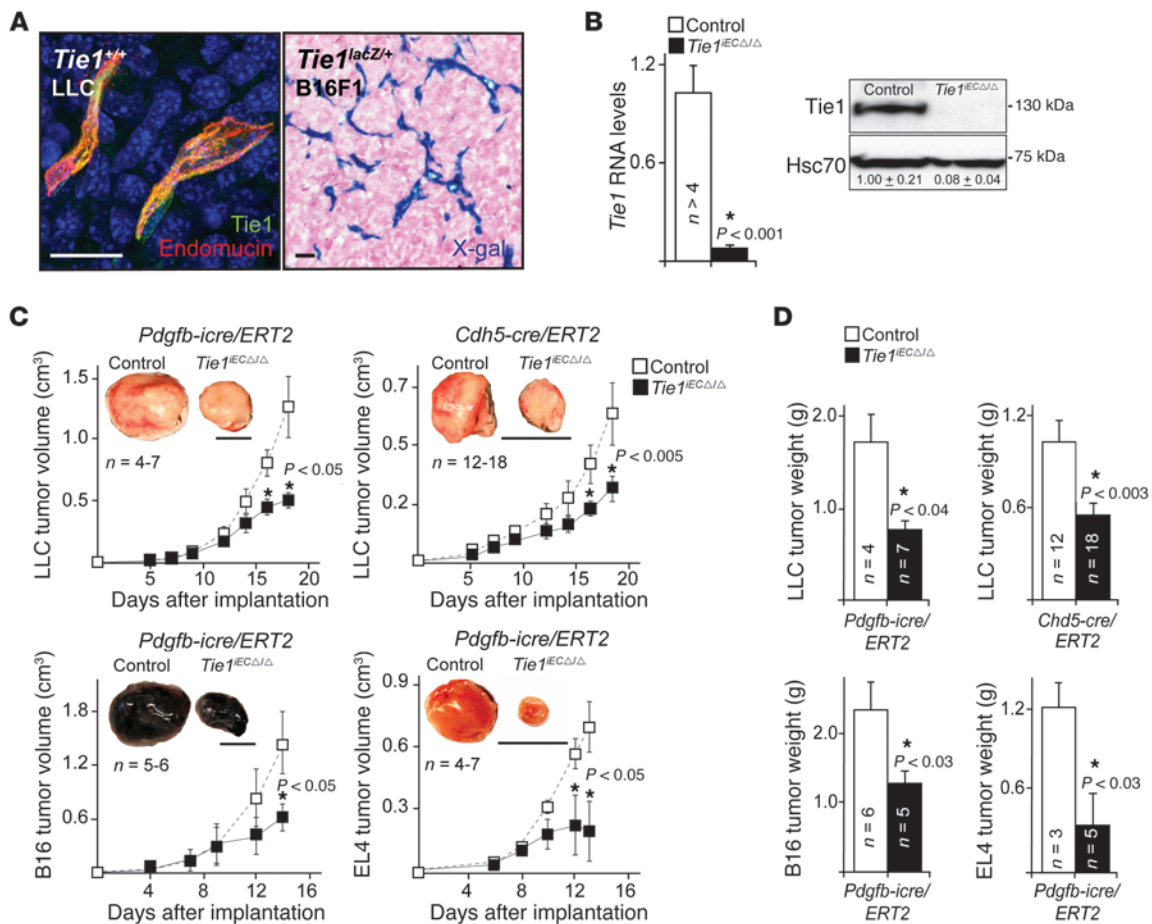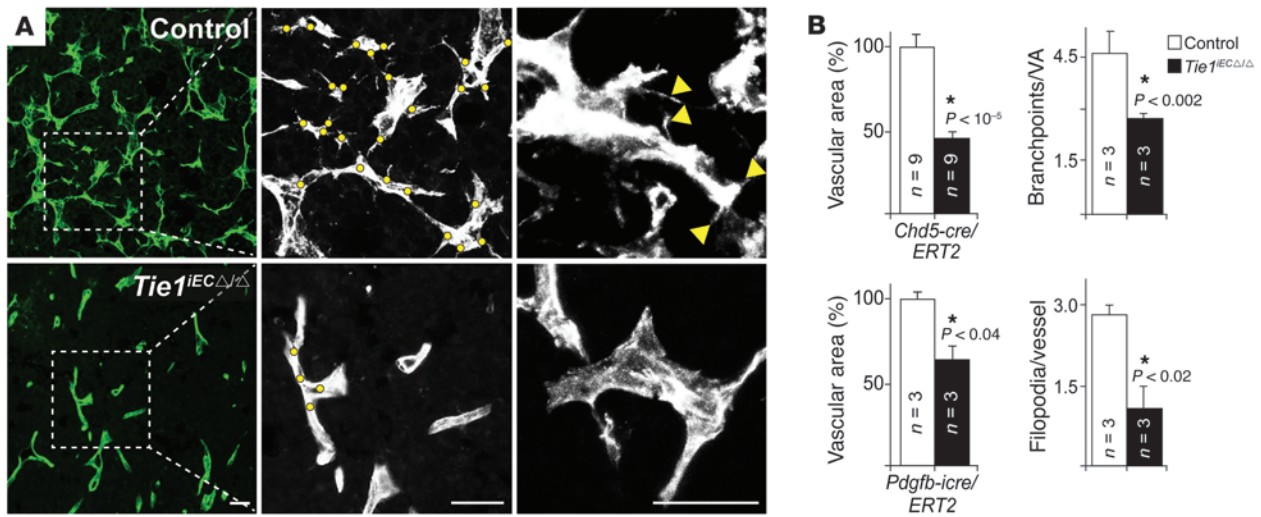
Figure 1
Endothelial Tie1 deficiency inhibits tumor growth. (**A**) Tie1 expression in the LLC (Tie1, green; endomucin, red; DNA, blue) and B16F1 (X-gal, blue) tumor vasculatures in $Tie1^{+/+}$ and $Tie1^{lacZ/+}$ mice, respectively. (**B**) $Tie1$ mRNA expression analyzed by quantitative RT-PCR ($P < 10^{-5}$) and Tie1 and Hsc70 Western blots from control and $Tie1^{iEC\Delta/\Delta}$ lung extracts. Numbers denote relative Tie1 levels. $n = 7$–10 lungs/genotype. (**C**) Growth curves of LLC, EL4, and B16F10 (B16) tumors. (**D**) Tumor weight at termination. Scale bars: 20 μm (**A**); 10 mm (**C**). Error bars denote SEM. Significant differences are shown by asterisks, with P values indicated.

DNA, mRNA, and protein levels in all $PGK\text{-}Cre/Tie1^{flox/flox}$ (referred to herein as $Tie1^{\Delta/\Delta}$) embryos analyzed (Supplemental Figure 1, C–F, and Supplemental Figure 2). $Tie1$ mRNA and protein levels in samples of $PGK\text{-}Cre/Tie1^{flox/+}$ ($Tie1^{\Delta/+}$) embryos were about half of those seen in WT and $Tie1^{flox/flox}$ control samples (Supplemental Figure 1, E and F). At E14.5, $Tie1^{\Delta/\Delta}$ embryos exhibited a pronounced edematous phenotype and hemorrhages (Supplemental Figure 1A), resembling transgenic embryos in which ubiquitous Tie1 deletion is achieved by insertion of the $LacZ$ gene into the $Tie1$ locus (5, 21). The $Tie1^{\Delta/\Delta}$ mice died by E17.5 (data not shown).

Examination of histological sections of the thoracic region, stained for the blood vascular endothelial marker endomucin and the lymphatic endothelial markers Prox1 and Lyve1, showed the presence of mispatterned first lymphatic structures that have been previously described as lymph sacs, in $Tie1^{\Delta/\Delta}$ but not control embryos at E14.5 (Supplemental Figure 3). This phenotype was similar to that reported for embryos that express homozygous hypomorphic Tie1 alleles (22). We visualized the developing lymphatic vasculature in more detail by planar illumination–based ultramicroscopy of whole-mount immunostained E12.5 embryos

(24) in which Tie1 had been deleted from endothelial cells (referred to herein as $Tie1^{iEC\Delta/\Delta}$) (Supplemental Table 1 and Supplemental Methods). Analysis of 3D reconstructions of images obtained by optical sectioning of embryos stained for Pecam1 and Prox1 showed that the first lymphatic structures, recently described as peripheral longitudinal lymphatic vessel (PLLV) and primordial thoracic duct (pTD) (24), were present in anatomically correct positions in the E12.5 $Tie1^{iEC\Delta/\Delta}$ embryos. However, these structures appeared abnormally dilated, and the superficial lymphatic vessels, which extend dorsally from the PLLV, were disrupted when compared with those of control embryos (Supplemental Figure 1B). These results are consistent with our previous analysis of Tie1 hypomorphic and $Tie1^{\Delta/\Delta}$ embryos at E14.5 (Supplemental Figure 3 and ref. 22) and indicate that Tie1 has an endothelial cell–autonomous role in lymphatic vasculature development.

Endothelial Tie1 deletion inhibits tumor angiogenesis and growth. We next assessed the function of Tie1 during tumor angiogenesis. Previous in situ hybridization studies have shown elevated $Tie1$ mRNA expression in the vascular endothelium of malignant human tumors compared with normal vessel endothelium (19,

Figure 2
Endothelial Tie1 deficiency inhibits tumor angiogenesis. (**A**) Endomucin immunofluorescence for LLC tumor blood vessels (green or white). Dots denote branchpoints; arrowheads denote filopodia. (**B**) Vessel density (expressed as area percentage), branchpoints per vessel area (VA), and filopodia per vessel. Scale bars: 100 μm. Error bars denote SEM. Significant differences are shown by asterisks, with *P* values indicated.

25). We confirmed Tie1 expression in angiogenic tumor vessels by immunohistochemical staining and by using heterozygous *Tie1^lacZ/+* mice, in which the endogenous *Tie1* locus drives *LacZ* gene expression and thus unambiguously marks transgene-expressing cells (Figure 1A). Interestingly, immunohistochemical staining showed variable levels of Tie1 expression in the tumor vasculature. We crossed *Tie1^flox/flox* mice with *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* mice (Supplemental Methods). This enabled inducible Tie1 deletion by endothelial-specific Cre recombinase activation after tamoxifen administration (Figure 1B and Supplemental Table 1). We implanted Lewis lung carcinoma (LLC), B16F10 melanoma (B16), or EL4 leukemia/lymphoma (EL4) cells to the *Tie1^iECΔ/Δ* mice and found significantly reduced tumor growth compared with controls (Figure 1, C and D).

Immunohistochemical staining indicated that Tie1 expression and blood vessel density were reduced in tumors grown in *Tie1^iECΔ/Δ* versus control mice (Supplemental Figure 4 and Figure 2, A and B). Notably, whereas LLC tumor vessels in control mice tended to grow in a branching honeycomb-like pattern, those in *Tie1^iECΔ/Δ* mice showed significantly decreased density of angiogenic sprouts and filopodial extensions (Figure 2, A and B). These results indicated that Tie1 is crucial for tumor growth and angiogenesis.

As Tie1 has been reported to promote endothelial cell survival in the late phase of angiogenic capillary growth during mouse embryonic development (21), we assessed endothelial cell apoptosis in the tumor vasculature. Apoptosis was significantly increased in *Tie1^iECΔ/Δ* tumor vessels (Figure 3, A–F). Electron microscopy indicated that the degenerating endothelial cells were occasionally associated with small intravascular fibrin deposits in *Tie1^iECΔ/Δ* tumors (Figure 3, G–J, and Supplemental Figure 5). However, we found no significant differences in endothelial cell proliferation between *Tie1^iECΔ/Δ* and control tumors (Supplemental Figure 6).

We also analyzed parameters of vessel maturation, integrity, and functionality in the tumors. The percentage of blood vessels coated with NG2 proteoglycan–positive pericytes or α-SMA–positive smooth muscle cells was similar in *Tie1^iECΔ/Δ* and control tumors

(Supplemental Figure 7). Intravascular perfusion with *Lycopersicon esculentum* (tomato) lectin showed a decreased proportion of perfused tumor vessels, and pimonidazole hydrochloride staining indicated a trend toward increased tumor hypoxia, in *Tie1^iECΔ/Δ* versus control tumors (Figure 4, A–F). Furthermore, tumor cell apoptosis was increased in *Tie1^iECΔ/Δ* mice (Figure 4, G–L). Thus, inhibition of tumor angiogenesis by Tie1 deletion was associated with reduced endothelial cell viability in the tumor vessels as well as increased tumor hypoxia and tumor cell death.

Endothelial Tie1 has been suggested to play a role in inflammation, and inflammatory cells regulate tumor angiogenesis and growth (20, 26). Therefore, we investigated whether Tie1 deletion affects endothelial expression of leukocyte adhesion receptors or leukocyte recruitment to the tumor stroma. P-selectin and intercellular adhesion molecule 1 (Icam1) expression were reduced in *Tie1^iECΔ/Δ* tumor endothelium, but there were no significant differences in leukocyte (CD45), monocyte/macrophage (F4/80), or granulocyte (Lys6/Gr-1) recruitment to the tumors (Supplemental Figures 8 and 9). These results suggest that inflammatory cells do not contribute substantially to the decreased tumor growth in *Tie1^iECΔ/Δ* mice.

Tie1 deletion does not harm the healthy vasculature or renal function. It was previously shown that stringent blocking of VEGF signaling in healthy adult mice for 2 weeks results in significant capillary regression in certain organs (27, 28). As adult *Tie1^iECΔ/Δ* mice exhibited increased endothelial cell apoptosis in tumor isografts, we next analyzed the effect of Tie1 deficiency on normal healthy vessels. We used the *Pdgfb-icre/ERT2* deletor strain to induce endothelial Tie1 deletion in 8- to 10-week-old mice. After a deletion period of 3 weeks (corresponding to that in the tumor models), we analyzed the blood vasculature in the lungs, kidneys, heart, thyroid, salivary glands, small intestine, trachea, and skin. Quantitation of Pecam1 and endomucin staining indicated that blood vessel density was not significantly different in *Tie1^iECΔ/Δ* versus control tissues (Supplemental Figure 10, A and B). Analysis of interendothelial adherens junctions of blood capillaries immunostained for cadherin 5 (Cdh5; also known as VE-cadherin) and quantitation of the diam-
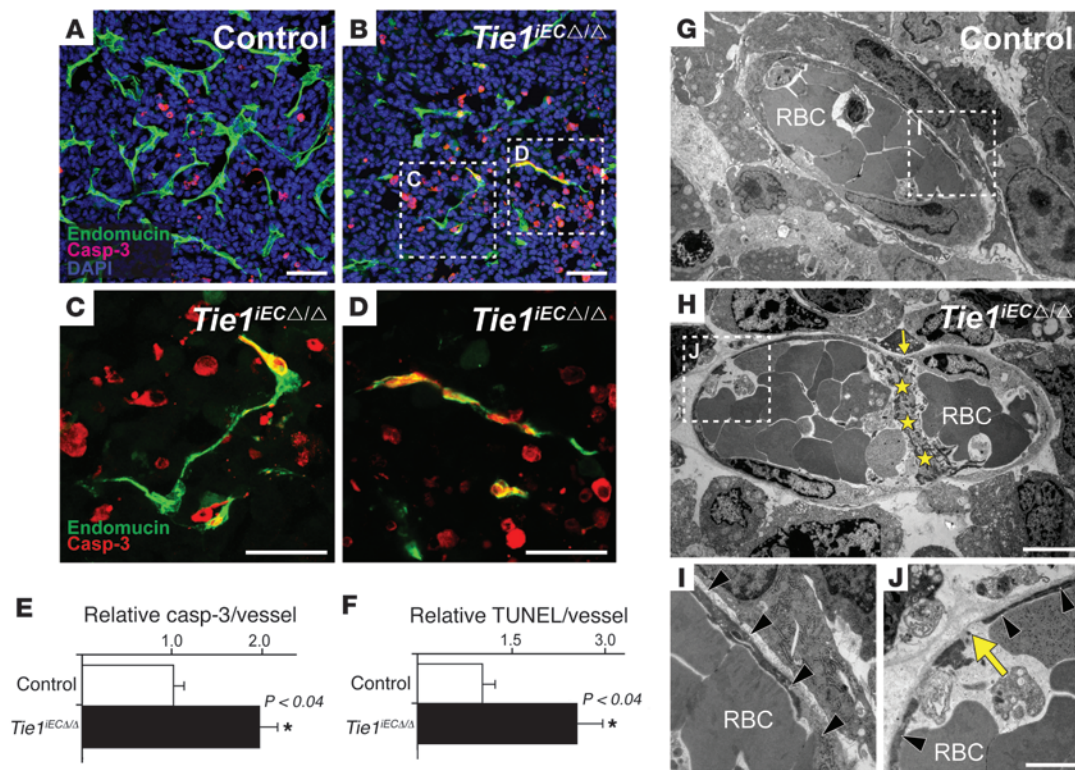
Figure 3
Endothelial Tie1 deficiency increases endothelial cell apoptosis in tumor blood vessels. (**A** and **B**) Endomucin (green), caspase-3 (red), and nuclear (blue) staining. (**C** and **D**) Higher-magnification views of boxed regions in **B**. (**E**) Quantification of caspase-3/endomucin and (**F**) TUNEL/endomucin double-positive endothelial cells per tumor vessel. (**G–J**) TEM of control and $Tie1^{iEC\Delta/\Delta}$ tumor blood vessels. (**I** and **J**) Higher-magnification views of boxed regions in **G** and **H**. Black arrowheads denote continuous endothelial cell layer; yellow arrows denote sites where no endothelial cell is apparent; yellow stars denote fibrin. RBC, red blood cell. Scale bars: 50 μm (**A–D**); 5 μm (**G** and **H**); 2 μm (**I** and **J**). Error bars denote SEM. Significant differences are shown by asterisks, with *P* values indicated.

eter of postcapillary venules in the tracheal mucosa did not reveal statistically significant differences between $Tie1^{iEC\Delta/\Delta}$ and control mice (Supplemental Figure 10, C–H). In addition, analysis of lungs by H&E and Masson's trichrome staining and by transmission EM (TEM) did not reveal substantial changes between $Tie1^{iEC\Delta/\Delta}$ and control mice (Supplemental Figure 10, I–M).

The ultrastructural features of renal glomerular capillaries, such as endothelial cell fenestrations, basement membrane thickness, or podocyte foot processes were not altered by either endothelial-specific or ubiquitous (*Rosa26/creERT* deletor; ref. 29) Tie1 deletion (3 weeks and 5 months, respectively; Supplemental Figure 11A, Supplemental Table 1, and Supplemental Methods). In line with this, Tie1 deletion did not alter blood urea nitrogen or creatinine concentrations in serum. Moreover, no significant changes were observed in polypeptides in urine when comparing control versus Tie1-deficient mice (Supplemental Figure 11, B and C), which suggests that Tie1 is not necessary for the maintenance of kidney function under basal conditions. Thus, despite the effects of Tie1 deficiency on tumor angiogenesis, normal vessels were not significantly affected in various adult $Tie1^{iEC\Delta/\Delta}$ mouse tissues after a 3-week deletion period.

Endothelial Tie1 deletion does not improve antiangiogenic therapy directed against VEGF or VEGFR-2. Recent results have suggested that blocking Angpt2 increases the efficacy of antiangiogenic therapy directed against the VEGF/VEGFR-2 pathway (30–32). We tested whether endothelial Tie1 deletion in combination with blocking antibodies against VEGF or VEGFR-2 provides additive inhibition of tumor angiogenesis and growth. The VEGFR-2 blocking antibody DC101 (33) and the VEGF blocking antibody mcr84 (34) were both capable of significantly inhibiting angiogenesis and growth of LLC, EL4, and B16F1 tumors in control mice. Although Tie1 deletion was equally effective in reducing tumor growth, no additive antiangiogenic effects were seen when either antibody was used in combination with endothelial Tie1 deletion (Supplemental Figure 12). Interestingly, when VEGF was injected and allowed to circulate for 10 minutes, decreased VEGFR-2 protein was detected in $Tie1^{iEC\Delta/\Delta}$ versus control lung lysates. The decreased VEGFR-2 levels may help to explain the reduced endothelial cell survival in the tumors and the ineffectiveness of VEGF signaling inhibition in $Tie1^{iEC\Delta/\Delta}$ mice (Supplemental Figure 13).

Previous studies assessing the safety of very-high-affinity anti-VEGF antibodies showed some renal and hepatic damage, including glomerulosclerosis and loss of glomerular structural integrity (35). We thus analyzed kidney glomeruli in the tumor-bearing mice treated with placebo or mcr84 antibody, which has a low toxicity profile in mice (34). Morphological and immunohistochemical analysis showed no statistically significant differences in glomerular tufts, Bowman's space, or vascular density in the kidney glomeruli (Supplemental Figure 14). Furthermore, the liver histology in H&E staining was unaltered, and total body weights were similar in
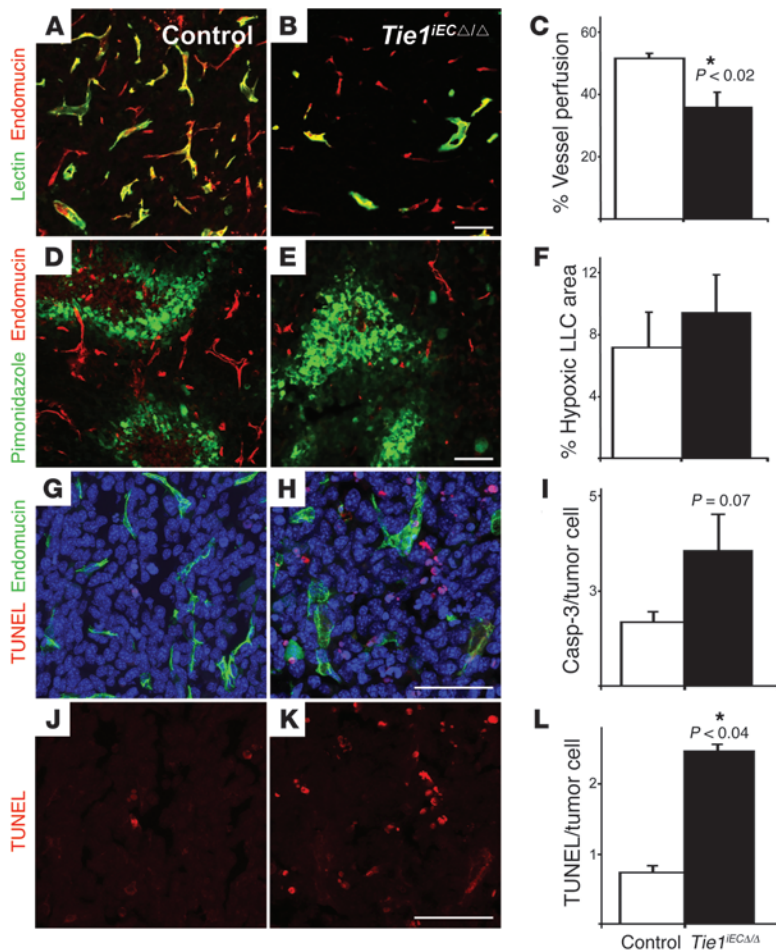
Figure 4
Vessel perfusion, hypoxia, and apoptosis in tumors of control and $Tie1^{iEC\Delta/\Delta}$ mice. (**A** and **B**) LLC tumor sections from mice injected i.v. with biotinylated *L. esculentum* lectin 10 minutes before tumor excision at day 18 after tumor cell implantation. Lectin (green) staining marks functional tumor blood vessels. (**C**) Vessel perfusion, determined as percent of lectin-positive area relative to percent of endomucin-positive area per field. $n = 3$–6 tumors/genotype (*Pdgfb-icre/ERT2* deletor). (**D** and **E**) LLC tumor sections from mice injected i.p. with pimonidazole (hypoxia marker) 1.5 hours before tumor excision. Pimonidazole (green) staining marks the hypoxic area. (**F**) Percent hypoxic area relative to total field area. $n = 9$ tumors/genotype (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors). Note the trend toward increased hypoxia in $Tie1^{iEC\Delta/\Delta}$ tumors. (**G** and **H**) Staining for TUNEL (red), endomucin (green), and DAPI (blue). (**J** and **K**) TUNEL staining alone from **G** and **H**, shown for better visualization. (**I** and **L**) Tumor cell apoptosis indices, calculated as ratio of (**I**) cleaved Caspase-3 and (**L**) TUNEL fluorescent cells to total DAPI-positive tumor cells per field. $n = 3$–6 tumors/genotype (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors). Scale bars: 100 μm. Error bars denote SEM. Significant differences are shown by asterisks, with P values indicated.

all mice throughout the experiment (data not shown). Thus, in our tumor model, the combination of endothelial Tie1 deletion and mcr84 antibody treatment did not induce kidney or liver damage (Supplemental Figure 14 and data not shown).

Effect of Tie1 deletion on Tie2 signaling pathways in vivo. Previous in vitro studies have suggested that silencing of endothelial Tie1 increases Angpt2-induced Tie2 phosphorylation and that VEGF enhances Tie2 responsiveness to Angpt1 by modulating the Tie1/Tie2 ratio on the cell surface (36, 37). However, in our experiments, total Tie2 phosphotyrosine was not significantly altered in the lungs of Tie1-deleted mice, even in the presence of VEGF, which was injected i.v. and allowed to circulate for 10 minutes (Supplemental Figure 15A). Interestingly however, phosphorylation of Tie2 at Y1106, which is known to bind the Dok-R protein and to promote Angpt1-mediated endothelial cell migration (38), was decreased when Tie1 was deleted (Supplemental Figure 15B).

Combinatorial targeting of Tie1 and angiopoietins results in additive tumor growth inhibition. The dominant Tie2 ligand in tumors is Angpt2, which is produced by the angiogenic tumor endothelium (39, 40). We thus tested whether combinatorial targeting of Tie1 and Angpts provides additive inhibition of tumor angiogenesis and growth. Because we could not identify a tumor isograft model that is sensitive to anti-Angpt2 antibodies (our unpublished observations), we used an adeno-associated virus (AAV) vector to deliver a soluble form of the extracellular domain (ECD) of mouse Tie2 (referred to herein as mTie2-ECD; Figure 5A), which binds

Angpt1 and Angpt2 and blocks their activity (13, 41). In agreement with previous reports (32, 41), mTie2-ECD treatment inhibited the growth of LLC tumor isografts in mice (Figure 5, B and C). However, the antitumor effect was significantly greater when mTie2-ECD was used in combination with endothelial Tie1 deletion (Figure 5, B and C). Compared with mTie2-ECD alone, the combinatorial targeting decreased the length and increased the width of the vessels in the tumor sections and tended to increase pericyte recruitment (Figure 5D and data not shown).

Endothelial Tie1 deletion inhibits postnatal retinal angiogenesis. The mouse retina provides an excellent opportunity to study postnatal angiogenesis and has been widely used for that purpose (42). We used β-galactosidase staining of retinas from heterozygous $Tie1^{+/lacZ}$ mice at P5 to demonstrate Tie1 expression in retinal endothelial cells, including the tip cells of the endothelial sprouts at the vascular front (Figure 6, A and B, and Supplemental Figure 16, A–D).

To delete Tie1 in the retinal endothelium, we first confirmed that administration of 4-hydroxytamoxifen (OHT) resulted in effective Cre recombinase activation in the retinal endothelial cells, using *Pdgfb-icre/ERT2* mice bred with the *Rosa26-R* reporter strain (43–45). OHT treatment from P1 to P4 induced strong Cre activation in the whole retinal vascular bed, as detected by β-galactosidase staining in P5 retinal endothelial cells of the *Pdgfb-icre/ERT2;Rosa26-R* pups (Supplemental Figure 16, E–H). However, although $Tie1^{iEC\Delta/\Delta}$ pups developed without major signs of distress until sacrifice at P5 or P6, analysis of their retinas revealed a sta-
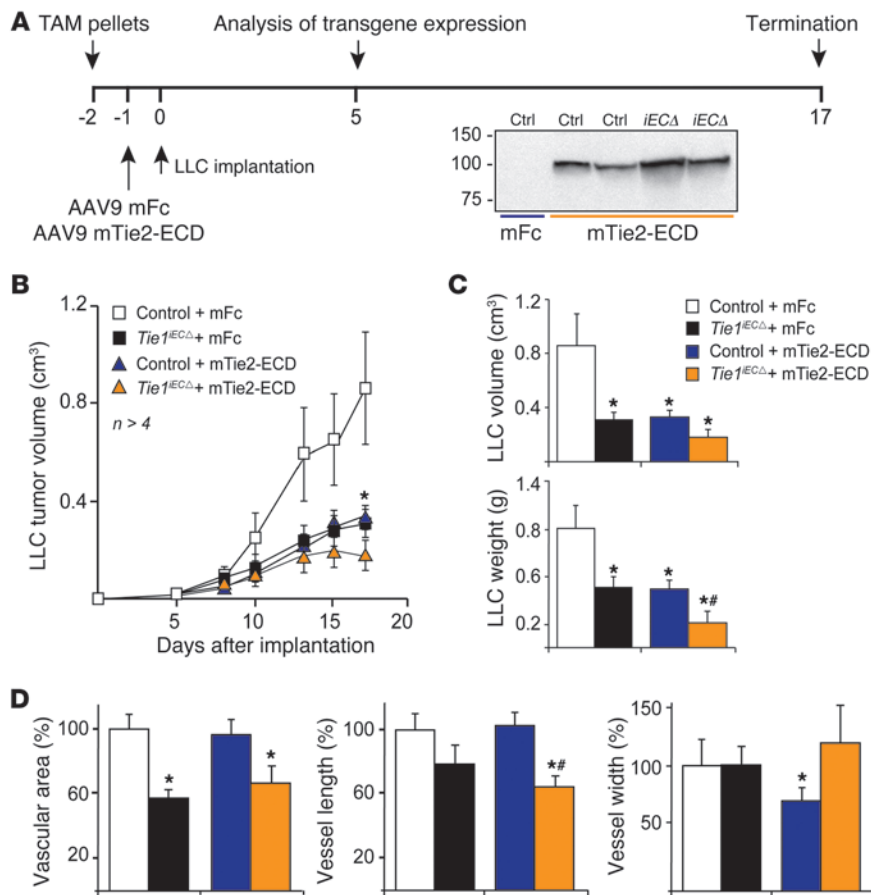
Figure 5
Combinatorial targeting of Tie1 and Angpts results in greater inhibition of tumor growth than targeting either alone. (**A**) Experimental settings for induction of Tie1 deletion by tamoxifen (TAM) pellets, soluble extracellular domain of murine Tie2 (mTie2-ECD) or soluble murine Fc gamma receptor (mFc) expression, and LLC tumor implantation. (**B**) LLC tumor growth curves. (**C**) Tumor volume and weight at 18 days. (**D**) Vascular area, vessel length, and vessel width (expressed as a percentage of control plus mFc). Error bars denote SEM. *$P < 0.05$ versus control plus IgG; #$P < 0.05$ versus control plus mTie2-ECD.

tistically significant decrease in vessel area, vessel branchpoints, and the distance of the migratory angiogenic vessel front from the optic nerve (Supplemental Figure 17). Notably, no significant retinal phenotype was observed in pups in which the Tie1 deletion was only 80%–85% complete (data not shown).

To maximize the extent of Tie1 deletion, we combined Tie1 conditional and constitutive targeted alleles (Figure 6, C–I). Tamoxifen-induced Tie1 deletion in *Cdh5(PAC)-cre/ERT2;Tie1*$^{flox/-}$ and *Pdgfb-icre/ERT2;Tie1*$^{flox/-}$ pups (referred to herein as *Tie1*$^{iECΔ/-}$) resulted in near-complete Tie1 ablation, and the resulting retinal phenotype was severely aggravated compared with that of *Tie1*$^{iECΔ/Δ}$ pups (Figure 6, C–I, and Supplemental Figure 17). Interestingly, in some *Tie1*$^{iECΔ/-}$ pups, we also observed the presence of endothelial cell tufts (Figure 6E), a feature not seen in *Tie1*$^{iECΔ/Δ}$ pups. Furthermore, the filopodial extensions of the tip cells were significantly reduced, and in some *Tie1*$^{iECΔ/-}$ pups, the migratory front of the retinal vasculature was almost devoid of endothelial sprouts (Figure 6H and Supplemental Figure 18). Further analysis of the vessels revealed a few pericyte-positive (i.e., NG2 staining), but endothelium-negative (i.e., lacking isolectin B4 [IB4] staining) cellular structures, particularly in capillary branches extending from the arterioles of the *Tie1*$^{iECΔ/-}$ retinas (Supplemental Figure 18), suggestive of occasional vessel branch regression. Nevertheless, the retinal vasculature seemed normal when Tie1 was deleted between P7 and P9 and analyzed at P17 (data not shown). These results indicate that Tie1 deficiency delays normal development of the postnatal retinal vasculature during angiogenesis, but

does not affect the vascular remodeling phase. Furthermore, the reduced vascular area and decreased number of angiogenic sprouts and filopodial extensions observed in the Tie1-deficient postnatal retinas resembled changes observed in the tumor vasculature in the Tie1-deficient adult mice.

The Notch pathway is activated in Tie1-deficient retinal endothelial cells. The expression of Tie1 in retinal endothelial tip cells and the retinal phenotype of Tie1-deleted mice prompted us to analyze whether the Tie1 deletion is associated with changes in Notch ligand Delta-like 4 (Dll4) signaling. Dll4 is mainly expressed in the angiogenic tip cells, and it activates Notch signaling in the stalk cells to suppress their migration and sprouting (46, 47). Quantitative RT-PCR using retinal extracts showed that the *Tie1*$^{iECΔ/Δ}$ and *Tie1*$^{iECΔ/-}$ retinas expressed significantly increased amounts of mRNA encoding *Dll4* and the downstream Notch signaling molecules Hairy enhancer-of-split related with YRPW motif 1 (*Hey1*) and Notch-regulated ankyrin repeat protein (*Nrarp*), as well as the hypoxia-regulated angiogenic factors *Vegf* and *Angpt2* (Figure 7A). *Dll4* mRNA and protein levels, and the intracellular domain of Notch (NCID), which is cleaved upon its activation, were also significantly upregulated in *Tie1*$^{iECΔ/Δ}$ and *Tie1*$^{iECΔ/-}$ lung extracts (Figure 7, B and H, and Supplemental Figure 19). Furthermore, whole-mount staining indicated significantly increased endothelial Dll4 abundance in *Tie1*$^{iECΔ/-}$ versus control retinas at P6 (Figure 7, C–E). These results indicate that the Notch pathway is activated in the Tie1-deficient retinas, consistent with the suppression of the migratory and sprouting phenotype of the retinal vasculature.
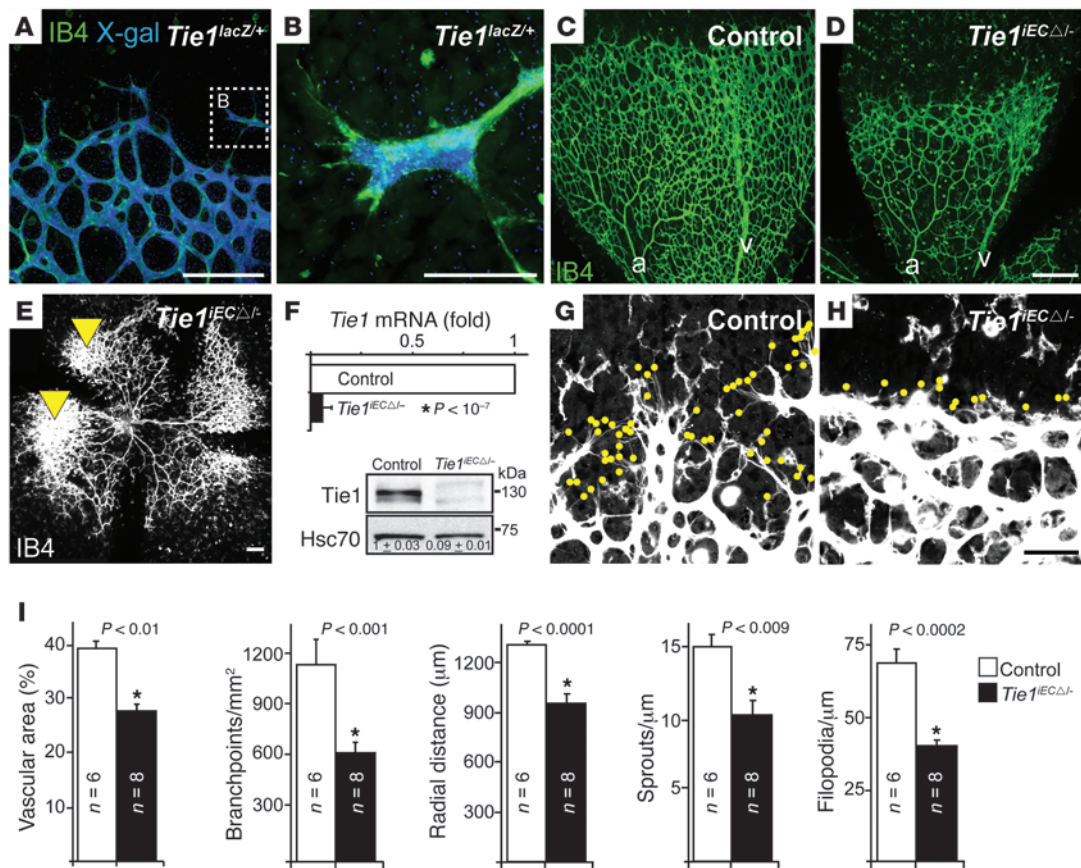
Figure 6
Endothelial Tie1 deficiency inhibits postnatal retinal angiogenesis. (**A**) β-galactosidase staining of a *Tie1lacZ/+* retina at P6. (**B**) A Tie1-expressing tip cell. (**C**–**E**) IB4–stained P6 retinas. a, arteriole; v, venule. Arrowheads denote vascular tufts. (**F**) Quantitative RT-PCR and Western blot of lungs from control and *Tie1iECΔ/–* (*Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors) P6 pups. Numbers denote mean densitometric readouts of relative Tie1 levels. *n* = 3–4 lungs/genotype. (**G** and **H**) High-magnification images of the angiogenic front. Dots denote filopodia. (**I**) Quantification of vascular area, branchpoints, radial distance from optic nerve, sprouts, and filopodia. Scale bars: 100 μm (**A**, **C**–**E**, **G**, and **H**); 50 μm (**B**). Error bars denote SEM. Significant differences are shown by asterisks, with *P* values indicated.

To further analyze the involvement of the Notch pathway in the Tie1-deficient phenotype, we used the γ-secretase inhibitor DAPT, which inhibits Notch cleavage and signaling (46). Interestingly, *Tie1iECΔ/–* and control pups had an almost identical retinal hypervascularization phenotype at P6, after a 48-hour treatment with DAPT (Figure 7, F and G). DAPT treatment also prevented the increase of NCID in *Tie1iECΔ/–* pups (Figure 7H). These results suggest that increased Notch activation contributes to the Tie1-deficient retinal phenotype.

Combinatorial targeting of Tie1 and Angpt2 results in additive inhibition of retinal angiogenesis. Anti-Angpt2 blocking antibodies inhibit retinal angiogenesis, and Angpt2 expression is highly selective for retinal endothelial tip cells, where we found Tie1 expression, but little or no Tie2 expression (13, 48, 49). We tested whether Tie1 deletion in combination with anti-Angpt2 blocking antibodies could provide additive inhibition of retinal angiogenesis. We deleted Tie1 in pups as described above and simultaneously administered anti-Angpt2 or IgG isotype control antibodies. At P5, as expected, both IgG-treated *Tie1iECΔ/Δ* pups and anti-Angpt2–treated control pups showed a statistically significant decrease in the distance of the vascular front from the optic nerve compared with IgG-treated

controls (Figure 8, A–E). Surprisingly however, significant additive inhibition of vascular front migration was observed when anti-Angpt2 antibodies were used in *Tie1iECΔ/Δ* pups (Figure 8, B, D, and E). These results showed that the combinatorial treatment with anti-Angpt2 blocking antibodies increases the antiangiogenic effects of Tie1 deletion on retinal sprouting angiogenesis, in line with our results on tumor angiogenesis and growth, in which combinatorial targeting of Tie1 and Angpt2 via soluble murine Tie2-ECD were additive.

Discussion
Here we report that deleting Tie1 specifically from the vascular endothelium inhibited tumor angiogenesis and growth, to the same extent as blocking antibodies targeted against VEGF or its major signal transducing receptor, VEGFR-2. To our knowledge, this is the first study showing that Tie1, a receptor for which no growth factor ligands have yet been identified, functions as a positive regulator of pathological angiogenesis and that Tie1 targeting has beneficial antitumor effects. Tumors grown in Tie1-deficient mice had decreased vessel density, vascular perfusion, and endothelial cell survival, and this was associated with decreased
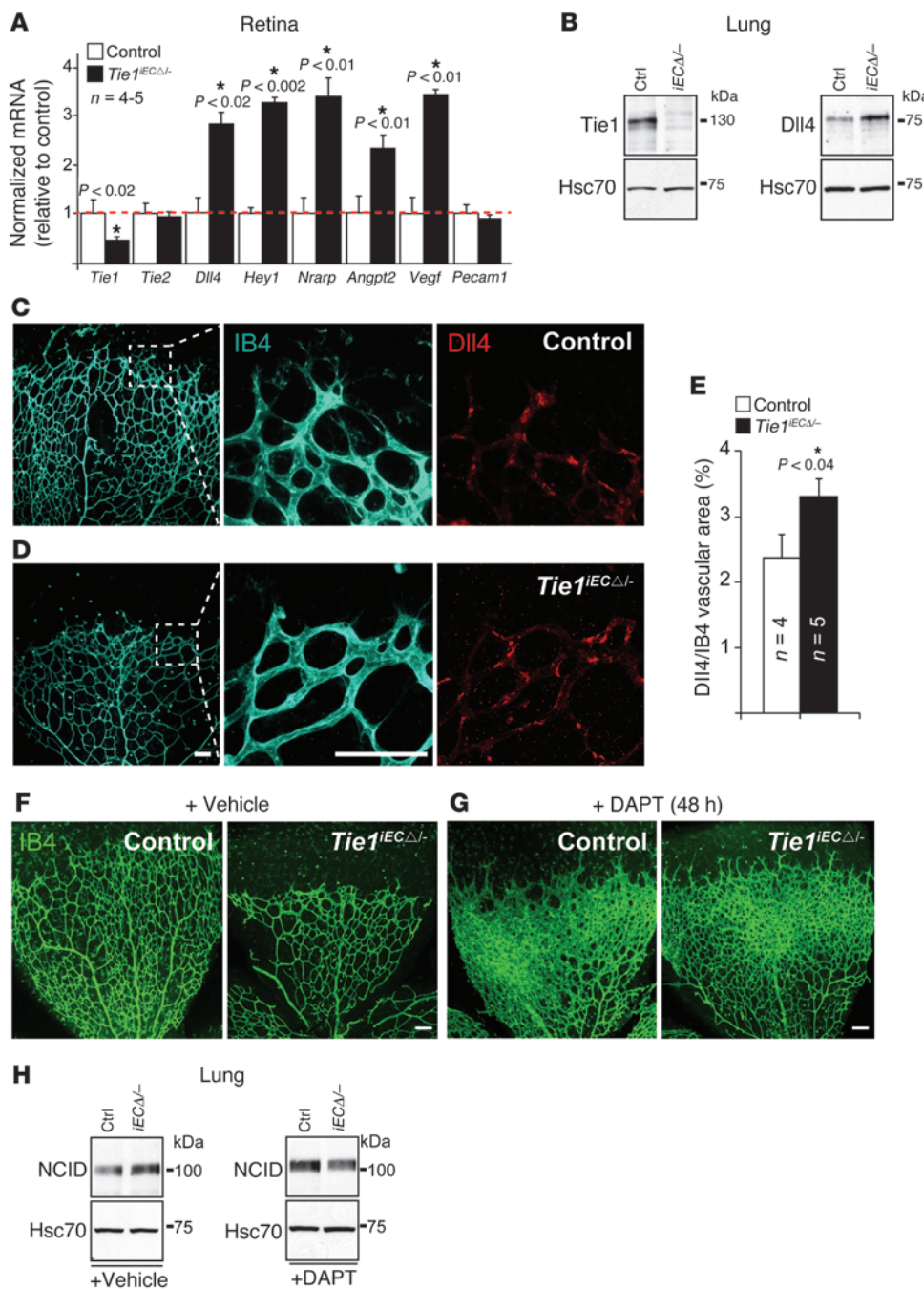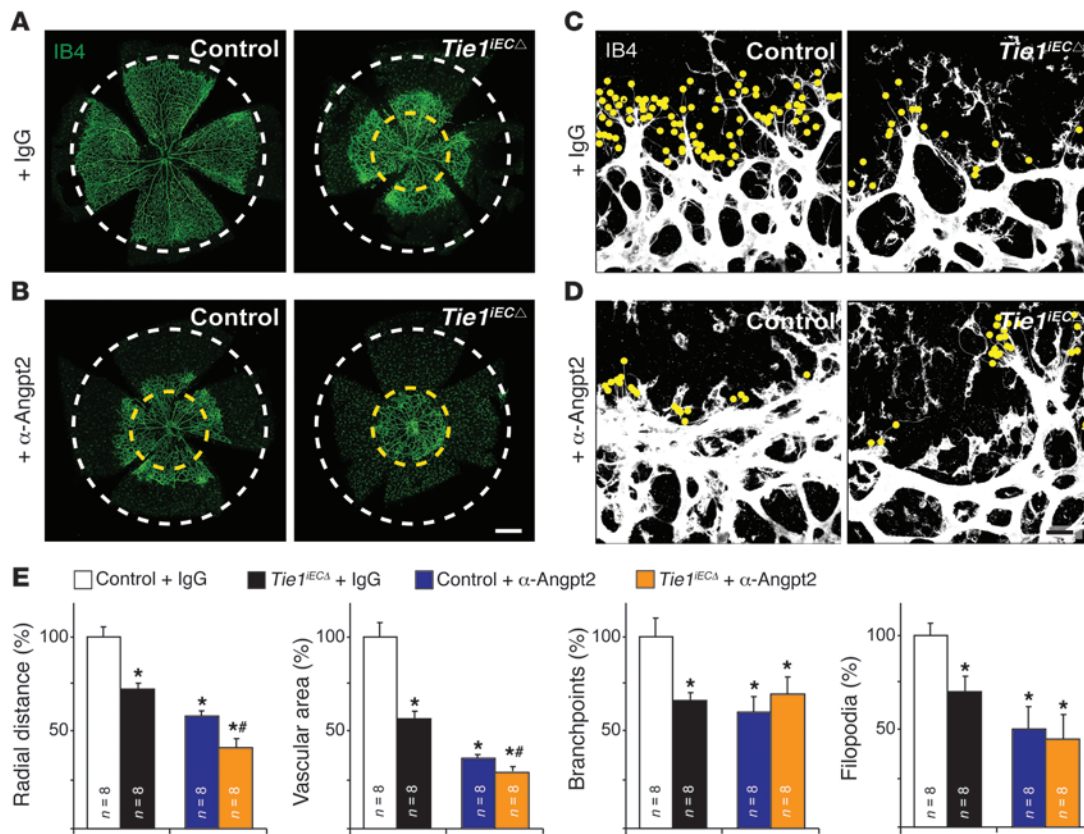
Figure 7
Tie1-deficient retinal phenotype involving upregulation of Dll4/Notch is abolished by Notch inhibitor. (**A**) Quantitative RT-PCR analysis of control and *Tie1$^{iEC\Delta/-}$* (*Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors) retinas at P6. mRNA levels were normalized to *Cdh5* to compensate for the decreased vascular area in the *Tie1$^{iEC\Delta/-}$* retinas and expressed relative to control levels (assigned as 1; red dashed line). (**B**) Representative Western blot analysis of lungs (see Supplemental Figure 19 for quantification). *n* = 5–8 (control); 6–14 (*Tie1$^{iEC\Delta/-}$*). (**C** and **D**) IB4 (cyan) and Dll4 (red) staining of (**C**) control and (**D**) *Tie1$^{iEC\Delta/-}$* angiogenic retinal fronts. (**E**) Quantification of Dll4-positive endothelium (expressed as a percentage). (**F** and **G**) IB4 (green) staining of retinas from control and *Tie1$^{iEC\Delta/-}$* (*Pdgfb-icre/ERT2* deletor) P6 pups treated with (**F**) vehicle or (**G**) DAPT for 48 hours. (**H**) Representative Western blotting for Notch1 cleaved intracellular domain (NCID) from vehicle- and DAPT-treated lungs. *n* = 3–4 lungs/genotype. Scale bars: 50 μm. Error bars denote SD (**A**) or SEM (**E**). Significant differences are shown by asterisks, with *P* values indicated.

tumor cell survival. The tumor vessels in *Tie1$^{iEC\Delta/\Delta}$* mice showed reduced numbers of angiogenic sprouts and filopodial extensions, resembling the delayed development of the postnatal retinal vasculature in *Tie1$^{iEC\Delta/-}$* mice.

VEGF- and Angpt2-mediated angiogenesis is involved in retinal and choroidal diseases, the most common causes of loss of vision in the developed countries (50). Angpt2 is expressed in the mouse retina between P0 and P7, when the superficial retinal vessels develop; after P7, its expression shifts to the regions of the developing intermediate and deep capillary vascular beds (9, 50, 51). Retinal vascular development is defective in Angpt2-knockout mice, and we and others have recently shown that anti-Angpt2 blocking antibodies

inhibit physiological retinal angiogenesis (9, 13, 49). Although the success of the anti-VEGF approach has been remarkable, some concerns exist about the long-term effects of VEGF antagonism in the eye (52). Therefore, new targets and their combinations for safer and more effective therapy are still needed. To our knowledge, our report is the first to show that targeting Tie1 significantly inhibits retinal angiogenesis and that, importantly, combinatorial treatment with anti-Angpt2 blocking antibodies increases this effect.

Notch and VEGF pathway interactions have been thoroughly studied in retinal sprouting angiogenesis, but only 1 study thus far has analyzed the Dll4/Notch crosstalk with angiopoietin/Tie2 signaling (53). We showed here that Dll4/Notch signals were

Figure 8
Combinatorial targeting of Tie1 and Angpt2 results in greater inhibition of retinal angiogenesis than targeting either alone. (**A** and **B**) IB4-stained retinas from control and either *Tie1^{iECΔ/Δ}* or *Tie1^{iECΔ/–}* (*Tie1^{iECΔ}*) P5 pups treated with (**A**) human IgG isotype (IgG) or (**B**) anti-Angpt2 antibodies. White dashed circles denote average control angiogenic front; yellow dashed circles denote average anti-Angpt2–treated *Tie1^{iECΔ}* angiogenic front. (**C** and **D**) High-magnification images of the angiogenic front. Dots denote filopodia. (**E**) Radial distance from optic nerve, vascular area, branchpoints, and filopodia (expressed as a percentage of IgG-treated control). Scale bars: 100 μm. Error bars denote SEM. *$P < 0.05$ versus control plus IgG; #$P < 0.05$ versus control plus anti-Angpt2.

increased in the absence of Tie1, inhibiting retinal angiogenesis even in the presence of increased amounts of the main hypoxia-induced angiogenic drivers, such as VEGF and Angpt2. Furthermore, blocking the Notch pathway abolished the Tie1 deletion phenotype in the developing retina, which suggests that increased Notch signaling is, at least in part, responsible for the inhibition of retinal angiogenesis in the Tie1-deleted mice. This is consistent with previous studies showing that vascular endothelial cells transfected with Dll4 grow poorly even in the presence of VEGF and that upregulation of the Notch pathway in the tumor vessels results in inhibition of tumor neovascularization and growth (54).

The combination of Tie1 inhibition with Angpt antagonists could potentially aid the development of drugs not only for the treatment of ocular diseases, but also for tumor therapy. In line with this, *Tie1^{iECΔ/Δ}* mice bearing LLC tumors treated with a soluble form of the Tie2 receptor, which blocks Angpt activity, resulted in a stronger antitumor effect than targeting Tie1 or Angpt alone. Considering that the Angpt1/Angpt2 balance in the context of the tumor environment is strongly shifted toward Anpgt2 expression (55, 56), one could argue that the additive antitumor effects are likely due to the combinatorial targeting of Tie1 and Anpgt2. Notably, the Angpt1/Angpt2 blocking pepti-

body has already entered clinical phase III trials for the treatment of ovarian cancer, and Angpt2 blocking antibodies are tested in phase I/II trials in many different cancers (www.clinicaltrials.gov). The development of Tie1 inhibitors would be a worthwhile goal, as they would probably synergize with the Angpt blocking agents in tumor therapy.

Tie1 has been shown to interact with Tie2 in endothelial cell-cell and cell-substratum contacts, where Angpt1 mediates activation of the complex (16–18). Although some reports have suggested that Tie1 may inhibit Tie2 activation, we did not find significantly increased Tie2 phosphorylation in lung lysates from the Tie1-deleted mice. Instead, phosphorylation of Tie2 at Y1106 appeared to be reduced in Tie1-deleted versus control mice. The Tie2^{Y1106} phosphopeptide is known to bind Dok-R, which mediates Angpt1-induced endothelial cell migration (38), suggesting that reduced Tie2 phosphorylation at Y1106 may contribute to the phenotype of the retinal and tumor blood vessels in Tie1-deleted mice. In line with this, Angpt1 was recently found to be required for postnatal retinal vascularization (10). However, as exogenous Angpt2 did not affect Tie2 phosphorylation at Y1106 in our experiments, blocking Angpt2 may inhibit another signaling pathway, for example, one mediated by the integrins (49). It should be noted that

endogenous Angpt2 is produced by endothelial cells in hypoxic tissues, and autocrine Angpt2 stimulation of Tie2 may mediate a proangiogenic function in conditions of vascular stress (57, 58).

The current antiangiogenic inhibitors based on targeting VEGF signaling are effective in treating age-related macular degeneration, but thus far, their utility in cancer has been limited to prolonging disease-free survival in patients with a few tumor types (3). Our present results indicate that Tie1 targeting inhibits tumor angiogenesis by decreasing endothelial cell survival and subsequently decreasing vascular density and vessel perfusion, while not affecting the normal vessels in various organs. The overall healthy condition of the Tie1-deleted mice is consistent with the conclusion that their possible vascular changes would have to be relatively subtle. Furthermore, recent experiments have shown that atherosclerosis is inhibited by partial Tie1 deletion (20), which argues that decreased Tie1 function might be beneficial not only in cancer, but also in atherosclerosis patients. Thus, our present findings elucidate a previously unknown regulatory system for angiogenesis and propose Tie1 as a potential new target for anti-angiogenic therapy that potentially holds clinical relevance now, more than 20 years after its isolation (59), when we first suggested — based on its enhanced expression pattern in the tumor vasculature (19) — the possible involvement for this interesting receptor in tumorigenesis and other angiogenesis-dependent diseases, such as diabetic retinopathy, atherosclerosis, and arthritis.

Methods

Further information can be found in Supplemental Methods.

Statistics. At least 3 mice were analyzed per time point and treatment. 2-tailed paired or equal-variance Student's *t* test or 1-way ANOVA was used for statistical analysis. A *P* value less than 0.05 was considered statistically significant.

Study approval. Mice were maintained in the Meilahti Experimental Animal Center and in the Laboratory Animal Centre of University of Oulu according to IACUC and IRB guidelines. The National Animal Experiment Board in Finland approved animal experiments used in this study.

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