Numerous studies have suggested a link between the angiogenic FGF and VEGF signaling pathways; however, the nature of this link has not been established. To evaluate this relationship, we investigated VEGF signaling in ECs with disrupted FGF signaling in vitro and in vivo. ECs lacking FGF signaling became unresponsive to VEGF, caused by downregulation of VEGF receptor 2 (VEGFR2) expression after reduced Vegfr2 enhancer activation. FGF mediated VEGFR2 expression via activation of Erk1/2. Transcriptional analysis revealed that Ets transcription factors controlled VEGFR2 expression in an FGF- and Erk1/2-dependent manner. Mice with defective FGF signaling exhibited loss of vascular integrity and reduced vascular morphogenesis. Thus, basal FGF stimulation of the endothelium is required for maintenance of VEGFR2 expression and the ability to respond to VEGF stimulation and accounts for the hierarchic control of vascular formation by FGFs and VEGF.

**Results**

**FGF signaling controls EC responsiveness to VEGF.** To test the hypothesis that FGF signaling controls EC responsiveness to VEGF and thus regulates VEGF function, we used a cytoplasmic truncated form of FGF receptor 1 (FGFR1) able to heterodimerize with all FGFRs as a dominant-negative construct (FGFR1DN), thereby suppressing overall FGF signaling (7, 9, 10). Bovine aortic ECs (BAECs) transduced with Ad vector encoding FGFR1DN (Ad-FGFR1DN) showed, as expected, decreased Erk1/2 and Akt phosphorylation in response to FGF stimulation; however, the response to VEGF-A was equally impaired in these cells (Figure 1A). Western blot analyses of VEGF receptor expression demonstrated a marked reduction in levels of VEGFR2, but not VEGFR1 (Figure 1A). Reduced VEGFR2 expression by FGF inhibition was also confirmed in Ad-FGFR1DN–transduced primary mouse aortic ECs (MAECs; Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI44762). Western blot analyses of VEGF receptor expression demonstrated a marked reduction in levels of VEGFR2, but not VEGFR1 (Figure 1A). Reduced VEGFR2 expression by FGF inhibition was also confirmed in Ad-FGFR1DN–transduced primary mouse aortic ECs (MAECs; Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI44762DS1), which indicates that this regulation is not specific to BAECs.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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To confirm that other means of FGF inhibition have a similar effect on VEGFR2 expression, we used soluble FGF receptors (sFGFRs), which are able to bind and trap FGF ligands in the extracellular space, and knockdown of FRS2, a key FGFR signaling regulator. We previously evaluated the efficacy of sFGFRs, and sFGFR1-IIIc, with its ability to bind many FGF ligands, is the most potent FGF inhibitor (7). Similar to Ad-FGFR1DN, Ad–sFGFR1-IIIc reduced Vegfr2 mRNA in BAECs in a dose-dependent manner (Supplemental Figure 1, B and C). Knockdown of FRS2α in human umbilical artery ECs also resulted in decreased VEGFR2 expression (Supplemental Figure 1D).

We then tested whether FGF signaling is equally necessary for the maintenance of VEGFR2 expression in microvascular ECs. Transduction of mouse lung microvascular ECs with either Ad-FGFR1DN or Ad–sFGFR1-IIIc resulted in a decrease in VEGFR2 levels similar to that observed in BAECs or human umbilical artery ECs (Supplemental Figure 1, E–G).

We next tested whether FGF signaling controls VEGFR2 expression at the transcriptional or posttranscriptional level. Quantitative analyses of Vegfr2 mRNA from Ad-FGFR1DN–transduced BAECs demonstrated a dose-dependent decrease in mRNA abundance, whereas the control virus had no effect (Figure 1C), suggestive of a primary transcription effect. At the same time, VEGFR2 protein half-life was not altered by suppression of FGF signaling (Supplemental Figure 2, A and B). Moreover, inhibition of proteasomal degradation using MG132 did not have any effect on VEGFR2 protein levels in these settings (Supplemental Figure 2C).

FGF-induced Erk1/2 activation mediates VEGFR2 expression. To further analyze the mechanism of FGF-mediated regulation of VEGFR2 expression, we investigated downstream signaling after FGFR activation. Upon binding to FGFR, FGF triggers activation of 3 major signaling pathways: Ras-MAPK, PI3K-Akt, and PLCγ-PKC (6). Whereas pharmacological inhibition of MEK1 and MEK2 by U0126 markedly
attenuated VEGFR2 expression in a dose- and time-dependent manner (Figure 1, D and E, and Supplemental Figure 3, A and B), inhibition of PI3K by LY294002 had no effect (Supplemental Figure 3C). To study the involvement of Erk1/2 in regulation of VEGFR2 expression, we used Ad vectors encoding Erk2 fused to the low-activity form of its upstream regulator, Mek1. Whereas the Mek1-Erk2 (ME) hybrid protein is retained in the cytoplasm because of the presence of a nuclear export sequence, mutation of the 4 leucines in the export sequence to alanines (Mek1-Erk2–LA [ME-LA]) dramatically increases its catalytic activity with concomitant nuclear translocation (11, 12). Transduction of Ad–ME-LA, but not Ad-GFP or Ad-ME, into FGFR1DN-expressing BAECs restored Vegfr2 mRNA and protein expression to control levels (Figure 1, F and G), which indicates that regulation of VEGFR2 expression by FGF is mediated through an Erk1/2-dependent signaling mechanism.

Transcriptional regulation of FGF-induced VEGFR2 expression. Transcriptional control of VEGFR2 expression is complex and realized by multiple mechanisms (13, 14). Since Erk1/2 is known to activate several transcription factors by increasing their phosphorylation (15), we measured transcriptional activity of a reporter construct containing VEGFR2 first intron enhancer and minimal promoter, (B) Luciferase reporter assay using BAECs transduced with Ad-null or Ad-FGFR1DN and transfectected with the WT Vegfr2 enhancer or minimal promoter constructs carrying Ets binding site mutations (Pea3, G56T_G57T; Ets1, G303T_G304T; FOX:ETS, C378A_C379A). (C) Decreased enhancer activity by mutation of the FOX:ETS site was not rescued by constitutively active Erk2. (D) ChIP assay of Ets1 and Etv2 binding to VEGFR2 enhancer in BAECs (untransfected or transfected with Myc-tagged Etv2). Input DNA, sample representing total input chromatin (1%). (E–H) Quantitative RT-PCR analysis of total RNA isolated from BAECs transduced with Ad-null or Ad-FGFR1DN. Values denote abundance relative to that of Ad-null (assigned as 1). (I–L) Quantitative RT-PCR analysis of total RNA isolated from BAECs transduced with Ad-ME or Ad–ME-LA. *P < 0.05 versus respective control or as indicated by brackets, Student’s t test.
containing Vegfr2 minimal promoter and the enhancer sequences expressed in BAECs. Introduction of Ad-FGFR1DN resulted in significant reduction in enhancer activity compared with no-Ad or empty Ad (Ad-Null) control that was rescued by expression of ME-LA (Figure 2A). As Erk1/2 is thought to act by phosphorylating Ets transcription factors, we next sought to identify the cis-acting elements in the Vegfr2 promoter/enhancer affected by FGF-dependent Erk1/2 activation. To this end, we mutated the GG tandem in the core GGAA sequence in 3 Ets binding sites in the Vegfr2 enhancer (Pea3 binding motif, G56T_G57T; Ets1 motif, G303T_G304T; and FOX:ETS composite motif, C378A_C379A). Enhancer activity assays revealed that only the mutation in the FOX:ETS motif attenuated the activity to a level similar to that achieved by FGF inhibition (Figure 2B). Moreover, the reduced enhancer activity observed in the presence of the mutant FOX:ETS motif was not restored by addition of the constitutively active form of Erk (Figure 2C), which strongly suggests that activation of this FOX:ETS site is directly downstream of Erk1/2 and critically important for Vegfr2 expression.

To evaluate the capability of Ets family transcription factor binding to the endogenous Vegfr2 enhancer, we performed ChIP assays. We confirmed the binding of Ets1 and Etv2 to the FOX:
ETS site of the VEGFR2 enhancer sequence in BAECs (Figure 2D) and MS1 cells (Supplemental Figure 4A). Ets1 Thr38 is an Erk1/2 phosphorylation site thought to be involved in Ets activation (16). Phosphorylation of this site was decreased in BAECs lacking FGF signaling (Supplemental Figure 4B). Treatment with U0126 also decreased Thr38 phosphorylation (Supplemental Figure 4C), demonstrating a link to Erk1/2 signaling. Thus, Ets transcription factors are capable of controlling VEGFR2 transcription via the FOX:ETS motif of the VEGFR2 enhancer and that the FGF-Erk1/2 pathway is required for this regulation.

The possible involvement of FOX:ETS in FGF-dependent regulation of VEGFR2 expression suggests that other endothelial genes whose expression is controlled via the same locus may also be affected. To test this, we examined expression of Tie2, Notch4, and Cdh5 (encoding VE-cadherin) in primary ECs transduced with Ad–FGFR1DN. There was a significant decline in both Tie2 and Notch4 expression, whereas Cdh5 expression was not changed (Figure 2, E–H). The latter finding is in agreement with our previous study showing that in the absence of FGF signaling, VE-cadherin distribution is severely perturbed, but its expression levels are unchanged (7).

Figure 4
Postischemic neovascularization is impaired in Ad–sFGFR1-IIIc–transduced mice. (A) Laser Doppler analysis of hindlimb perfusion. Ad-Null, Ad–sFGFR1-IIIc, or Ad–sFGFR3-IIIb (5 × 10^10 viral particle in all cases) was injected 7 days prior to the induction of hindlimb ischemia (n = 6 per group). Changes in perfusion are shown as a ratio of right to left hindlimb (R/L). Data (mean ± SEM) were analyzed by Student’s t test. (B) Micro-CT reconstruction at 16-μm resolution of the calf portions of a mouse hindlimb 21 days after femoral artery ligation. Scale bar: 273 μm × 52 μm. (C) Quantitative analysis of micro-CT images in the calf, presented as total number of vascular structures in 250 z axis slices (n = 4 per group). Data are mean ± SEM. (D) SDF-1α expression in the small arteries of the ischemic region. Hindlimb ischemia was induced in control and Ad–sFGFR1-IIIc–transduced mice; 48 hours later, gastrocnemius muscle of the ischemic and nonischemic leg was harvested and subjected to immunostaining for SDF-1α and α-SMA. Scale bars: 20 μm. (E) Quantitative real-time PCR analysis of Vegfr2 expression using total RNA isolated from muscle tissues of C57BL/6 mice injected with Ad-Null or Ad–sFGFR1-IIIc 7 days after ischemia induction (n = 3 per group). *P < 0.05, **P < 0.01 versus Ad-Null.
Figure 5
Lack of endothelial FGF signaling impairs posts ischemic tissue recovery and arteriogenesis. (A) Tissue loss in FGFR1DN mice. After induction of FGFR1DN expression, hindlimb ischemia was produced by ligation of the right femoral artery in control and FGFR1DN mice. Photographs were taken 7 days after induction of ischemia. (B) Tissue loss score (0, healthy; 1, black nails; 2, black toes; 3, toe loss; 4, foot loss) at day 7. Data were analyzed by Wilcoxon rank-sum test (n = 20 [control]; 14 [FGFR1DN]; 12 [Ad–sFGFR1-IIIc and Ad–sFGFR3-IIib]). Boxes denote interquartile range; lines within boxes denote median; symbols within boxes denote mean; whiskers denote 5th and 95th percentile; bars denote minimum and maximum. (C) Apoptosis in the ischemic muscle of FGFR1DN mice. 3 days after induction of ischemia, gastrocnemius muscle was taken and stained for TUNEL. Apoptotic nuclei (green) were widely distributed throughout muscle cells of FGFR1DN mice. Scale bars: 20 μm. (D) Percent TUNEL+ apoptotic cells relative to DAPI+ cells (n = 3 per group). (E) Laser Doppler analysis of perfusion in mice after hindlimb ischemia. Changes in perfusion are shown as a ratio of right to left hindlimb (n = 5 per group). Data are mean ± SEM. (F) Micro-CT reconstruction at 16-μm resolution of the calf and thigh portions of mouse hindlimbs 21 days after femoral artery ligation. Scale bar: 332 μm × 96 μm. (G) Quantitative analysis of micro-CT images in the calf, presented as total number of vascular structures in 250 z axis slices (n = 3 per group). Data are mean ± SEM. *P < 0.05, **P < 0.01 versus respective control.
To further explore the role of FGF-driven Erk1/2 activation in these settings, we analyzed expression of the same genes after transduction with Ad–ME-LA. In agreement with FGFR1DN suppression data, Tie2 and Notch4 expression were induced by ERK, whereas Cdh5 expression remained unchanged (Figure 2, I–L). A global analysis of angiogenic gene expression using a small-scale angiogenesis gene array after Ad-FGFR1DN transduction identified a number of genes affected by FGF signaling, including secreted angiogenic factors, matrix-degrading enzymes, and integrins (Supplemental Figure 5, A–C).

**FGF-driven VEGFR2 expression is required for angiogenesis.** To determine whether the transcriptional regulation of Vegfr2 by FGF-Erk1/2 signaling plays a role in angiogenic processes in vivo, we examined VEGFR2 expression in mice with defective FGF signaling by using sFGFR1-IIIc, which we previously demonstrated to virtually shut down FGF signaling (7). At 7 days after intravenous injection of Ad–sFGFR1-IIIc, Vegfr2 mRNA levels in adductor muscle were significantly decreased compared with those in mice injected with Ad-Null (Figure 3A), which suggests that ongoing FGF signaling is necessary for basal expression of VEGFR2 in vivo. This effect was specific to VEGFR2, since Vegfr1 mRNA levels were not changed (Figure 3B).

To assess the physiologic significance of this reduction in VEGFR2 expression, Matrigel plugs containing either FGF2 or VEGF-A were implanted subcutaneously in mice injected with
either Ad–sFGFR1-IIIc or Ad-Null 7 days prior. Blockade of FGF signaling not only attenuated the angiogenic response induced by FGF2, but also inhibited the angiogenic response induced by VEGF-A (Figure 3, C and D), as would be expected given reduced VEGFR2 expression in these settings. To further confirm this, a rescue experiment was performed using Ad-VEGFR2. VEGFR2 expression in the Matrigel plug using a constitutive, non–FGF-dependent promoter restored the VEGF-induced angiogenic response in the absence of FGF signaling (Figure 3, E and F). Moreover, the VEGF-A–induced angiogenic response in these settings was also rescued by expression of the ME- LA construct, but not the control ME construct (Figure 3G), further demonstrating that Erk1/2 activation plays an important role in FGF-induced regulation of VEGFR2 expression.

**FGF signaling is required for adult arteriogenesis.** We next tested the role of FGF-VEGF signaling interplay in arteriogenesis. Hindlimb ischemia studies were carried out in mice expressing either sFGFR1-IIIc (which binds FGF1, FGF2, FGF4, FGF5, FGF6, and FGF8) or sFGFR3-IIIb (FGF1, FGF9, and FGF20) traps (17). Laser Doppler perfusion imaging demonstrated that blood flow recovery started 3 days after ligation in control animals and was essentially complete by day 28, in agreement with previous publications (18). Flow recovery was significantly impeded in mice with sFGFR1-IIIc, but not sFGFR3-IIIb, FGF traps (Figure 4A). Micro-CT imaging demonstrated reduced arteriogenesis in Ad–sFGFR1-IIIc–injected mice that was limited to smaller arteries (Figure 4, B and C). VEGF-mediated SDF-1 expression in the perivascular region plays an important role in promotion of arteriogenesis in this setting (19). Indeed, SDF-1α was abundantly expressed in perivascular spaces in ischemic tissue in control mice, but its expression was markedly impaired in mice suppressed with FGF signaling (Figure 4D). To examine whether these effects are associated with reduced VEGFR2 expression, we determined Vegf2 mRNA expression in Ad–sFGFR1-IIIc–injected and control mice. In agreement with in vitro data, there was a marked decrease in Vegf2 mRNA expression not only in normal muscle, but also in ischemic muscle (Figure 4E). Thus, shutdown of FGF signaling in the vasculature caused a decreased arteriogenic response accompanied by downregulation of VEGFR2 expression and reduced VEGF signaling. Since inhibition of FGF signaling by sFGFR traps may not be limited to ECs, we developed a genetic model of endothelial-specific shutdown of FGF signaling using tetracycline-regulated, endothelial-specific expression of a FGFRI1DN construct (referred to herein as TRE-FGFR1DN). To test the activation of FGFR1DN expression by doxycycline withdrawal, TRE-FGFR1DN was expressed in tTA-expressing MEF-3T3 cells. High promoter activity, as measured by β-galactosidase activity, was seen in the absence of doxycycline and was almost completely suppressed in its presence (Supplemental Figure 6A). Expression of FGFR1DN in the absence of doxycycline was confirmed by Western blotting (Supplemental Figure 6A, inset).

The litter size and body weight of Tie2-tTA/TRE-FGFR1DN double-transgenic mice (FGFR1DN mice) in the “off” mode were similar to those of controls, which indicates that suppression of FGFR1DN expression was successfully achieved during fetal development and in the perinatal growth period. Because expression of the particular type of Tie2 promoter used in this study is very low in normal adult tissue and significantly stimulated in ischemic tissue (20), we used a hindlimb ischemia model. At 3 days after ischemia induction, there was marked upregulation of endothelial-specific FGFR1DN expression in the affected leg, while it remained undetectable in unaffected muscles (Supplemental Figure 6B). FGFR1DN expression became apparent 24 hours after induction of ischemia, with peak expression at 48 hours (Supplemental Figure 6C).

Induction of hindlimb ischemia after activation of endothelial FGFR1DN expression led to a dramatic phenotype. As early as 3 days after femoral artery ligation, FGFR1DN mice, but not control mice, began exhibiting toe loss; by day 7, frequent foot loss and severe muscle atrophy were apparent (Figure 5, A and B). Moderate tissue loss was observed in Ad–sFGFR1-IIIc–injected, but not Ad–sFGFR3-IIIb–injected, mice (Figure 5B), and TUNEL staining demonstrated widespread apoptosis of skeletal muscle in the ischemic limb of FGFR1DN mice (Figure 5, C and D). Blood flow analysis using laser Doppler perfusion imaging showed virtually no recovery in FGFR1DN mice, whereas control mice recovered normally (Figure 5E). Micro-CT analysis of arterial vasculature 21 days after induction of ischemia demonstrated a significant reduction in the number of arterial structures 64 μm in diameter or smaller, while the number of larger arteries was not affected (Figure 5, F and G). These results suggest that inhibition of the endothelial FGF signaling induced by FGFR1DN expression results in profound disruption of the arteriogenic response. Early observations in FGFR1DN-expressing mice suggested the presence of edema and hemorrhage in the ischemic limb. Micro-CT imaging at day 3 demonstrated extravasations of contrast (Figure 6A), confirming impaired vessel integrity. To further evaluate this, we obtained FITC-dextran angiograms in the lower adductor group muscle in the ischemic region of FGFR1DN mice and controls. Inhibition of FGF signaling was associated with dextran extravasations (Figure 6B), suggestive of disruption of vessel integrity. In line with the observed loss of arterial integrity, there was a substantial increase in skeletal muscle Evans blue dye retention in FGFR1DN mice compared with control mice (Figure 6C). As in other models, suppression of FGF signaling in these mice was associated with a marked reduction in Vegf2 mRNA expression in the ischemic tissue (Figure 6D). Further confirmation of reduced VEGF signaling was shown by profound blunting of a normal increase in tissue cGMP levels (Supplemental Figure 5D).

Inflammatory cells can mediate neovascularization by secreting angiogenic factors in response to angiogenic stimuli. Both FGF and VEGF are capable of recruiting monocytes to the site of new vessel formation, thus augmenting angiogenic response. To test the possibility that FGF inhibition suppresses inflammatory responses, thereby resulting in reduction of VEGFR2 expression and new vessel formation, we evaluated the contribution of inflammatory components to the vascular phenotype observed in our mouse models. In the Matrigel plug, infiltration of CD45+ cells was reduced in Ad–sFGFR1-IIIc–injected mice (Supplemental Figure 7, A and B). In contrast, the presence of inflammatory cells in the adductor region of the ischemic leg was comparable in both control and FGFR1DN mice (Supplemental Figure 7, C and D).

**Discussion**

Our present data indicate that (a) FGF-induced Erk1/2 activation is required for Ets activation, (b) subsequent Ets binding to the FOX-ETS motif in the first intron enhancer of the Vegfr2 gene plays an important role in VEGFR2 expression, and (c) this FGF regulation of VEGFR2 expression plays a critical role in adult neovascularization and vascular integrity (Figure 6E). Early observation pointed to critical interplay and synergism of FGF and VEGF systems in angio-
genic assays, with a combination of VEGF and FGF2 inducing a far stronger in vitro angiogenic response than either growth factor by itself (21). This was thought to be due to an induction of VEGFR2 expression by FGF2 while VEGF induction of FGFR expression was not seen (22). This has been attributed to the involvement of Erk and PKC signaling, but not yet been demonstrated (23). Our data demonstrate, for the first time to our knowledge, that Erk1/2 activation is critical for FGF-dependent regulation of VEGFR2 expression. Moreover, our results indicate that basal endothelial FGF signaling is critically required for VEGF actions that are important for both vessel formation and maintenance. This is in agreement with a prior observation of reduced VEGFR2 expression in Fgfr1−/−embryoid bodies. Surprisingly, however, vascular development in these embryoid bodies was increased (24), perhaps as a result of excessive VEGFR1 activity. In our present study, the consequence of VEGFR2 reduction was attenuation of angiogenic and arteriogenic responses in adult mice.

In many experimental settings, FGF-driven angiogenesis is blocked by VEGF inhibition, which suggests that FGF controls angiogenesis upstream of VEGF by modulating VEGF function (1, 4, 25–29). This hierarchical regulation appears to play a role similarly in lymphangiogenesis, as FGF2-induced lymphatic growth is inhibited by the blockade of VEGFR3 signaling (30).

Although we demonstrated FGF-VEGF signaling crosstalk in ECs, identifying specific FGF ligands and their source is a more complex issue, since numerous FGFs, including angiogenic (FGF1, FGF2, FGF4, and FGF5) and endocrine (FGF19, FGF21, and FGF23) factors, circulate in the bloodstream. Many of these FGFs can bind to the same FGF receptor subtypes, leading to a high level of redundancy. Given the critical role of FGF-VEGF crosstalk, such redundancy provides obvious evolutionary advantages. In addition to the interaction with the VEGF system, FGFs can control functions of other growth factors and chemokines, such as PDGF, HGF, and MCP-1 (31–34). Although the precise mechanisms of these interactions remain obscure, it is interesting to speculate that FGFs can modulate multiple neovascularization events.

We found that continuous FGF stimulation was necessary for the maintenance of VEGFR2 levels and that in its absence, Vegfr2 expression rapidly declined, leading to reduced production of NO, impaired angiogenesis and arteriogenesis, and, eventually, loss of vascular integrity. This is accomplished at the molecular level by FGF-dependent Erk1/2 activation in the endothelium that in turn leads to Ets family transcription factor binding to the Ets binding site in the recently described FOX:ETS composite site in the Vegfr2 enhancer (8).

Endothelial enhancers usually have multiple conserved Ets sites, which occur in clusters. In the case of Vegfr2, the 3′ region of the first intron (+3,437/+3,947) contains an autonomous enhancer that drives endothelium-specific Vegfr2 expression (35). Although the enhancer contains at least 3 sites that can bind Ets family members (Pea3, Ets1, and FOX:ETS sites), only the mutation of the Ets core binding sequence in the FOX:ETS site significantly reduced the enhancer’s transcriptional activity, which suggests that this is a key site of action. The FOX:ETS site can bind several Ets family members, including Etv2, Ets1, and, likely, Ets2. Etv2 is particularly interesting in light of recent studies showing its involvement in regulation of endothelial-specific gene expression in embryos (8, 36, 37). The contribution of Etv2 to VEGFR2 expression and embryonic vascular development — through activation of VEGFR2 promoter, but not VEGFR2 enhancer — has been previously demonstrated (36). Etv2 expression is significantly downregulated in adult mice, suggestive of a difference between embryonic and adult angiogenesis (38). Genetic studies show that although neither Ets1-null nor Ets2-null mice exhibit vascular defects, mutation of both Ets1 and Ets2 results in embryonic lethality as a result of impaired endothelial survival and angiogenesis (39–41). Therefore, it is reasonable to speculate that VEGFR2 expression is controlled by Ets family members in a dynamic fashion. Given the short half-life of VEGFR2 protein, as shown by us and others (ref. 42 and the present study), this finely tuned transcriptional regulation of VEGFR2 expression enables ECs to quickly adjust to changes in the extracellular environment, especially when vessel growth is required.

The role of FGF signaling in the maintenance of VEGFR2 expression is particularly interesting in this context, as augmented and reduced FGF input to the endothelium will lead to increase and decrease, respectively, in VEGFR2 levels, thereby modulating its sensitivity to VEGF stimulation. Our demonstration of FGF-dependent maintenance of VEGFR2 expression is consistent with a prior study demonstrating the loss of vascular integrity in the absence of FGF signaling (7) and with studies implicating VEGF signaling in EC survival (43).

FGF-dependent regulation of expression is not limited to VEGFR2. Since the FOX:ETS motif is involved in regulation of a number of endothelial-specific genes (8, 44), other genes may be affected as well. Indeed, we observed a reduction in Tie2 and Notch4 expression after suppression of FGF signaling, whereas VE-cadherin expression was not affected. The lack of regulation of VE-cadherin expression by the FOX:ETS motif is not surprising, since it is likely that not all FOX:ETS sequences are regulated in the same manner (their regulation is likely to be context dependent), and the relative importance of this motif may vary in different genes.

Although anti-VEGF therapies can cause vessel disintegration and regression not only in many experimental settings, but also in human clinical trials (45), the vasculature quickly rebounds once VEGF inhibition is withdrawn (46, 47). A combination of FGF and VEGF signaling inhibition may have a far more profound effect on the vasculature, as the rebound in vascular growth is not likely to occur in the absence of VEGFR2 expression.

In summary, FGF signaling is critically required for the maintenance of VEGFR2 expression, and its inhibition has profound effects on VEGF-dependent biological processes.

Methods

Study approval. Mice were maintained in the Animal Research Center at Yale University. All animal experiments were performed under a protocol approved by the IACUC of Yale University.

Cell culture and Ad transduction. BAECs and human umbilical artery ECs (Lonza) as well as MAECs and mouse lung microvascular ECs (isolated as previously described; ref. 48) were cultured at 37°C in 5% CO2 in EGM-2 MV medium (Lonza) on plates coated with fibronectin (10 μg/ml). Ad vectors were transduced at MOI 10–100 pfu/cell for Ad-FGFR1DN or 100–500 viral particles/cell for Ad-ME and Ad–ME-LA. The infection medium was replaced 4–6 hours later with normal growth medium. cGMP was measured with a cGMP EIA kit (Cayman Chemical).

VEGFR2 enhancer assay and VEGFR2 enhancer mutants. BAECs were transduced with Ad-Null or Ad-FGFR1DN and then transfected with plasmids the next day using Lipofectamine 2000 (Invitrogen). Both transductions and transfections were carried out in triplicate, and each experiment was repeated at least twice. 48–72 hours later, cells were harvested and assayed
using Dual-Luciferase Reporter Assay System (Promega). Absorbance ratios of Firefly to Renilla luciferases were normalized to the Ad-null control. Mouse VEGFR2 enhancer/partial promoter construct in pGL2 vector (gift of C. Patterson, University of North Carolina, Chapel Hill, North Carolina, USA; ref. 49) was used for luciferase assay. In mutagenesis studies, the consensus Ets binding core sequence GGAGA (T/G) and A/TTCC (antisense) were mutated to TTA/A and T/TTCC, respectively. Mutations were introduced at the Pea3 site (ACCCAAAGGAG, G567_G577), Ets1 site (GCAAGAAAAAC, G303T_G304T), and FOX/ETS site (TTCTTCTGTATCG, C377A_A379A) with the QuickChange site-directed mutagenesis kit (Stratagene). The VEGFR2 enhancer sequence was based on GenBank accession no. AF061804 (35).

**Immunostaining.** Muscles from ischemic lower legs were embedded in OCT compound (Sakura Finetek) and frozen to −80 °C. Cryostat tissue sections (5–10 μm) were stained with anti-CD31, -HA, -SDR-1f, and −α-smooth muscle actin antibodies. IgGw was used to show antibody specificity.

**Hindlimb ischemia model and laser Doppler perfusion imaging.** Mouse hindlimb ischemia was induced as described previously (18). Data are reported as a ratio of ischemic (right) to nonischemic (left) hind limb blood flow (18).

**Angiography and micro-CT imaging.** Angiography and micro-CT analysis were carried out as described previously (18). Data were expressed as a vascular segment number, representing the total number of vessels of specified diameter counted in 250 sections for calf in 3D micro-CT images.

**Permeability assays.** 0.5% Evans Blue (200 μl) was injected into the retroorbital space and allowed to circulate for 30 minutes. The mice were then sacrificed, blood was drained, and the adductor group muscle was excised and dried at 55 °C. Evans Blue in tissues was extracted with formamide for 24 hours at 55 °C, and its fluorescence at 607 nm was measured by a fluorescence reader (Bio-Tek) (7). To visualize vascular leakiness, 3 days after induction of hindlimb ischemia, 500 μl FITC plus 2 μl dextran (Sigma-Aldrich), 10 mg/ml in 0.1% PBS, was injected into the left carotid artery. Dextran extravasation was monitored with a fluorescence stereomicroscope.

**Ad-FGFR infection and in vivo Matrigel plug assay.** C57BL/6 mice were injected with 5 × 10^10 viral particles of Ad−sFGFR1−Hic or Ad−Null together with 10 μm antennapedia peptide (gift of W. Sessa, Yale University; ref. 50).

After 7 days, mice were injected subcutaneously with 0.5 ml Matrigel containing FGF2 (200 ng/ml) or VEGF-A (100 ng/ml) with heparin (10 U). Ad−Flag-mVEGFR2, Ad−ME, or Ad−ME−LA (0.85 × 10^8 viral particles) were added in the Matrigel as described previously (51). 7 days later, mice were euthanized, and Matrigel plugs were stained with a monoclonal CD31 antibody, DylkDDKKTag antibody (Cell Signaling), and CD45 antibody (becton Dickinson). Randomly acquired frames (368.5 μm × 368.5 μm) were analyzed by counting CD31+ vessels using NIH Image software.

**Mouse angiogenesis array.** Mouse primary lung ECs were transduced with Ad−Null or Ad−FGFR1DN (MOI 50; n = 3 per group) for 24 hours. Cells were then harvested, and total RNA was collected using RNeasy Plus Mini Kit (Qiagen) and converted to cDNA using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). PCR array was performed using RT² Profiler Array Kits from SABiosciences. Array results were deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/ accession no. GSE28483).

**Statistics.** Unless otherwise indicated, data are presented as mean ± SD. Statistical analysis was performed with 2-tailed Student’s t test or Wilcoxon rank-sum test. Differences were considered statistically significant for P values of 0.05 or less.

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7. Murakami M, et al. The FGF system has a key role in regulating hindlimb ischemia, 500 μl FITC plus 2 μl dextran (Sigma-Aldrich), 10 mg/ml in 0.1% PBS, was injected into the left carotid artery. Dextran extravasation was monitored with a fluorescence stereomicroscope.

8. Ad−FGFR infection and in vivo Matrigel plug assay. C57BL/6 mice were injected with 5 × 10^10 viral particles of Ad−sFGFR1−Hic or Ad−Null together with 10 μm antennapedia peptide (gift of W. Sessa, Yale University; ref. 50).


