Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK

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Focal adhesion kinase (FAK) plays a critical role during vascular development because knockout of FAK in endothelial cells (ECs) is embryonic lethal. Surprisingly, tamoxifen-inducible conditional knockout of FAK in adult blood vessels (inducible EC–specific FAK knockout [i-EC-FAK-KO]) produces no vascular phenotype, and these animals are capable of developing a robust growth factor–induced angiogenic response. Although angiogenesis in wild-type mice is suppressed by pharmacological inhibition of FAK, i-EC-FAK-KO mice are refractory to this treatment, which suggests that adult i-EC-FAK-KO mice develop a compensatory mechanism to bypass the requirement for FAK. Indeed, expression of the FAK-related proline-rich tyrosine kinase 2 (Pyk2) is elevated and phosphorylated in i-EC-FAK-KO blood vessels. In cultured ECs, FAK knockdown leads to increased Pyk2 expression and, surprisingly, FAK kinase inhibition leads to increased Pyk2 phosphorylation. Pyk2 can functionally compensate for the loss of FAK because knockdown or pharmacological inhibition of Pyk2 disrupts angiogenesis in i-EC-FAK-KO mice. These studies reveal the adaptive capacity of ECs to switch to Pyk2-dependent signaling after deletion or kinase inhibition of FAK.

Introduction

Vascular remodeling requires a complex interplay between growth factor receptors, extracellular matrix components, and integrin receptors, making these attractive targets for antiangiogenic therapy. Key intermediary proteins function in a membrane-proximal manner to integrate extracellular signals and promote intracellular signal transduction required for vasculogenesis and angiogenesis. One of these intracellular proteins is the cytoplasmic tyrosine kinase FAK, which is activated by growth factor receptors or integrin clustering and is critical for the assembly of a variety of signaling complexes (Mitra and Schlaepfer, 2006). FAK expression is essential for blood vessel development because global (Ilic et al., 1995) or endothelial cell (EC)-specific (Shen et al., 2005; Braren et al., 2006) knockout of FAK results in embryonic lethality with vascular defects. Interestingly, overexpression of FAK has the opposite effect, as transgenic mice overexpressing FAK in ECs show enhanced angiogenic responses to skin wounds and muscle ischemia (Peng et al., 2004). Together, these studies point to FAK as a critical factor for developmental and pathological angiogenesis. Indeed, control of FAK signaling has been suggested as a potential anticancer therapy and several FAK inhibitors have recently been developed (Slack-Davis et al., 2007; Roberts et al., 2008). However, it is not clear whether FAK inhibitors target ECs or impact angiogenesis directly.

Because conditional knockout of FAK from the endothelium produces a lethal phenotype, the role of FAK during vascular remodeling in vivo has not been fully addressed. Here, we report that tamoxifen-inducible, Cre-mediated FAK deletion from adult endothelium is surprisingly not lethal due to functional compensation by the FAK-related protein proline-rich tyrosine kinase 2 (Pyk2). This compensatory switch from FAK to Pyk2 occurs in blood vessels and in cultured human ECs, promoting vascular hemostasis and preserving integrin-mediated signaling during vascular remodeling events.

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Abbreviations used in this paper: BAEC, bovine aortic endothelial cell; bFGF, basic fibroblast growth factor; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; i-EC-FAK-KO, inducible EC–specific FAK knockout; Pyk2, proline-rich tyrosine kinase 2; shRNA, short hairpin RNA; WT, wild type.

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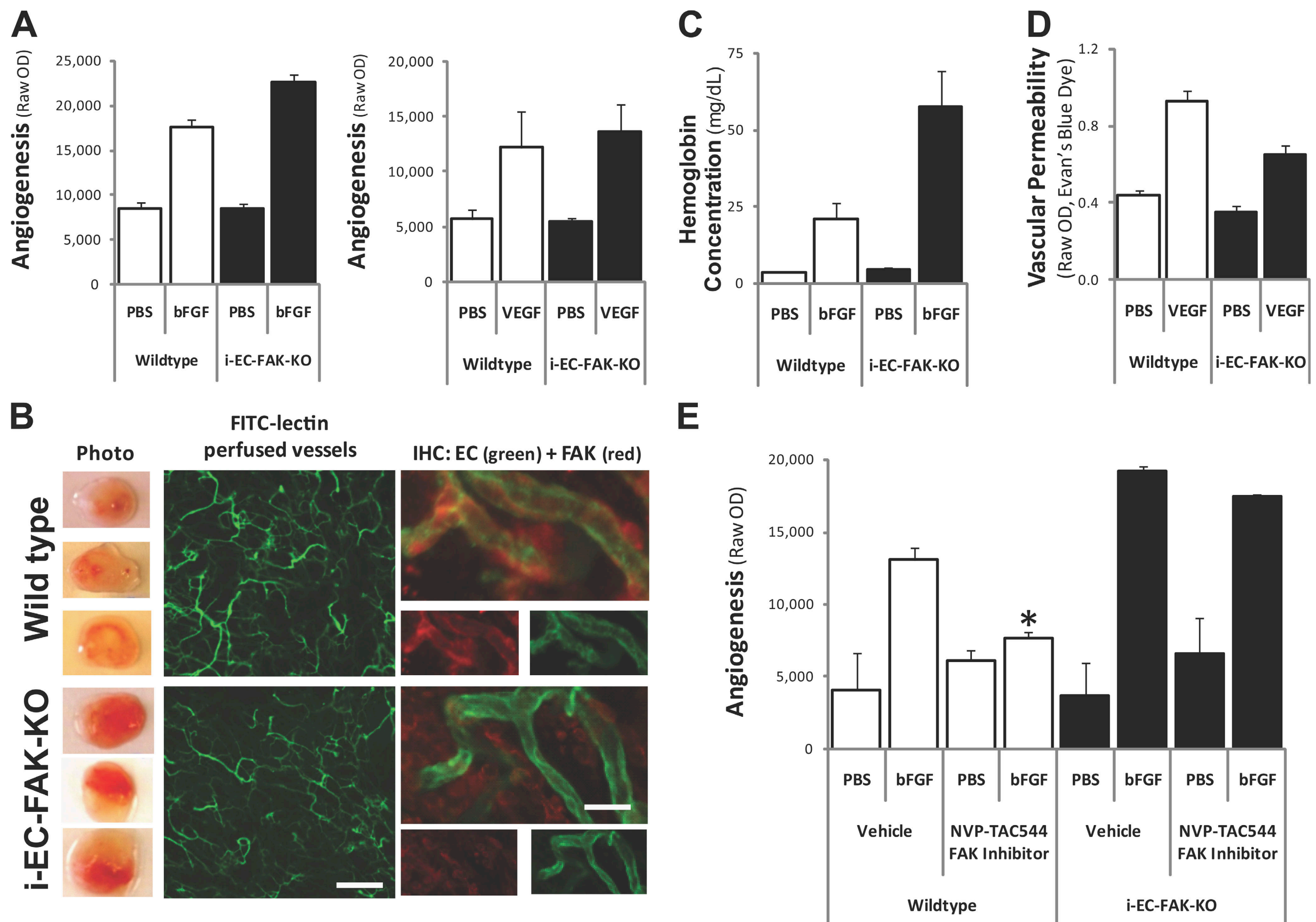

Figure 1. **Robust growth factor–induced angiogenesis in i-EC-FAK-KO mice.** Matrigel containing PBS, bFGF, or VEGF was injected subcutaneously to assess angiogenesis in vivo. (A) Angiogenic responses to bFGF or VEGF were equal or more robust in i-EC-FAK-KO compared with the WT, quantified by FITC-lectin content. n = 7–15 each. (B, left) FITC-lectin–perfused blood vessels within Matrigel from the WT and i-EC-FAK-KO appear similar. Bar, 50 µm. (right) Staining for FAK (red) and EC markers (green) confirms the lack of FAK on i-EC-FAK-KO vessels. Bar, 5 µm. (C) Hemoglobin concentration is increased in i-EC-FAK-KO plugs, which is consistent with their bloodier appearance. n = 4 each. (D) i-EC-FAK-KO mice showed 30% less VEGF-induced leakage in the skin compared with the WT, which suggests that their robust angiogenic response was not caused by increased leakage. n = 11 each. (E) Treatment with the NVP-TAC544 FAK inhibitor blocked the angiogenic response induced by bFGF in WT but not i-EC-FAK-KO. n = 4 each; *, $P < 0.05$ versus vehicle. All graphs show mean ± SEM.

Results and discussion

Generation of mice with inducible, conditional FAK knockout

To assess the postdevelopmental role of FAK in adult blood vessels, we used a Cre/loxP strategy to create an inducible, conditional knockout of FAK in ECs. Floxed FAK mice containing two loxP sites flanking exon 3 of the FAK gene (Shen et al., 2005) were crossed with End-SCL-Cre-ER(T) mice containing tamoxifen-inducible Cre-ER(T) driven by the 5′ endothelial enhancer of the stem cell leukemia locus (Gothert et al., 2004). At 5 wk of age, littermates of FAK fl/fl;Cre(+) and FAK fl/fl;Cre(−) mice were treated with 2 mg tamoxifen every 2 d for 2 wk to generate "wild-type" (WT) mice (tamoxifen-treated mice with no Cre expression and thus no FAK deletion) and inducible EC–specific FAK knockout ("i-EC-FAK-KO") mice (tamoxifen-induced EC-specific Cre expression resulting in FAK deletion).

Robust angiogenic response in i-EC-FAK-KO mice

In contrast to previous EC-specific FAK knockout models with embryonic lethality (Shen et al., 2005; Braren et al., 2006), knockout of FAK in adult endothelium did not produce an overt phenotype in mice of either gender. This finding prompted us to challenge these mice with angiogenic growth factors to assess the role of FAK during angiogenesis. Matrigel containing basic fibroblast growth factor (bFGF) or VEGF was implanted subcutaneously into mice to induce neovascularization. After 5 d, mice were injected with FITC-lectin to label ECs and the plugs were removed and homogenized to quantify the FITC-lectin content. Surprisingly, either bFGF or VEGF elicited a robust angiogenic response in i-EC-FAK-KO mice that was equivalent to or greater than that observed in WT mice (Fig. 1 A). Although neovascularization was evident by both EC-specific FITC-lectin binding and labeling with EC markers, vessels within i-EC-FAK-KO plugs did not stain positive for FAK (Fig. 1 B). This result

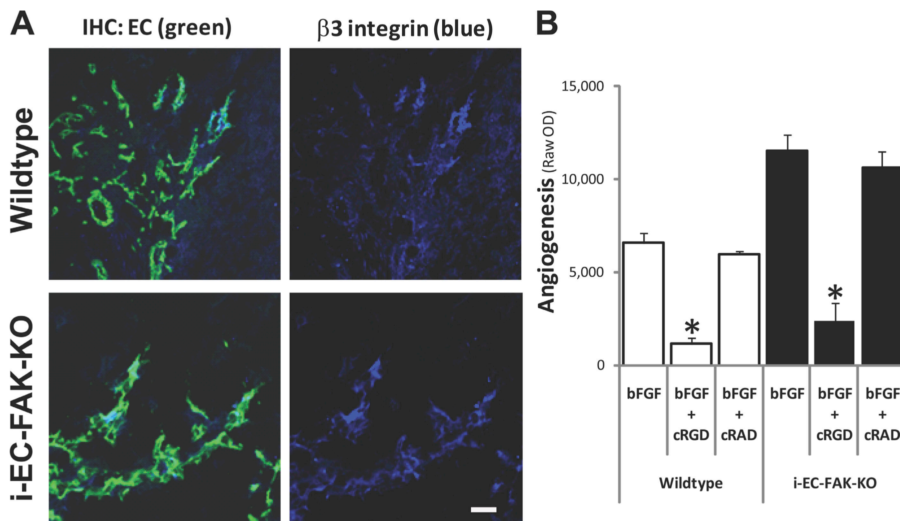

Figure 2. **Integrin requirement for angiogenesis despite loss of EC FAK expression.** (A) β3 integrin (blue) is expressed on vessels (green) within Matrigel plugs from WT or i-EC-FAK-KO mice. Bar, 5 µm. (B) The selective αvβ3 integrin antagonist cRGD-fK blocked the angiogenic response equivalently for both genotypes. *n* = 4 each group; *, $P < 0.05$ versus bFGF. Graph shows mean ± SEM.

confirms the loss of EC FAK expression in i-EC-FAK-KO mice and specifically on the newly forming vessels within the Matrigel plugs. The Matrigel plugs from i-EC-FAK-KO mice appeared bloodier and had a higher hemoglobin concentration than the WT (Fig. 1, B and C). However, local VEGF injection to the skin induced a slightly lower vascular leak response in i-EC-FAK-KO mice (Fig. 1 D). Thus, the more robust angiogenic response in i-EC-FAK-KO mice does not appear to be a function of VEGF-induced vascular leak.

Antiangiogenic activity of a FAK kinase inhibitor

Small molecule FAK kinase inhibitors can suppress tumor cell proliferation and as such are now undergoing clinical evaluation as anticancer drugs (Shi et al., 2007; Slack-Davis et al., 2007). In addition to direct effects on tumor cells, it is not known whether these inhibitors also suppress angiogenesis by targeting FAK activity in ECs, pericytes, inflammatory cells, or stromal cells. Here, we report that daily treatment with a FAK kinase inhibitor (NVP-TAC544; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200710038/DC1) in WT mice resulted in an 80% decrease in bFGF-mediated angiogenesis but had no effect in i-EC-FAK-KO mice (Fig. 1 E). Because this ATP-competitive inhibitor blocks FAK kinase activity in all cell types involved in the angiogenic response, our results indicate that FAK kinase activity specifically within ECs is critical for angiogenesis in WT animals and that targeting FAK in ECs accounts for the antiangiogenic activity of this drug. Thus, a compensatory signaling pathway in ECs may be operative accounting for a FAK-independent angiogenic response in i-EC-FAK-KO mice.

Angiogenesis is integrin-dependent in i-EC-FAK-KO mice

Because integrins do not possess intrinsic enzymatic activity, signal transduction requires association with and activation of membrane-proximal proteins such as FAK, which transmits signals from integrins and initiates intracellular signaling pathways (van Nimwegen and van de Water, 2007). Because integrin αvβ3 is expressed selectively on angiogenic EC in vivo (Brooks et al., 1994) and inhibitors of αvβ3 abolish angiogenesis in response to growth factors or tumors (Brooks et al., 1995), we examined the role of this integrin during angiogenesis in the presence or absence of FAK. In Matrigel plugs from WT and i-EC-FAK-KO mice, bFGF-stimulated blood vessels showed intense αvβ3 staining, indicating that FAK is not required for αvβ3 expression on angiogenic EC (Fig. 2 A). To assess the efficacy of αvβ3 antagonism, Matrigel plugs were implanted containing bFGF along with a cyclic RGD-fK peptide that selectively blocks αvβ3 function (Dai et al., 2000) or a control cyclic RAD-fK peptide. We found that the cRGD-fK peptide blocked angiogenesis in both genotypes (Fig. 2 B), which suggests that integrin-mediated signals can be transmitted through alternative intracellular components during angiogenesis in the absence of EC FAK.

Pyk2 expression is increased on normal and angiogenic blood vessels in i-EC-FAK-KO mice

Pyk2 is a cytoplasmic tyrosine kinase structurally related to FAK. FAK and Pyk2 have been proposed to promote both distinct and overlapping signaling events, which appear to vary by cell type. Although FAK is ubiquitously expressed, Pyk2 is selectively enriched in certain cell types, including hematopoietic cells (Tang et al., 2002). Although Pyk2 has recently been linked to EC functions including VE-cadherin–mediated adhesion (van Buul et al., 2005; Allingham et al., 2007), cell migration (Avraham et al., 2003), angiogenic sprouting (Tang et al., 2002; Matsui et al., 2007), and integrin-mediated signaling (Lamalice et al., 2007), the role of Pyk2 during vascular remodeling in vivo remains poorly understood. Although Pyk2 expression is enhanced in embryonic fibroblasts isolated from FAK null mice (Sieg et al., 1998), no changes in Pyk2 levels have been detected within tissues from conditional FAK knockout mice with the exception of conditional knockout in osteoblasts (Kim et al., 2007).

To determine if Pyk2 may account for the compensatory angiogenic response observed in adult i-EC-FAK-KO mice, tissues from WT or i-EC-FAK-KO mice were examined using

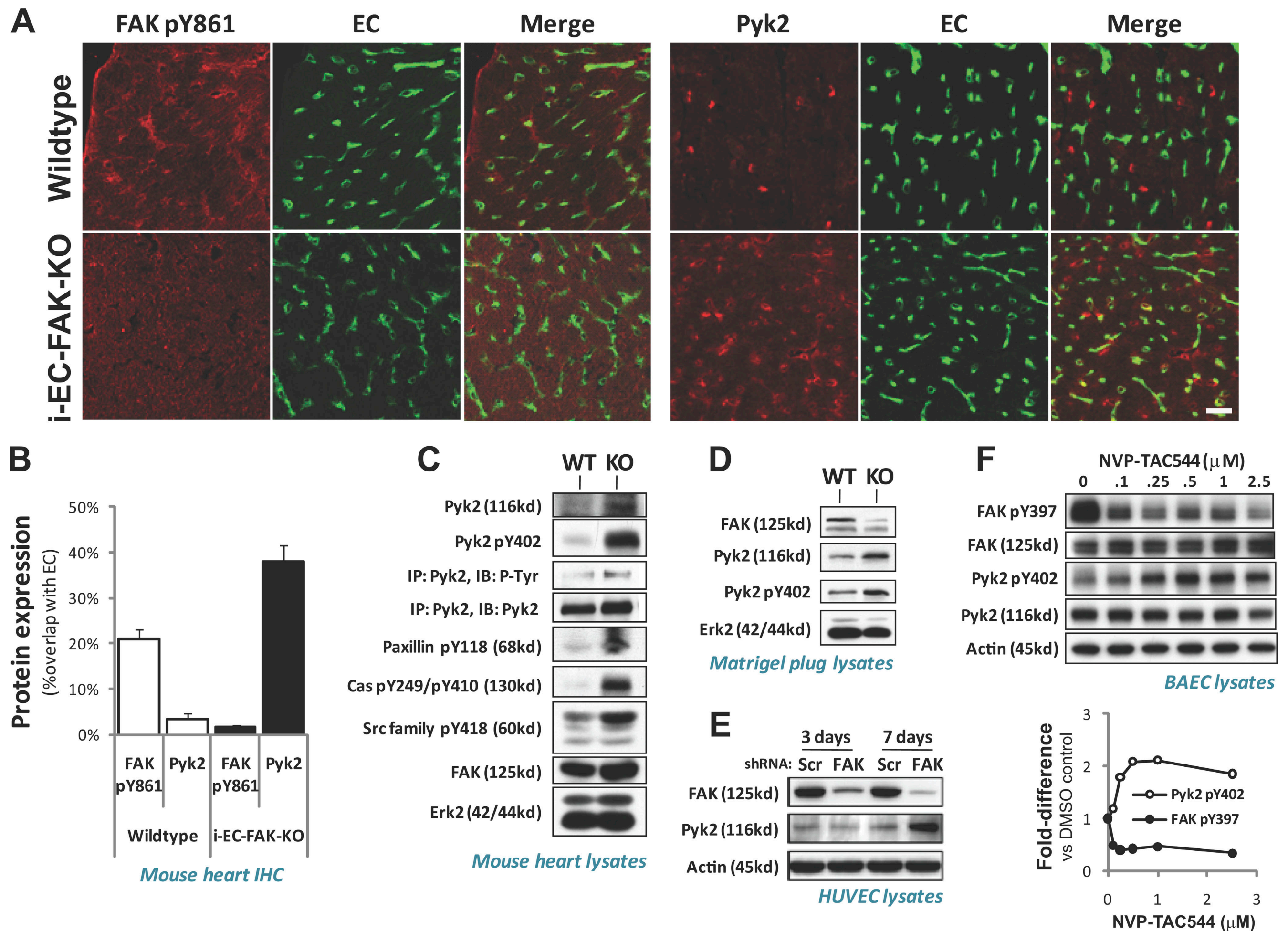

Figure 3. **Pyk2 is up-regulated in the absence of FAK.** (A and B) FAK pY861 staining (a marker for activated FAK) appears on blood vessels in the WT but not i-EC-FAK-KO heart. Although minimally detected on blood vessels in the WT heart, Pyk2 expression on ECs is elevated in i-EC-FAK-KO. Graph represents mean ± SEM. Bar, 10 µm. (C) Expression of Pyk2, phosphorylation of Pyk2 on its autophosphorylation site Y402, and phosphorylation of FAK/Pyk2 substrates was increased 3- to 10-fold in heart lysates from i-EC-FAK-KO mice. (D) Pyk2 and pY402 were elevated in angiogenic Matrigel plugs from i-EC-FAK-KO mice. (E) Primary human ECs (HUVECs) were treated with shRNA for FAK or a nonsilencing scramble shRNA control "Scr." Pyk2 expression was elevated after FAK deletion. (F) BAECs treated with the NVP-TAC544 FAK inhibitor for 1 h showed dose-dependent FAK blockade (pY397) and a surprising increase in Pyk2 (pY402) phosphorylation.

immunohistochemistry (Fig. 3 A). As expected, FAK was ubiquitously expressed on all cell types in the heart (unpublished data). In contrast, we found expression of FAK pY861 to be primarily associated with vascular structures in the WT heart, and this was reduced in the i-EC-FAK-KO heart (Fig. 3, A and B). Pyk2 expression in the WT heart was primarily restricted to lymphatic vessels and hematopoietic cells and was minimally detected on ECs (Fig. 3, A and B). In contrast, we observed a 10-fold increase in the percentage of Pyk2-positive ECs within i-EC-FAK-KO mice (Fig. 3 B). We confirmed this increase in Pyk2 expression and activated Pyk2 phosphorylated at Y402 by immunoblotting mouse hearts lysates (Fig. 3 C). Pyk2 may be constitutively active in these tissues because we also measured elevated phosphorylation of the FAK/Pyk2 substrates Src pY418, p130Cas pY249/pY410, and paxillin pY118 (Wu et al., 2008). These substrates downstream of integrin ligation and FAK activation play a role in cell migration and invasion during angiogenesis and tissue remodeling (Mitra and Schlaepfer, 2006). Increased phosphorylation of these substrates supports our finding of a more robust angiogenic response in i-EC-FAK-KO mice (Fig. 1). To investigate signaling within angiogenic tissues directly, Matrigel plugs were lysed and processed for immunoblotting. The cells infiltrating the Matrigel plugs growing within i-EC-FAK-KO mice showed decreased FAK along with increased Pyk2 expression and pY402 phosphorylation compared with the WT (Fig. 3 D). Together, our results suggest that Pyk2 expression increases after the loss of FAK on blood vessels in vivo. This Pyk2 response appears to be an overcompensation resulting in robust angiogenesis and vascular leakage, possibly because of constitutively increased Pyk2 activity.

Pyk2 compensation for loss of FAK also occurs in human ECs

To investigate whether a similar compensatory mechanism might take place in human ECs, we knocked down FAK in primary human umbilical vein ECs (HUVECs) using short hairpin RNA (shRNA) and monitored expression of Pyk2 over time. Compared with a nonsilencing scramble control shRNA (Scr), FAK shRNA

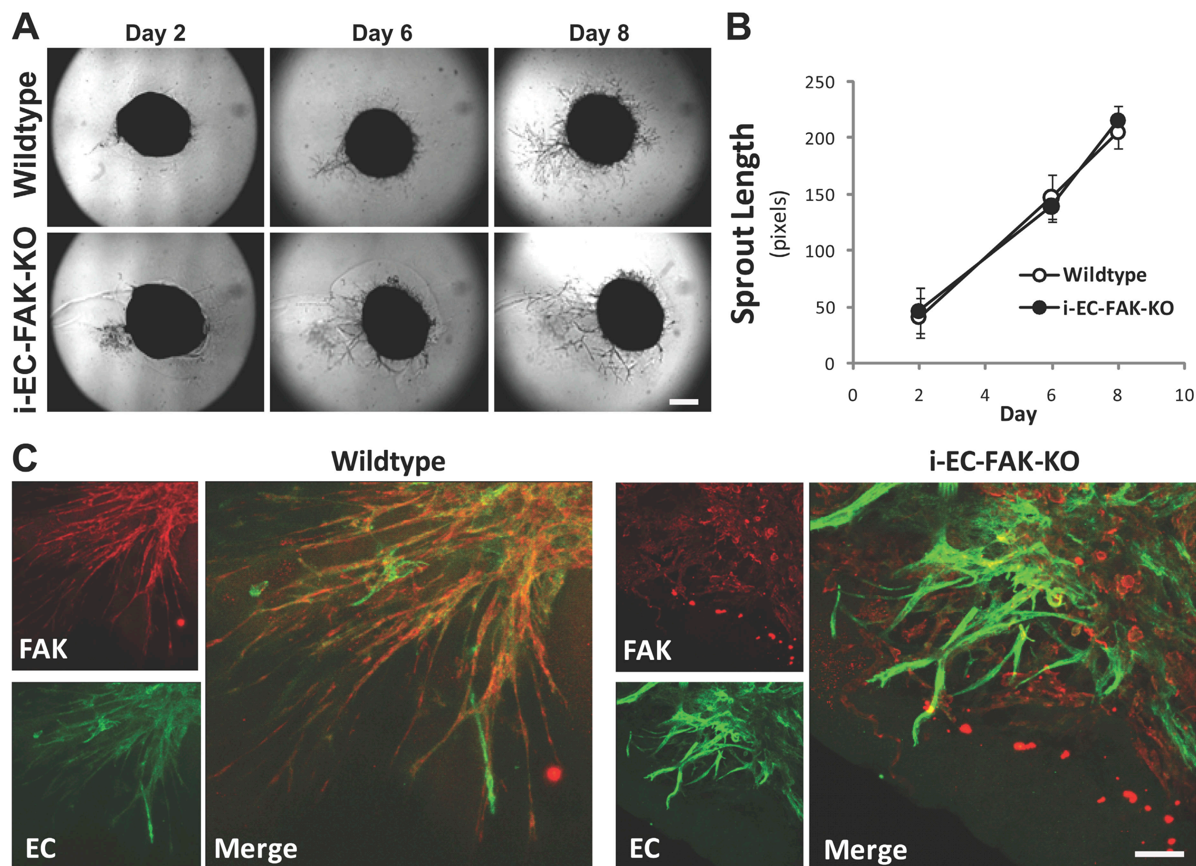

Figure 4. **Ex vivo sprouting of aortic explants is normal in i-EC-FAK-KO mice.** (A and B) Aortic ring explants from WT and i-EC-FAK-KO mice were grown as ex vivo cultures to assess sprouting. The mean sprout length at several time points was similar between genotypes, suggesting a normal angiogenic response to serum. *n* = 6 each; Graph shows mean ± SEM. (C) Immunohistochemical staining for FAK (red) and EC (green) confirms lack of FAK protein expression on vessels sprouting from rings isolated from i-EC-FAK-KO mice. Bars: (A) 0.5 mm; (C) 80 µm.

induced an 85–90% knockdown of FAK along with a threefold induction of Pyk2 expression at 7 d (Fig. 3 E). These findings are consistent with the increase in Pyk2 we observed in i-EC-FAK-KO mice, a model in which Cre-mediated FAK deletion occurs gradually during a 2-wk tamoxifen treatment period. The fact that compensatory Pyk2 expression increases only after 3–7 d may explain the lethality associated with EC FAK deletion during development (Shen et al., 2005; Braren et al., 2006), a situation in which the loss FAK cannot be compensated for by Pyk2 on a timescale to allow embryonic survival.

FAK inhibition activates Pyk2

Typical ECs express low levels of Pyk2, as observed for the mouse heart (Fig. 3, A–C) and HUVECs (Fig. 3 E). In contrast, bovine aortic ECs (BAECs) express significant levels of endogenous Pyk2 (Fig. 3 F). Treatment of these Pyk2-positive ECs with the NVP-TAC544 FAK inhibitor induced a dose-dependent decrease in FAK pY397 phosphorylation along with a surprising increase in Pyk2 pY402 phosphorylation (Fig. 3 F). In vitro kinase assays confirm that this inhibitor blocks FAK while increasing Pyk2 in BAECs (Fig. S1 C). Although it is possible that this inhibitor may interact with Pyk2, leading to its activation, we observed similar Pyk2 activation in mouse embryonic fibroblasts treated with two chemically distinct FAK inhibitors (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200710038/DC1). Together, our in vitro and in vivo studies using genetic and pharmacological approaches suggest that Pyk2 activity increases upon FAK deletion or inhibition and that a balance of FAK/Pyk2 activity may influence EC growth or survival during angiogenesis.

Pyk2 can functionally compensate for loss of FAK during angiogenesis

To further manipulate FAK and Pyk2 function during angiogenesis, we grew sections of mouse aorta in a 3D Matrigel culture to examine EC sprouting over time. Consistent with our in vivo results (Fig. 1), we observed equivalent ex vivo sprouting between genotypes, and immunohistochemical staining confirmed the absence of FAK on ECs sprouting from i-EC-FAK-KO explants (Fig. 4). If Pyk2 does compensate for FAK deletion, then a Pyk2 blockade should impair the angiogenic response in i-EC-FAK-KO mice. To test this using a knockdown approach, aortic rings were cultured in the presence of lentiviruses expressing GFP along with shRNA for FAK, Pyk2, or a scrambled control. Immunoblotting confirmed equivalent GFP expression and shRNA-mediated knockdown (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200710038/DC1), and the scramble control shRNA did not affect the angiogenic response (not depicted). In WT aortic rings, shRNA-mediated knockdown of FAK reduced sprouting but knockdown of Pyk2 had no

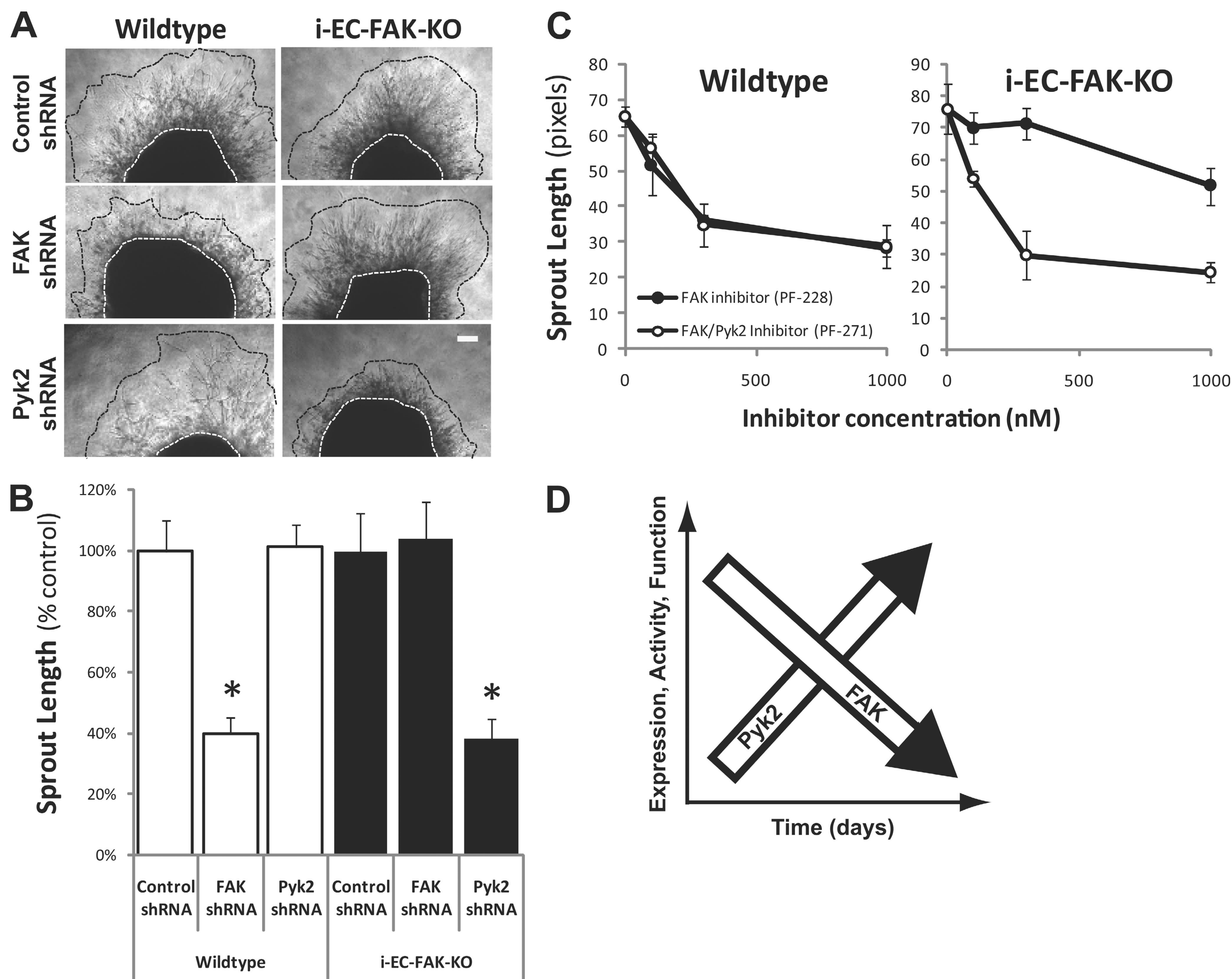

Figure 5. **Pyk2 can functionally compensate for loss of FAK during angiogenesis.** (A and B) Knockdown of FAK (but not Pyk2) slowed the rate of sprouting in WT aortic explants, indicating FAK plays the primary role during angiogenesis in normal blood vessels. For i-EC-FAK-KO, knockdown of Pyk2 (but not FAK) inhibited the angiogenic response. Broken lines indicate sprout length. Bar, 0.25 mm. (C) Sprouting of WT aortic explants was dose-dependently blocked by either a pharmacological inhibitor selective for FAK (PF-228) or a dual FAK/Pyk2 inhibitor (PF-271). Only the dual FAK/Pyk2 inhibitor (PF-271) blocked sprouting in i-EC-FAK-KO explants. $n = 6$ each; *, $P < 0.05$ versus control shRNA or vehicle. Graphs show mean ± SEM. (D) Our results suggest a necessity for FAK and/or Pyk2 expression in ECs that can preserve the angiogenic response in i-EC-FAK-KO mice.

effect, confirming that FAK (and not Pyk2) plays a primary role in angiogenesis within normal blood vessels (Fig. 5, A and B). In i-EC-FAK-KO vessels, knockdown of FAK had no effect but knockdown of Pyk2 disrupted sprouting (Fig. 5, A and B).

In the knockout mouse tissues, FAK is deleted and Pyk2 is up-regulated for the duration of the experiment; thus, the initial sprouting and invasion into the Matrigel matrix in vivo (Fig. 1) or in vitro (Fig. 4) must occur in the absence of FAK. In contrast, for shRNA-mediated FAK knockdown, FAK expression gradually decreases and compensatory Pyk2 expression gradually increases after several days as we observed for cultured ECs (Fig. 3 E). Accordingly, in aortic sprouting experiments using shRNA-mediated FAK knockdown, we found the largest differences between groups between 4 and 7 d, after which time the rate of sprouting was equivalent (unpublished data). Another significant difference between these models is the fact that shRNA impacts all cell types, whereas the knockout mouse has selective FAK deletion within ECs. The shRNA-mediated FAK knockdown results are consistent with our observations for sprouting in vivo (Fig. 1 E), in which pharmacological FAK inhibition delivered to all cell types did not negatively affect the angiogenic response in i-EC-FAK-KO mice. Together, these studies strongly suggest that EC FAK expression is responsible for the antiangiogenic properties of FAK inhibitors in WT mice, whereas Pyk2 expression is responsible for angiogenesis in i-EC-FAK-KO mice.

To further test this, aortic explants were treated daily with an ATP-competitive inhibitor selective for FAK but not Pyk2 (PF-228; Slack-Davis et al., 2007) or a dual inhibitor of FAK/Pyk2 (PF-271; Roberts et al., 2008). The effects of these inhibitors on FAK and Pyk2 tyrosine phosphorylation are shown in Fig. S2. In aortic vessels isolated from WT mice, both inhibitors reduced the amount of angiogenic sprouting to a similar extent (Fig. 5 C). However, only the dual FAK/Pyk2 inhibitor could

reduce sprouting in aortic explants isolated from i-EC-FAK-KO mice (Fig. 5 C). Thus, we have demonstrated using both genetic and pharmacological approaches that increased Pyk2 expression and kinase activity compensate for the loss of FAK within i-EC-FAK-KO mice during angiogenesis. These findings reveal that Pyk2, typically involved in hematopoietic signaling, can adapt to assume the function of FAK within ECs to preserve the angiogenic response in vivo.

Conclusions

We have demonstrated that although normal ECs express low levels of Pyk2, increased Pyk2 expression and activity occurs upon loss of FAK both in i-EC-FAK-KO mice and in cultured ECs treated with FAK shRNA (summarized in Fig. 5 D). In cells with endogenous Pyk2, Pyk2 activity dose-dependently increases upon a 1-h treatment with pharmacological FAK inhibitors (Figs. 3 F, S1 C, and S2). Our findings support the hypothesis that ECs have the capacity to use Pyk2 signaling, but Pyk2 may be suppressed by FAK under normal conditions. Accordingly, the mechanism by which Pyk2 is up-regulated after FAK blockade warrants further investigation. We recently reported that Pyk2 expression is elevated in primary mouse and human fibroblasts upon FAK silencing and linked to enhanced p190RhoGEF expression leading to deregulated RhoA activation, elevated focal adhesion formation, and enhanced cell proliferation (Lim et al., 2008). Future work will reveal whether fibroblasts, ECs, or even carcinoma cells may share similar pathways of FAK/Pyk2 regulation and how these systems may contribute to the angiogenic response during cancer. This is especially relevant to the further development of dual FAK/Pyk2 inhibitors as anticancer or, potentially, antiangiogenic drugs.

Materials and methods

Mice

All mice were maintained on a C57BL6 background. Floxed FAK mice containing two loxP sites flanking exon 3 of the FAK gene (Shen et al., 2005) were crossed with mice harboring tamoxifen-inducible Cre-ER(T) driven by the 5′ endothelial enhancer of the stem cell leukemia locus (provided by C.G. Begley, University of Western Australia, Perth, Australia; Gothert et al., 2004). Age-matched littermates of FAK fl/fl;Cre(+) and FAK fl/fl;Cre(−) mice were treated with 2 mg tamoxifen (Sigma-Aldrich) every 2 d for 2 wk to generate WT mice (tamoxifen-treated mice with no Cre expression) and i-EC-FAK-KO mice (tamoxifen-induced EC-specific Cre expression and FAK deletion).

Inhibitors and shRNA

The cyclic RGD-fK peptide that binds integrin $\alpha v\beta 3$ with high potency and selectivity was synthesized as described previously (Dai et al., 2000). The FAK inhibitor NVP-TAC544 (Garcia-Echevarria et al., 2004, 2005) provided by T. Honda (Novartis, Tsukuba, Ibaraki, Japan) was solubilized in Cremophore DL/DMSO/ethanol (1:1:1) and injected i.p. with 40 mg/kg daily (~850 nM). The dual FAK/Pyk2 inhibitor PF-271 (Roberts et al., 2008) was synthesized according to available methods (Kath and Luzzio, 2004; Kath et al., 2005). The FAK inhibitor PF-228 (Slack-Davis et al., 2007) was provided by W.G. Roberts (Pfizer, Groton, CT). Both inhibitors were solubilized in DMSO at 1 mM. Lentiviral expression of scramble control, FAK, and Pyk2 shRNA has been described previously (Schlaepfer et al., 2007).

Primary antibodies

Antibodies were obtained from Santa Cruz Biotechnology, Inc. (FAK, paxillin, and Erk2), Cell Signaling Technology (Pyk2, p130Cas pY249/pY410, and Src pY416), BD Biosciences (Pyk2 and integrin $\beta 3$), Millipore (FAK, Pyk2 pY402, and paxillin pY118), Invitrogen (FAK pY397 and Pyk2 pY402), and Sigma-Aldrich (actin). Blood vessels were labeled for "EC markers" with a mix of rat anti–mouse antibodies recognizing Flk-1 (BD Biosciences), CD31 (BD Biosciences), VE-cadherin (BD Biosciences), and CD105 (Millipore).

In vivo angiogenesis

The Matrigel assay was performed to assess in vivo angiogenesis (Weis, 2007). In brief, mice were injected subcutaneously on the flank with 400 µl growth factor–reduced Matrigel (BD Biosciences) containing either sterile saline or 400 ng of human recombinant bFGF (Millipore) or VEGF (PeproTech). After 7 d, mice were injected intravenously with 20 µg FITC-conjugated lectin that binds selectively to mouse ECs (GSL I–BSL I; Vector Laboratories). The Matrigel plugs were removed, photographed, viewed whole-mount, and then fixed and stained for microscopy. Alternatively, plugs were homogenized and the fluorescence content was read at 620 nm (Tecan) or hemoglobin content was quantified using the QuantiChrom hemoglobin assay kit (BioAssay Systems).

In vivo permeability

A modified Miles assay was used to evaluate VEGF-induced leak in the skin as described previously (Eliceiri et al., 1999).

Ex vivo angiogenesis

The abdominal aorta was isolated using sterile technique and cut into 1-mm sections that were embedded in growth factor–reduced Matrigel and cultured using DME with 10% FCS and 30 ng/ml human recombinant VEGFA-165 (PeproTech) daily. Images were acquired on an inverted microscope (Axiovert 100; Carl Zeiss, Inc.) using a 20× 0.70 NA objective (Carl Zeiss, Inc.) and a SPOT RT camera (Model 2.2.1; Diagnostic Instruments, Inc.). Image J (National Institutes of Health) was used to measure sprout length.

Immunoblotting and immunostaining

Standard Western blotting and immunostaining of cells, whole-mount preparations, or frozen sections was performed. Secondary antibodies conjugated to Alexa Fluor 488, 568, or 647 (Invitrogen) were used for immunofluorescence. Images were acquired at room temperature using confocal microscopy (Nikon C1si with EZC1 acquisition software; Nikon) with Plan Apo 10× 0.45 NA air, Plan Apo 20× 0.75 NA air, and Plan Apo 60× 1.40 NA oil objective lenses (Nikon). Colocalization with EC markers was measured using MetaMorph 7 (MDS Analytical Technologies).

Statistical analysis

Graphs are presented as mean ± SEM, with statistical significance determined from a two-tailed Student's *t* test using $\alpha = 0.05$ and $P < 0.05$.

Online supplemental material

Fig. S1 provides the chemical structure and kinase profile for the NVP-TAC544 FAK inhibitor. Fig. S2 reveals the dose response of FAK inhibitors on FAK and Pyk2 tyrosine phosphorylation. Fig. S3 offers validation of the Pyk2 shRNA. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200710038/DC1.

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