Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis

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Focal adhesion kinase (FAK) is a critical mediator of signal transduction by integrins and growth factor receptors in a variety of cells including endothelial cells (ECs). Here, we describe EC-specific knockout of FAK using a Cre-loxP approach. In contrast to the total FAK knockout, deletion of FAK specifically in ECs did not affect early embryonic development including normal vasculogenesis. However, in late embryogenesis, FAK deletion in the ECs led to defective angiogenesis in the embryos, yolk sac, and placenta, impaired vasculature and associated hemorrhage, edema, and developmental delay, and late embryonic lethal phenotype. Histologically, ECs and blood vessels in the mutant embryos present a disorganized, detached, and apoptotic appearance. Consistent with these phenotypes, deletion of FAK in ECs isolated from the floxed FAK mice led to reduced tubulogenesis, cell survival, proliferation, and migration in vitro. Together, these results strongly suggest a role of FAK in angiogenesis and vascular development due to its essential function in the regulation of multiple EC activities.

Introduction

Blood vessel formation is fundamental to embryonic development and organogenesis, as well as to the pathogenesis of many diseases including coronary heart disease, diabetes, retinopathies, and cancer (Dvorak, 2003). Blood vessels are formed by two processes: vasculogenesis, whereby a primitive vascular network is established during embryogenesis from the pluripotent mesenchymal progenitors, and angiogenesis, in which the growth of new capillaries occurs from the preexisting vessels (Yancopoulos et al., 1998). Endothelial cells (ECs) play a pivotal role in both vasculogenesis and angiogenesis, and function as both transducers and effectors of local environmental signals for vessel formation. Multiple cell surface receptors and their ligands have been shown to play important roles in the regulation of blood vessel formation. These include proangiogenic growth factors and their receptors on EC, multiple integrins, and their ECM ligands (Yancopoulos et al., 1998; Hynes, 2002). However, much less is known about the roles and mechanisms of the intracellular signaling pathways triggered by these receptors in the regulation of angiogenesis and vasculogenesis.

FAK is a cytoplasmic tyrosine kinase that plays a key role in integrin-mediated signal transduction pathways (Parsons, 2003; Schlaepfer and Mitra, 2004). Integrin-mediated cell adhesion leads to FAK activation and autophosphorylation in a variety of cell types. Activated FAK associates with a number of Src homology 2 domain–containing signaling molecules including Src family kinases, p85 subunit of PI3K, phospholipase C-γ, and Grb7 (Parsons, 2003). FAK binding to Src family kinases contributes to the activation of both kinases, which leads to phosphorylation of several other sites on FAK and a number of other substrates including paxillin (Burridge et al., 1992; Schaller and Parsons, 1995), p130cas (Vuori et al., 1996; Ruest et al., 2001), and Shc (Schlaepfer et al., 1998). FAK and its interactions with these signaling molecules have been shown to trigger several downstream signaling pathways that regulate cell spreading and migration, cell survival, and cell cycle progression (Parsons, 2003; Schlaepfer and Mitra, 2004).

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Abbreviations used in this paper: CFKO, conditional FAK knockout; EC, endothelial cell; MEF, mouse embryonic fibroblast; PECAM-1, platelet-endothelial cell adhesion molecule-1.

Figure 1. **Generation of the floxed and total deletion FAK allele.** (A) Schematic of mouse FAK protein, targeting vector, genomic and the loxP modified FAK loci. Large filled triangles represent loxP sites. The relevant restriction sites (A, ApaI; S, SacI; and B, BamHI), position of probe (thick line) for Southern blotting, and primers (P1, P2, and P3) for PCR genotyping are indicated. Crosses of the mice with targeted allele with EII-a Cre mice result in three possible outcomes at the FAK loci (flox, Δ and neoΔ). (B and C) Southern blotting analysis of the tail DNA from representative mice after digests with ApaI (B) or SacI (C). (D and E) Cell lysates were prepared from lung (D) or embryos (E) of mice with various genotypes as indicated. They were then analyzed by Western blotting using anti-FAK or anti-vinculin, as indicated.
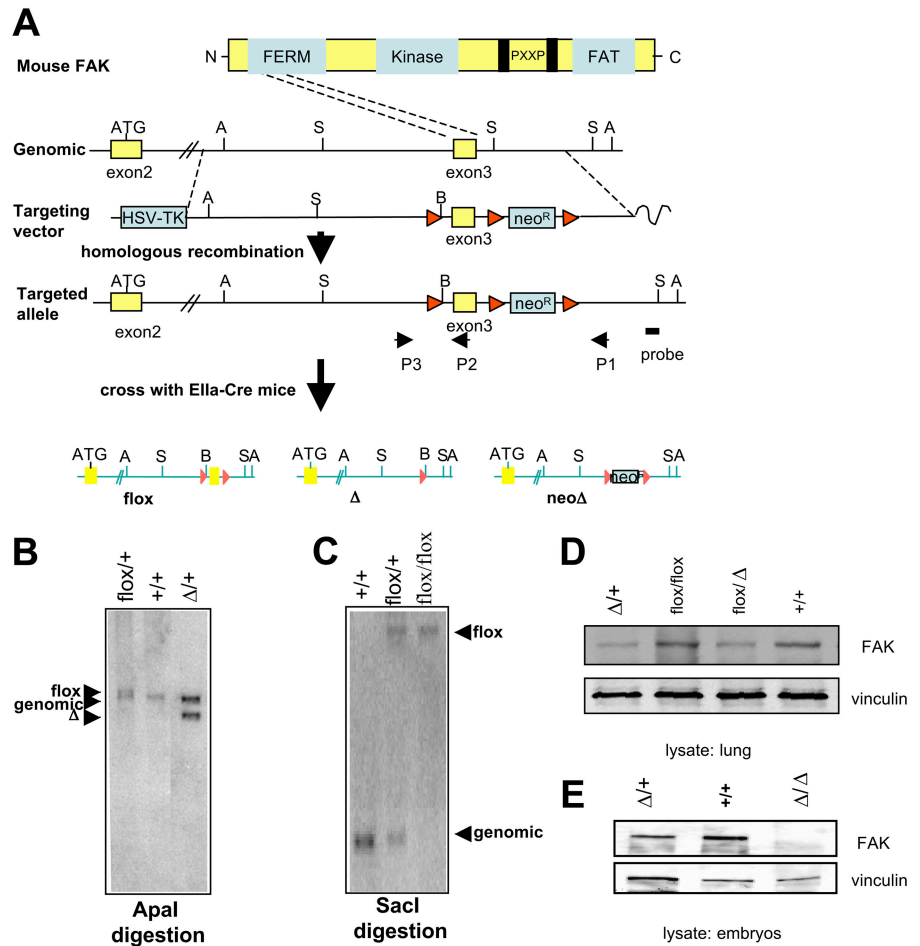
Consistent with its critical roles in vitro, FAK gene knockout in mice resulted in an embryonic lethal phenotype due to defects in the axial mesodermal tissues and cardiovascular system (Ilic et al., 1995). Both vasculogenesis and angiogenesis of the vasculature were impaired and neither a normal heart nor fully developed blood vessels were present in the FAK-null embryos. These results suggested a crucial role of FAK in the development of the vasculature. However, the relatively early (E8.5) embryonic lethality prevented analysis of the role of FAK in the late stage of embryonic development including angiogenesis in vivo.

A potential role of FAK in angiogenesis has also been suggested by a number of other studies. During the mouse embryo development, FAK expression became increasingly restricted to the blood vessels (Polte et al., 1994). Increased EC migration into a wounded monolayer was correlated with increased tyrosine phosphorylation and kinase activity of FAK (Romer et al., 1994). In addition, activation of VEGF receptor-2 by VEGF-A induced association of FAK with PI3-kinase, which is required for the stimulation of migration of porcine aortic ECs (Qi and Claesson-Welsh, 2001). Angiopoietin-1, another angiogenesis stimulator, also increased FAK phosphorylation during angiogenesis in vitro (Kim et al., 2000a). Lastly, several members of the integrin family play important roles in the regulation of angiogenesis (Eliceiri and Cheresh, 2001). A recent report also showed that formation of a signal-

ing complex containing FAK and integrin $\alpha v\beta 5$ in an Src-dependent manner is essential for VEGF-stimulated angiogenesis (Eliceiri et al., 2002). Given FAK's role in mediating signaling by integrins and growth factor receptors, these results also strongly suggest a potential role for FAK in vasculogenesis and angiogenesis.

To investigate the physiological role of FAK in vascular development and angiogenesis in vivo, we generated a strain of mice with FAK gene flanked by two loxP sites (floxed FAK). Analysis of the EC-specific deletion of FAK embryos and isolated ECs indicated a role for FAK in angiogenesis and vascular development due to its essential function in the regulation of multiple EC activities.

Results

Generation of floxed FAK mice

To enable ablation of the functional mouse *FAK* locus in a conditional manner, we inserted two *loxP* sites flanking its third coding exon followed by a neomycin resistance cassette and a third *loxP* site via homologous recombination in embryonic stem cells (Fig. 1 A). Cre-mediated deletion of exon 3 leads to a frame-shift mutation because of directly splicing from exon 2 (containing the ATG initiation codon) to exon 4, which produces a small truncated and nonfunctional peptide (~70 aa) lacking the majority of FAK sequences. Heterozygous mice

Table I. Genotypes of progeny from FAK$^{\Delta/+}$ heterozygous intercrosses

	E7.5	E8.5	E9.5	E10.5	E11.5	Born
+/+	4	11	16	12	10	47
$\Delta/+$	8	26	30	24	22	95
Δ/Δ	4	17	12	4	0	0
Total	16	54	58	40	32	143

with the targeted allele ($FAK^{flox-neo/+}$) were crossed with EIIa-Cre mice to remove the neomycin resistance cassette that may affect the normal expression of the targeted gene (hypomorphism) (Chen et al., 1993). This led to the generation of three types offsprings with deletion of neomycin resistance cassette ($FAK^{flox/+}$), exon 3 deleted ($FAK^{neo/+}$), or both ($FAK^{\Delta/+}$). The heterozygous floxed FAK ($FAK^{flox/+}$) and FAK-null ($FAK^{\Delta/+}$) mice were identified by PCR analysis of tail DNA (unpublished data) and confirmed by Southern blotting (Fig. 1, B and C). The identified $FAK^{flox/+}$ mice were then bred with C57BL/6J mice to segregate the floxed FAK allele from the EIIa-Cre transgene.

Matings between heterozygous $FAK^{flox/+}$ mice yielded homozygous floxed FAK mice ($FAK^{flox/flox}$) at the expected Mendelian ratio (unpublished data) that were viable, fertile, and did not show gross or histological abnormalities. To ensure normal expression of FAK gene flanked with *loxP* sequences, FAK protein expressions in various mice strains were analyzed by Western blotting. Fig. 1 D shows a similar expression level of FAK in the lungs from $FAK^{flox/flox}$ mice and wild-type mice ($FAK^{+/+}$). As expected, both $FAK^{\Delta/+}$ and $FAK^{flox/\Delta}$ mice showed reduced expression of FAK, presumably due to the removal of one FAK allele. Together, these results suggest that the *loxP* sequences flanking the exon 3 of FAK did not affect FAK expression or the normal development and function of mice (thus not a hypomorphic allele).

Previous studies showed that deletion of the second kinase exon resulted in FAK deficiency and early embryonic lethality (Ilic et al., 1995). To test the effect of exon 3 deletion in FAK, intercrosses of $FAK^{\Delta/+}$ heterozygous mice were performed. Near-Mendelian ratios of wild-type, heterozygous, and homozygous embryos were detected at E7.5 and E8.5. A decrease in the number of homozygous embryos was found at E9.5 and E10.5, and no $FAK^{\Delta/\Delta}$ embryos were detected at or beyond E11.5 (Table I and unpublished data). Analysis of lysates from the E8.5 embryos confirmed the absence of FAK in the $FAK^{\Delta/\Delta}$ embryos (Fig. 1 E). These results are consistent with the previous studies that inactivation of *FAK* leads to embryonic lethality in early embryogenesis (Ilic et al., 1995) (also see Fig. 2 A, d), although in our study the FAK gene is disrupted in a different location. They also confirmed that deletion of exon 3 leads to the loss of functional *FAK* locus in mice.

EC-specific FAK deletion in mice

To specifically inactivate the *FAK* gene in ECs, the floxed FAK mice were intercrossed with transgenic mice expressing Cre recombinase under the control of the Tie2 endothelial-specific promoter (Koni et al., 2001). Of the 161 viable offspring of the crosses between $FAK^{\Delta/+}$; Tie2Cre males to $FAK^{flox/flox}$ females analyzed, we obtained 52 $FAK^{flox/\Delta}$ progeny, 54 $FAK^{flox/+}$;
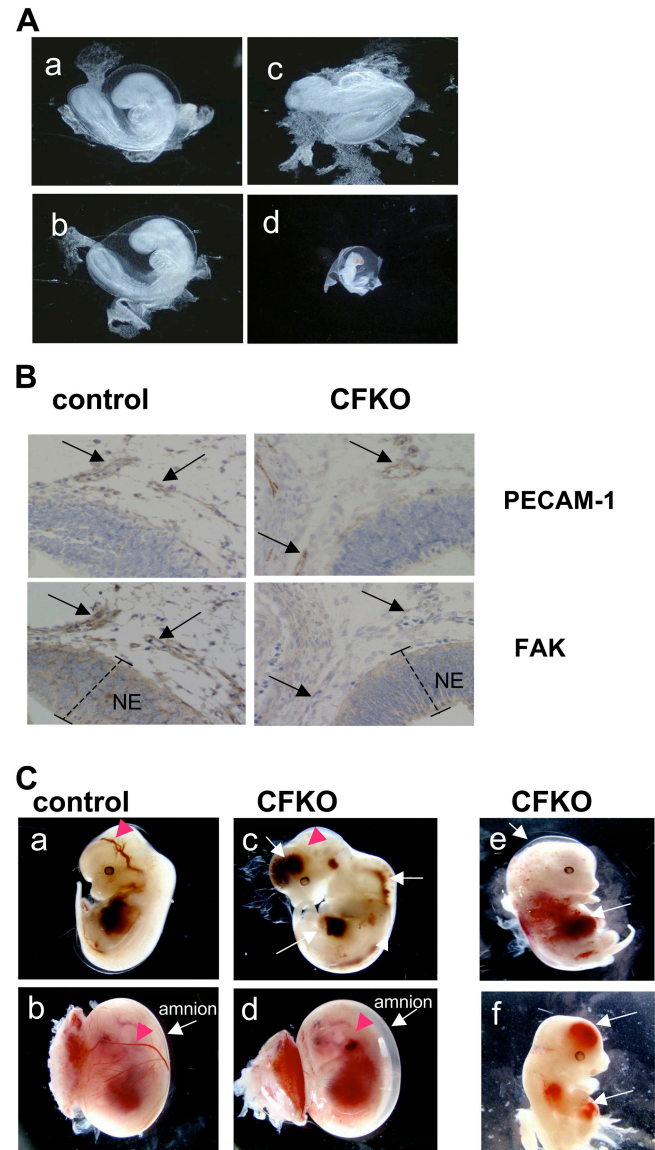

Figure 2. EC-specific FAK deletion in mice. (A) E8.5 embryos with +/+ (a), flox/Δ; Tie2Cre (CFKO) (b), flox/Δ (c), and Δ/Δ (d) genotypes. At this stage, CFKO embryos (c) are indistinguishable from control embryos (a and b), whereas FAK total KO embryos (d) showed a marked retardation in development and much reduced overall size. (B) Immunohistological analysis for PECAM-1 and FAK on adjacent brain sections of control and CFKO E9.5 embryos. Vascular ECs show positive staining with anti-PECAM-1 in both control and CFKO embryos (arrows). FAK is detected in the ECs of the control, but not CFKO, embryos (arrows). Neuroepithelium (NE, bracketed) are stained with FAK in both control and CFKO embryos. (C) Gross examination of whole embryos at E13.5 (a–d) or later (e and f), with (b and d) or without (a, c, e, and f) intact amnion and implantation sites. There are variable size hemorrhages (long arrows) in the CFKO embryos. Note the lack of vascular network in the head and amnion of the CFKO embryo when compared with the control littermates (red arrowheads). The amnion of CFKO embryos is thickened by edema (b and d, long arrows). The later embryos (e and f) are two examples of the representative range of abnormalities showing scattered hemorrhages and subcutaneous edema (e, long arrow), and smaller embryo with marked discoloration due to embryonic death and multifocal hemorrhages (f, long arrows).

Tie2Cre progeny, and 52 $FAK^{flox/+}$ progeny, but only 5 $FAK^{flox/\Delta}$; Tie2Cre (designated as conditional FAK knockout [CFKO]) progeny (Table II). Although the offsprings with three other

Table II. **Genotypes of progeny from crosses between $FAK^{flox/flox}$ and $FAK^{\Delta/+}$; Tie2Cre mice**

	E8.5	E9.5	E10.5	E11.5	E12.5	E13.5	E14.5	E15.5	E16.5	E17.5	Born
flox/Δ	8	11	35	24	30	41	24	28	11	8	52
flox/Δ; Tie2Cre (CFKO)	9	11	28	23	28	23	10	6	1	1	5
flox/+	10	10	36	25	33	42	25	28	12	7	54
flox/+; Tie2Cre	9	11	35	27	32	41	22	24	10	7	52
# Litter	4	6	16	13	17	24	11	13	7	3	29

genotypes are very close to the Mendelian ratio of this cross-ing (25% each), the 3% pups with CFKO genotype is signifi-cantly lower than the expected 25% of all pups, suggesting potential embryonic lethal phenotype for the majority of CFKO embryos.

To determine when these CFKO embryos die, embryos were examined from E8.5 to birth (see Table II). Near-Mendelian ratios of 1:1:1:1 were represented for the above four genotypes including CFKO embryos between E8.5 and E12.5 (Table II). CFKO embryos at E8.5 and E9.5 were indistinguishable from the control littermates in morphology and development (Fig. 2 A). At E10.5, ∼5% CFKO embryos showed developmental re-tardation (unpublished data); however, the majority of embryos at this stage and until E12.5 were seen normally in the overall gross appearance and development (unpublished data). This is in marked difference from the $FAK^{\Delta/\Delta}$ embryos that showed se-vere developmental retardation as early at E8.5 (Fig. 2 A). By E13.5, a decreased number of CFKO embryos (∼15.6%) than the expected 25% Mendelian ratio was found (Table II), and the embryos exhibited various defects (see below). At E14.5 and thereafter, a further deterioration and reduction in the number of CFKO embryos was seen (Table II and unpublished data). EC-specific FAK deletion was verified by immunohistological analysis of the CFKO embryo and its control littermate at E9.5. FAK expression was detected in ECs as marked by staining of platelet-endothelial cell adhesion molecule-1 (PECAM-1) in the control, but not CFKO embryos (Fig. 2 B). In contrast, FAK ex-pression was detected in the neuroepithelium in both the control and CFKO embryos, suggesting EC-specific inactivation of FAK gene in CFKO embryos. Together, these results suggest that EC-specific deletion of FAK leads to a late embryonic le-thality in the majority of the embryos. They also suggest that the total FAK KO mice likely have a broader deficiency than the EC-specific CFKO, which is responsible for the early em-bryonic lethal phenotype (Ilic et al., 1995).

Vascular defects in EC-specific CFKO embryos

Gross examination of CFKO embryos at E13.5 revealed ran-domly multifocal, randomly scattered, variably sized hemor-rhages up to 2 mm in diameter and the absence of normal su-perficial vasculature. There was thickening of the amnion due to edema present in the membranes and the prominent amniotic blood vessels were not evident (Fig. 2 C, a–d). At E14.5 and later, there were different degrees of lesion severity in the CFKO embryos that ranged from large multifocal superficial scattered hemorrhages and superficial edema to early embry-onic death characterized by discolored embryos with a fragile
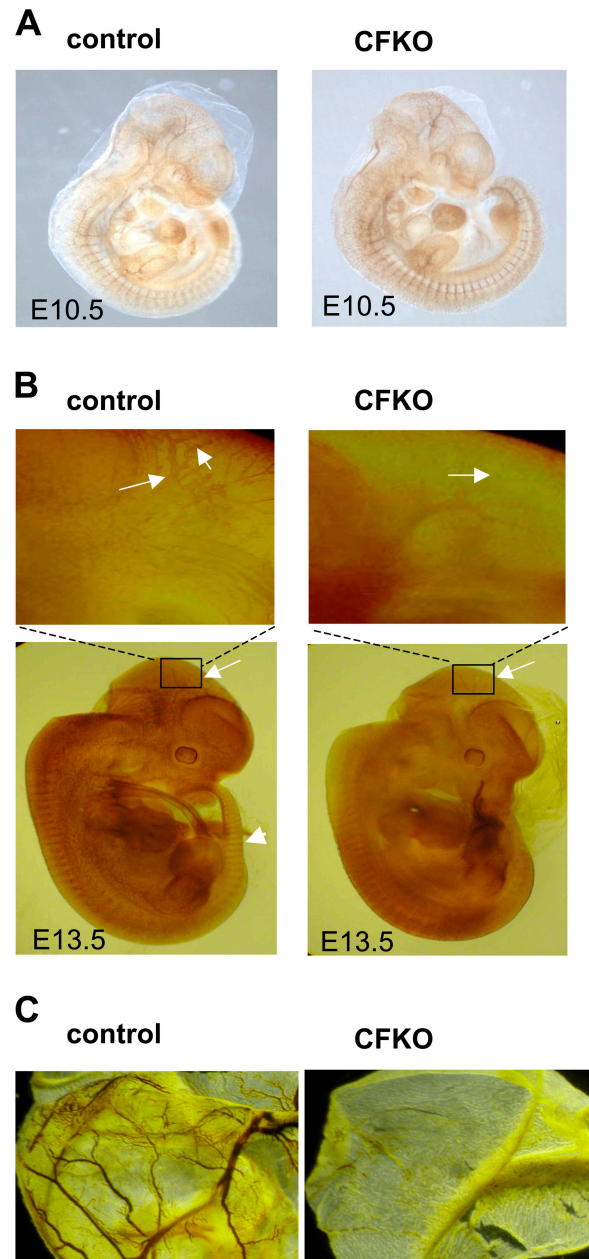

Figure 3. **Whole-mount staining of PECAM-1 of the embryos and yolk sacs.** (A) E10.5 embryos. There is no difference of vascular structures at this stage between the control and CFKO embryos. (B) E13.5 embryos. Typi-cal defects in the vascular network observed in the CFKO embryos are shown. There is lack of the superficial vascular network in the head region (long arrows), no clearly defined visceral outlines, and no clear definition of the axial skeleton at the coccigeal level (corresponding part marked by short arrow for the control embryo) when compared with the control embryo. (C) Characteristic decrease of superficial vasculature with absence of small vessel branch formation in the yolk sacs of CFKO embryos.
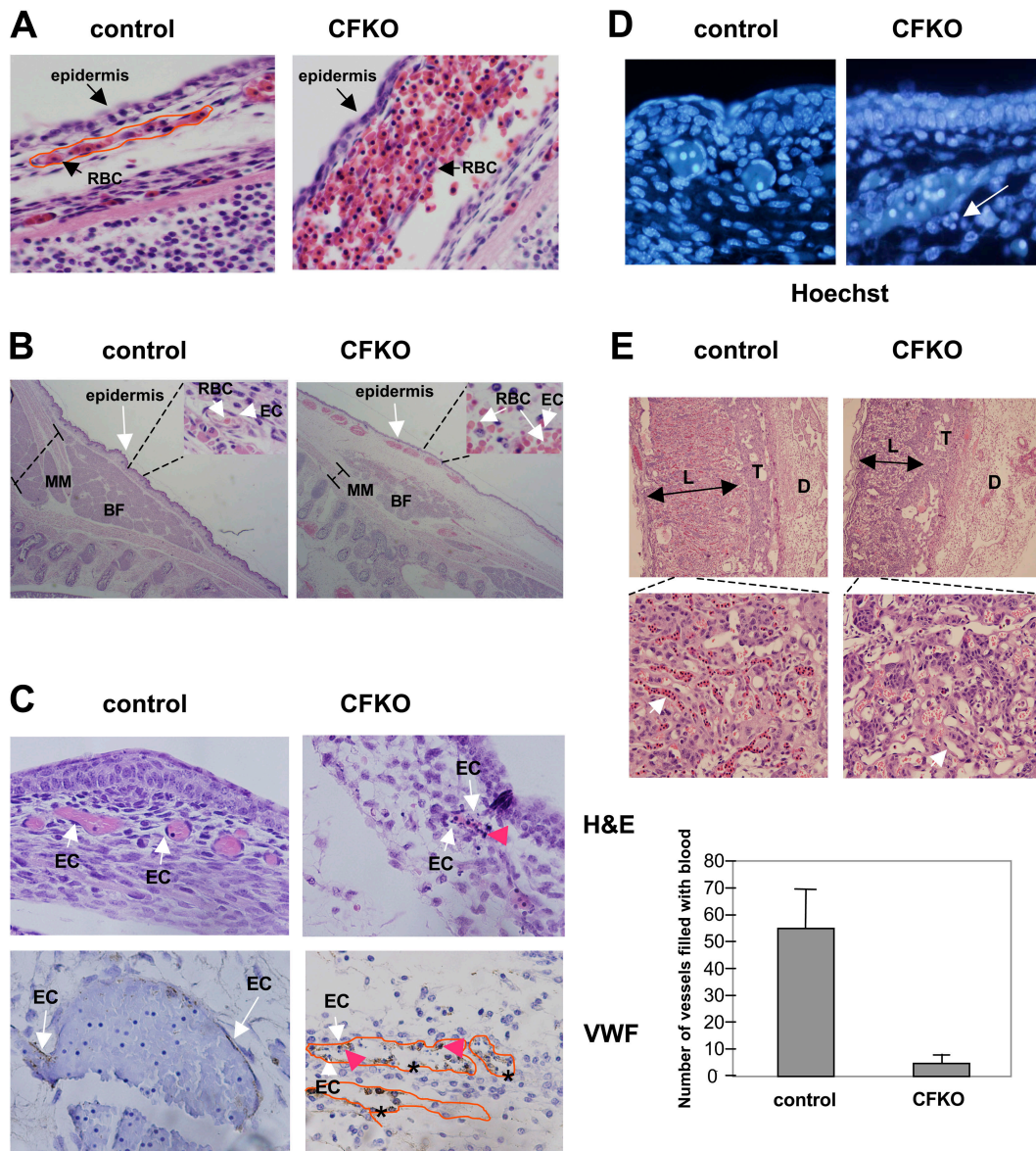
Figure 4. **Vascular lesions and associated defects of CFKO embryos and placentas.** (A) Histopathological sections of skin from the dorsum of control and CFKO embryos. There is marked expansion of the dermis and subcutaneous tissue of the CFKO embryos by hemorrhage and edema. RBC, red blood cells. (B) Histopathological section of a median plane at the level of the thoracolumbar region of control and CFKO embryos. Distinctive expansion of the subcutaneous tissue in the CFKO embryos due to edema and engorged capillaries with perivascular hemorrhages; the brown fat (BF) pads are smaller with pyknotic nuclei and scant eosinophilic cytoplasm. The muscle mass (MM) is reduced significantly (bracketed). Insets show RBC inside the EC-lined vessels in control embryos, but both inside and outside of the capillaries in CFKO embryos. (C) Hematoxylin and eosin staining and immunohistochemical staining with anti-vWF show the normal capillaries with RBC in the control embryos and collapsed vessels lined by defective ECs in the CFKO embryos. Some of the ECs are pyknotic with karyorrhectic debris (red arrowheads), others are swollen and rounded (arrows), and a few have sloughed into the lumen of the vessel. The collapsed vessels are outlined with lumina marked by asterisks. (D) Hoechst staining of control and CFKO embryos. Presence of apoptotic ECs in the CFKO (but not control) embryos are marked by the long arrow. (E) Histopathological sections from E13.5 embryos implantation site. Typical examples of decreased thickness of the labyrinth area in the CFKO embryos compared with the controls (top panels). The arterial canals and branches on the CFKO labyrinth have collapsed vessel outlines and the lumina are devoid of nucleated fetal RBCs (bottom panels and graph). D, decidua; L, labyrinth layer; T, trophoblast.

underdeveloped appearance (Fig. 2 C, e and f). These results suggest probable vascular defects in late embryogenesis in the CFKO embryos.

To test this possibility directly, we performed whole mount staining of PECAM-1 in the CFKO and control embryos at different embryonic stages. At E10.5, the vascular patterns were similar between the CFKO and control littermate embryos (Fig. 3 A). However, at E13.5 there was significantly reduced vascular network in the head region of the CFKO embryos when compared with control embryos (Fig. 3 B). There were no clear outlines of internal viscera and the axial skeleton in the CFKO embryos. In the yolk sac of E13.5 CFKO embryos, there was a marked decrease in sprouting angiogenesis and much fewer branched vessels compared with the controls (Fig. 3 C). Together, these results strongly suggest that inactivation of FAK in ECs leads to defects in vascular development

and angiogenesis, which are likely responsible for the hemorrhage, edema, and death in late embryogenesis.

## Increased EC death and blood vessel collapse in the CFKO embryos

Consistent with the findings of gross embryonic analysis (see Fig. 2 and Fig. 3), histological studies of the CFKO embryos at E13.5 or later showed marked thickening and expansion of the dermis by loosely arranged mesenchymal cells mixed with acellular pale esosinophilic material, when compared with the normal thin compacted dermis and subcutaneous tissue in the control embryos (Fig. 4, A and B). The affected subcutaneous tissue had numerous perivascular hemorrhages of variable size, whereas the control embryos had intact blood vessels (Fig. 4, A and B). We also observed that the lining ECs confined to the small capillaries of the CFKO embryos had pyknotic and karyorrhectic changes reflecting individual cell necrosis of ECs. Many ECs were rounded, detached, and sloughing into the lumen. The lumen of the affected blood vessels was collapsed (Fig. 4 C, right panels). In contrast, the lining ECs of the control embryos were flat and thin with elongated and intact nuclei. The structure of the blood vessels was intact and filled with nucleated red blood cells (Fig. 4 C, left panels). Staining of the subcutaneous sections with Hoechst revealed some apoptotic ECs in the CFKO embryos, but not in the control embryos (Fig. 4 D). Together, these results suggest that destruction of ECs and consequent collapse of blood vessels may be responsible for the extensive defects in angiogenesis and vascular development as a result of FAK inactivation in the ECs of CFKO embryos.

Defective vascularization of placenta in the CFKO embryos

A general developmental delay was evident based on the smaller overall size of the CFKO embryos compared with littermate controls. These defects and late embryonic lethality are consistent with deficiencies of angiogenesis and vascular development in the CFKO embryos and/or placenta. Thus, we analyzed vascularization in the CFKO placenta by histology. Fig. 4 E shows that the labyrinth layer of the CFKO placenta was markedly reduced in thickness as compared with the control littermates. The arterial canals and the canal branches of the CFKO placenta were devoid of nucleated fetal RBCs, and some of these blood vessels appeared collapsed. As the developing embryonic hearts appear normal (unpublished data), the lack of fetal RBCs is unlikely due to decreased perfusion from the fetal hearts, but rather caused by the defective vasculature in the placenta. Although it is not clear whether this placental insufficiency was the cause for the late embryonic lethality of CFKO embryos, these data together demonstrate a critical role of FAK for the development and function of endothelium in both embryos and placenta.

Deletion of FAK in isolated primary ECs results in reduced capillary formation and multiple cellular deficiencies in vitro

To further understand the mechanisms of the endothelial defects in CFKO embryos, we isolated primary ECs from homozygous
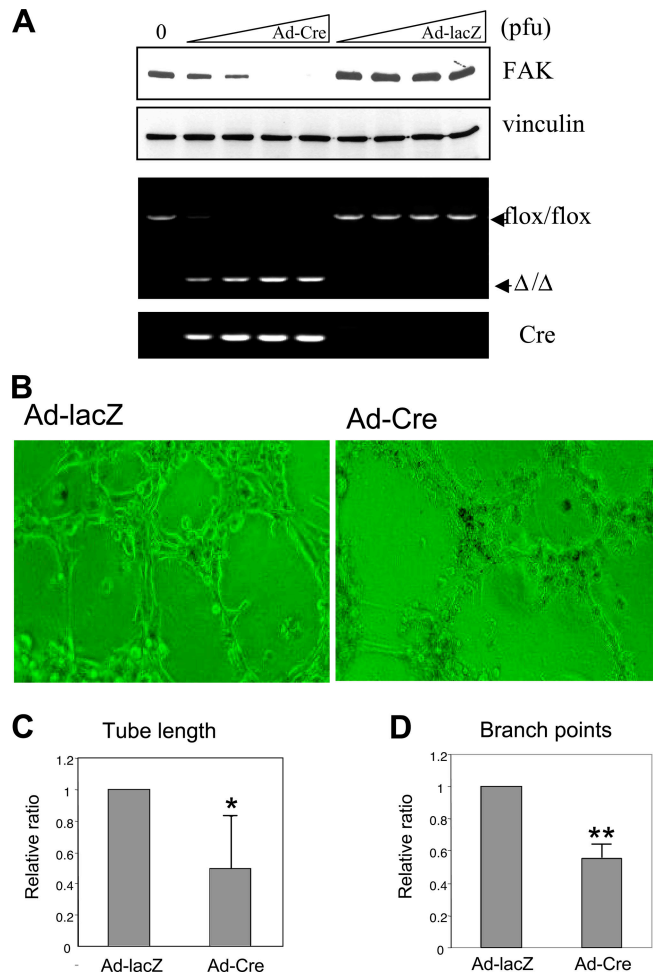
Figure 5. **Defective tubulogenesis of the isolated FAK$^{-/-}$ primary ECs.** (A) ECs isolated from homozygous floxed FAK mice (flox/flox) were infected with increasing amount of recombinant adenoviruses encoding Cre (Ad-Cre) or a control insert (Ad-lacZ), as indicated. Cell lysates were analyzed by Western blotting with anti-FAK or anti-vinculin (top two panels). Genomic DNA was analyzed by PCR (bottom two panels). (B–D) Primary ECs from floxed FAK mice and infected with Ad-Cre or Ad-lacZ were cultured on Matrigel as described in Materials and methods. Images of representative fields are shown in B. The length of the tubules (C) and branch points (D) were quantified from three independent experiments and shown as the relative ratio of the value + standard error. *, P = 0.023 and **, P = 0.017 in comparison to value from Ad-lacZ–induced cells.

floxed FAK mice. The isolated floxed FAK ECs were infected by recombinant adenoviruses encoding Cre recombinase (Ad-Cre). As shown in Fig. 5 A, Ad-Cre infection of the floxed FAK ECs led to a dose-dependent decrease in the expression of FAK protein concomitant with excision of exon 3 of FAK gene. As expected, infection of the cells with a control recombinant adenovirus encoding lacZ (Ad-lacZ) did not affect FAK protein expression or the flox allele of the FAK gene. These ECs are designated as CFKO and control ECs, respectively.

The effect of FAK inactivation in the isolated ECs was assessed by examining their ability to change morphology and form capillaries when cultured on Matrigel, which is a process mimicking sprouting and tube formation during angiogenesis in vivo. Fig. 5 B shows the significantly reduced formation of tubules of CFKO ECs compared with the control ECs. Quanti-
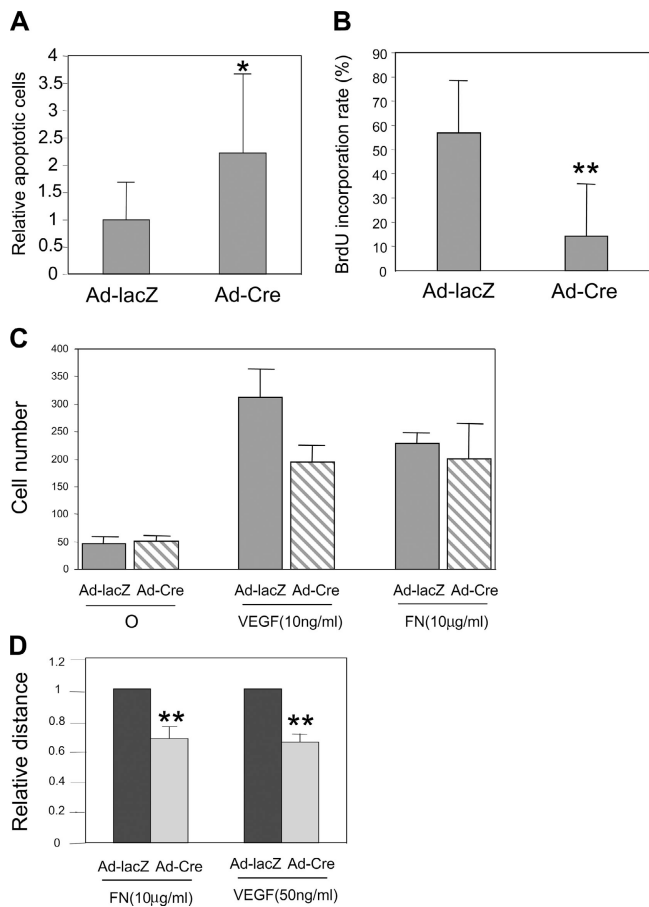
Figure 6. **Increased apoptosis, and reduced proliferation and migration of the isolated FAK$^{-/-}$ primary ECs.** Primary ECs from floxed FAK mice and infected with Ad-lacZ or Ad-Cre were measured for apoptosis using TUNEL assay (A), proliferation by BrdU incorporation assay (B), and migration in response to VEGF or FN by Boyden chamber assay (C) and wound closure assay (D), as described in Materials and methods. The mean + standard error from at least three experiments is shown. *, P = 0.014 and **, P < 0.001 in comparison to value from Ad-lacZ–infected cells.

tation of multiple experiments indicated that both the length of the tubules and the number of branch points were reduced in the CFKO ECs (Fig. 5, C and D). Interestingly, consistent with EC necrosis and apoptosis observed in the CFKO embryos (see Fig. 4, C and D), we noted that some of CFKO ECs, but few control ECs, appeared to be apoptotic under this condition. Indeed, the CFKO ECs showed reduced survival in serum-free condition when compared with the control ECs (unpublished data). TUNEL assay was then performed to test a possible role of FAK on EC apoptosis directly. Fig. 6 A shows that inactivation of FAK resulted in increased apoptosis of the primary ECs. These results suggested that increased apoptosis and decreased survival of ECs upon FAK KO may be responsible for the defective vascular development and associated hemorrhage and edema, and possibly also reduced angiogenesis in the CFKO embryos.

We also investigated the effects of FAK inactivation on the proliferation and migration of the primary ECs to determine the contribution of their possible changes to the in vivo vascular defects of CFKO embryos. Fig. 6 B shows a decreased cell

cycle progression of CFKO ECs upon serum stimulation in comparison to control ECs, as measured by the BrdU incorporation as described in Materials and methods. Analysis of cell migration using the Boyden chamber assays showed reduced migration of CFKO ECs in response to VEGF stimulation compared with control ECs. Surprisingly, however, little difference was observed between the CFKO and control ECs in their migration in response to FN (Fig. 6 C). The important role of FAK in FN-stimulated migration is well described for many cell types, including ECs in previous studies (Parsons, 2003; Schlaepfer and Mitra, 2004). Therefore, we further investigated migration of CFKO ECs using the potentially more physiologically relevant wound closure assays. Fig. 6 D shows that deletion of FAK reduced EC migration in response to both FN and VEGF. As both EC migration and proliferation are critical for angiogenesis, these data suggested that reduced proliferation and migration of ECs upon FAK deletion could both contribute to the defective angiogenesis in the CFKO embryos in vivo.

Differential requirement of FAK kinase activity in VEGF-stimulated EC migration

To gain more insights into the role of FAK in EC migration in response to VEGF and FN as well as its potential differential function in various cell types, we prepared recombinant adenoviruses encoding FAK (Ad-FAK) and its kinase-defective mutant (Ad-KD) and examined their ability to rescue cell migration deficiency upon deletion of endogenous FAK. The isolated floxed FAK ECs were infected sequentially by Ad-Cre and Ad-FAK, Ad-KD, or a control recombinant adenovirus Ad-GFP, as described in Materials and methods. As shown in Fig. 7 A, infection with Ad-FAK or Ad-KD, but not Ad-GFP, led to expression of the exogenous FAK in the CFKO ECs. Analysis of the exogenous FAK with anti-PY397 antibody (specific for the major FAK autophosphorylation site Y397) showed that FAK is phosphorylated at this site, whereas the KD mutant is not. As expected, reexpression of FAK rescued their deficiency in VEGF- and FN-stimulated migration (Fig. 7, B and C). Interestingly, however, reexpression of FAK KD mutant rescued CFKO EC migration in response to FN (Fig. 7 C), but not VEGF (Fig. 7 B). We also isolated mouse embryonic fibroblasts (MEFs) from the floxed FAK mice and examined their migration upon deletion of FAK via Ad-Cre infection (CFKO MEF). In contrast to results from ECs, deletion of FAK only affected MEF migration in response to FN, but not to VEGF (Fig. 7 D). As in the case of EC migration on FN, both wild-type and KD mutant FAK rescued CFKO MEF migration on FN (Fig. 7 E). Together, these results suggest that FAK may play differential roles in migration of EC and MEF and that FAK activity is required for VEGF-stimulated EC migration, whereas it is dispensable for FN-stimulated migration of either EC or MEF.

Reduced paxillin phosphorylation and decreased MAP kinase signaling in FAK-deficient ECs

Multiple targets and signaling pathways have been implicated in mediating regulation of cellular functions by FAK (Parsons,

Figure 7. Differential requirement of FAK kinase activity for VEGF-stimulated EC migration. (A–C) Primary ECs from floxed FAK mice were infected with Ad-Cre to delete endogenous FAK followed by infection of Ad-FAK, Ad-KD, or the control Ad-GFP as indicated. Aliquots of lysates were analyzed by Western blotting using anti-FAK, anti-pY397, or anti-vinculin, as indicated (A). The infected cells were subjected to wound closure assay in response to VEGF (B) or FN (C), as described in Materials and methods. The mean + standard error from at least three experiments is shown. *, $P < 0.005$; **, $P = 0.448$; and ***, $P = 0.012$ in comparison to value from Ad-GFP–infected cells. (D and E) MEFs were isolated from floxed FAK mice, and then infected with Ad-lacZ or Ad-Cre (D) or Ad-Cre followed by Ad-FAK, Ad-KD, or the control Ad-GFP (E), as indicated. The infected cells were then subjected to wound closure assays in response to VEGF (D as indicated) or FN (D as indicated and E). The mean + standard error from at least three experiments is shown. *, $P < 0.01$ and **, $P = 0.17$ in comparison to value from (D) Ad-lacZ– or (E) Ad-GFP–infected cells.
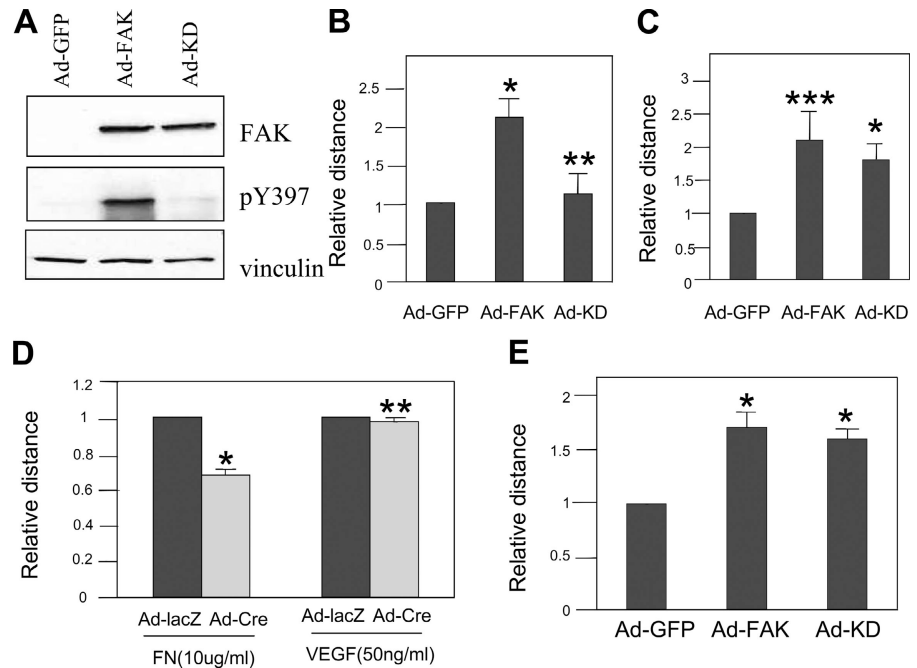
2003; Schlaepfer and Mitra, 2004). The activation states of several FAK targets were examined. As shown in Fig. 8 A, FAK expression is abolished in the CFKO ECs compared with the control ECs. In contrast to FAK-null cells from the total FAK KO embryos (Sieg et al., 1998), the FAK-related tyrosine kinase Pyk2 was not up-regulated in the absence of FAK in ECs. Furthermore, the major FAK target paxillin showed a significant decrease in phosphorylation at Tyr 118 in CFKO ECs that is critical for paxillin regulation of cell spreading and migration (Petit et al., 2000). Phosphorylations of JNK (C-Jun NH$_2$-terminal kinase) and Erk1/2 were also decreased in the CFKO ECs. Consistent with the absence of FAK and reduced paxillin phosphorylation, we found a marked reduction of tyrosine phosphorylation staining in focal contacts with a phosphotyrosine-specific antibody (PY20) (Fig. 8 B). Staining of focal contacts with anti-vinculin showed an increased number of focal contacts in CFKO ECs than control ECs, as observed previously in the fibroblasts from total KO embryos (Ilic et al., 1995), suggesting that reduced phosphotyrosine staining is not due to a reduction in focal contacts in the CFKO ECs, per se. Together, these data suggest that deficiencies in paxillin phosphorylation and JNK and Erk signaling may be responsible for the cellular defects in the isolated primary CFKO ECs in vitro and defective angiogenesis and vascular development of CFKO embryos in vivo.

EC-specific FAK transgene expression cannot rescue total FAK KO embryonic lethality

Together with the previous studies showing early embryonic lethality of FAK total KO embryos (Ilic et al., 1995), our results here suggested that FAK is required for both early and late embryogenesis perhaps in different cells/tissues. To further test this possibility, we examined whether restoration of FAK expression in ECs would rescue the early embryonic lethal phenotype of the total KO embryos. We have previously generated FAK transgenic mice with EC-specific expression of the FAK transgene under the control of Tie2 promoter/enhancer (Peng et al., 2004). These mice were crossed with $FAK^{\Delta/+}$ heterozygous mice to introduce Tie2-FAK transgene into the FAK-null background, which were then intercrossed for the generation of possibly rescued progeny with $FAK^{\Delta/\Delta}$; Tie2-FAK genotype. Of the 84 pups analyzed, we did not obtain any offspring with the rescued genotype, although the Mendelian ratio of this genotype for the crossing is 12.5%. These results suggest that introduction of EC-specific FAK gene could not rescue the early embryonic lethal phenotype of the total FAK KO embryos. They provide further support for the role of FAK in both early embryogenesis (perhaps for mesoderm cells) and vascular development in late embryogenesis.

Discussion

As a critical mediator of signaling by integrins and growth factor receptors, FAK has been implicated in playing an important role in the regulation and function of ECs by a number of studies in vitro (Romer et al., 1994; Kim et al., 2000a; Qi and Claesson-Welsh, 2001). Furthermore, vascular defects were observed in FAK total KO embryos and ECs from these embryos or cultured embryoid bodies (Ilic et al., 1995, 2003). However, the early embryonic lethality of the total KO mice precluded in vivo analysis of potential roles of FAK in angiogenesis, which is an integral part of vascular development in late embryogenesis and adult organisms. Using a conditional KO approach that specifically inactivates FAK gene in ECs, we found that FAK expression is required for the vascular development in late embryogenesis. Although they developed normally, including formation of the vascular structures in early embryogenesis, the CFKO

A

B
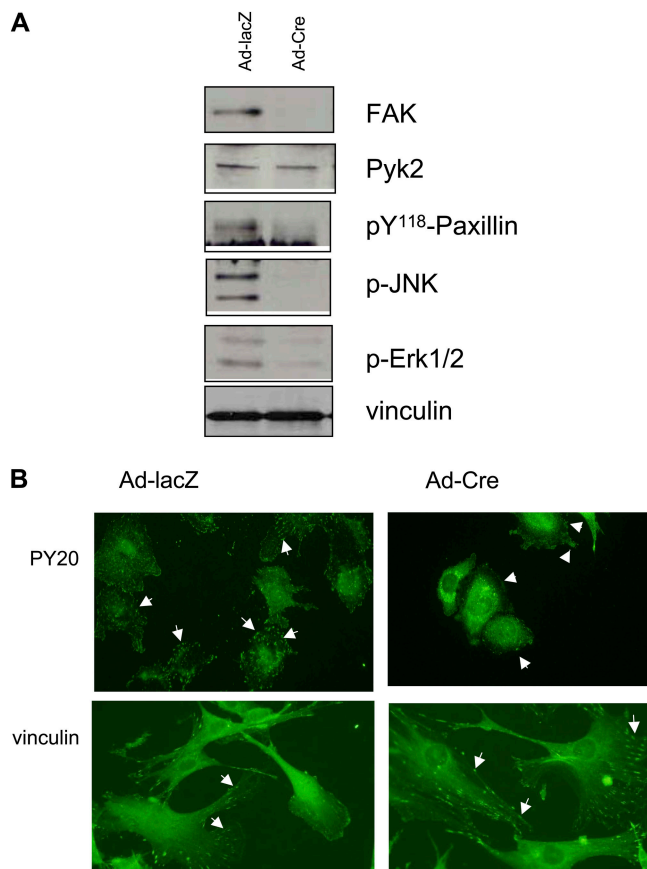
Figure 8. **Effects of FAK deletion on downstream targets in the isolated primary ECs.** (A) Cell lysates of primary ECs from floxed FAK mice and infected with Ad-lacZ or Ad-Cre were analyzed by Western blotting with various antibodies as indicated. (B) Immunofluorescent staining of the above ECs (see A) with anti-phosphotyrosine antibody PY20 (top panels) or anti-vinculin (bottom panels). Focal adhesions are marked by arrows.

embryos showed multiple defects in late embryogenesis including reduced blood vessel network in the superficial vasculature, hemorrhage, edema, developmental delay in the embryos, abnormalities of blood vessels in both yolk sac and placental labyrinth, and embryonic lethality. Furthermore, we showed that EC-specific expression of a FAK transgene could not rescue the early embryonic lethality of the total KO embryos. These studies demonstrate that FAK is required for angiogenesis and vascular development and integrity in late embryogenesis, and together with the previous total KO data (Ilic et al., 1995), they suggest that FAK plays a role in at least two different stages of embryonic development in multiple cell/tissue types.

The apparently normal vasculature of CFKO embryos at E10.5 suggested that FAK is not required for vasculogenesis during early embryonic development. This is somewhat surprising given the previous observation of extensive vascular defects in the FAK total KO mice (Ilic et al., 1995). One possibility is that the FAK gene is not completely deleted at this early stage of development. However, previous analysis suggested that both the endogenous Tie2 and Tie2-Cre transgenes were expressed as early as E7.5 in ECs and their precursor hemangioblasts (Schlaeger et al., 1995, 1997; Kisanuki et al.,

2001). Effective cleavage of floxed genes and associated vascular defects in early embryogenesis were also shown in recent studies using Tie2-Cre transgenic mice (Cattelino et al., 2003; Friedrich et al., 2004). Lastly, our data also showed that FAK is absent in ECs at or before E9.5. Therefore, this possibility is unlikely to explain the lack of vascular defects in CFKO during vasculogenesis, although we cannot completely exclude such a possibility. An alternative possibility is that abnormalities in other cells/tissues of total KO embryos (e.g., other tissues derived from the defective mesoderm) could be responsible for the deficiencies in vasculogenesis in these embryos. In contrast to the total KO embryos, vasculogenesis proceeded normally in the CFKO embryos described here because FAK is inactivated only in ECs, and these other cells/tissues are not affected in CFKO embryos. Another interesting possibility is that the defects in vasculogenesis observed in the total KO embryos are due to defects in mesoderm tissues from which ECs are derived. It is possible that one or more FAK-regulated gene could be inactivated in these tissues in the total KO embryo (but not in the CFKO embryos described here), which could then lead to the vasculogenesis defects, whereas inactivation of FAK itself in ECs would not. FAK has been shown to regulate expression of a variety of genes involved in the regulation of cell cycle progression and migration (Zhao et al., 2003). Interestingly, a recent paper showed that pleiotrophin is down-regulated in the early FAK total KO embryos (Ilic et al., 2003), which is a protein with multiple functions in development (Zhang and Deuel, 1999; Souttou et al., 2001). It is clear that future studies will be necessary to test these possibilities and identify potential key target genes and mechanisms involved in the FAK regulation of vasculogenesis in early embryonic development.

A role for FAK expression in ECs for angiogenesis and vascular development and integrity in late embryogenesis is suggested by our observation of multiple vascular defects in the CFKO embryos. Consistent with previous studies of FAK using HUVEC cells (Romer et al., 1994; Gilmore and Romer, 1996; Kim et al., 2000a; Qi and Claesson-Welsh, 2001; Ilic et al., 2003), we found that deletion of FAK in primary ECs led to increased apoptosis, reduced proliferation and migration, and reduced ability to form capillaries on Matrigel. As embryonic angiogenesis involves both EC proliferation and migration, the above cellular defects could contribute to the reduced angiogenesis in CFKO embryos in vivo. Interestingly, consistent with inactivation of FAK, tyrosine phosphorylation of paxillin at Tyr118 is significantly reduced in the CFKO ECs. Paxillin is a focal adhesion protein and major substrate for the FAK/Src complex, and has been shown to play important roles in the regulation of cell adhesion and migration (Turner, 1998; Petit et al., 2000; Schaller, 2004). We also observed decreases of JNK and Erk1/2 activities in CFKO ECs, which are consistent with previous studies showing regulation of cell cycle progression and migration by FAK via both of these pathways (Parsons, 2003; Schlaepfer and Mitra, 2004). Thus, reduced paxillin phosphorylation, JNK and/or Erk signaling could contribute to the reduced cell migration and proliferation in the primary CFKO ECs and defective angiogenesis in the CFKO embryos.

Our analysis of FAK and its kinase-defective mutant in their ability to rescue migration deficiency of primary ECs and MEFs suggested a potential kinase-independent function for FAK. We found that although FAK kinase activity is required for VEGF-stimulated EC migration, it is dispensable for FN-stimulated migration of either EC or MEF. The ability of KD mutant to rescue migration of EC and MEF on FN is consistent with our previous observation that it promoted migration of CHO cells as effectively as the wild-type FAK (Cary et al., 1996). This activity was attributed to trans-phosphorylation of the KD mutant by endogenous FAK in CHO cells, allowing it to function in a similar manner as wild-type and phosphorylated FAK. In the case of ECs and MEFs here, however, endogenous FAK was deleted from these cells and the KD mutant in ECs was not phosphorylated on Y397. Therefore, these results suggest that promotion of migration of both EC and MEF on FN by FAK is independent of its kinase activity. They reveal potentially differential roles of FAK in mediating cell migration on ECM such as FN and growth factors like VEGF. Future studies will be needed to understand the potential kinase-independent function of FAK as well as the mechanisms underlying a differential role of FAK in EC migration on FN and VEGF.

Cell–cell junctions play key roles in the regulation of ECs. Interestingly, in a recent study FAK has been suggested to play a role in N-cadherin function in HeLa cells (Schaller, 2004; Yano et al., 2004). Therefore, FAK inactivation in the ECs may also lead to defects in EC junctions mediated by VE-cadherin, which may lead to hemorrhage and edema in the CFKO embryos. Furthermore, the reduced CFKO EC survival and increased apoptosis observed in vitro may also contribute to a decreased vascular integrity and these phenotypes. Indeed, signs of EC death and collapsed vessels were also observed in the CFKO embryos in vivo. Given the multiple targets of FAK for different cellular functions, future studies will be necessary to further clarify the roles of various FAK downstream signaling pathways in the regulation of various EC functions in vitro and angiogenesis and vascular development in vivo. A potentially powerful approach is to use various FAK mutants defective in interaction with specific targets to rescue the EC and embryonic phenotypes of CFKO.

In addition to the various embryonic defects in late embryogenesis, we also observed defects in the CFKO placenta, which is a highly vascularized tissue and is also responsible for sustaining the development of the embryos. We found reduced thickness of the labyrinth layer, collapsed vessels, and lack of fetal RBCs in the vessels. Although the placenta defects could certainly contribute to the abnormalities in the CFKO embryos, it is not clear at present whether potential placenta insufficiency or the embryonic vascular defects (or both) are responsible for the late embryonic lethal phenotype in the majority of CFKO embryos. We noted, however, that the EC defects in the embryos appear to be more severe than those in the placenta (e.g., necrotic ECs in the embryos, see Fig. 4, C and D), possibly due to the intact maternal vessels that may provide nutrient support for the adjacent fetal ECs. This suggests that placenta insufficiency is not likely to be responsible for the defective

angiogenesis and vascular development in the embryos. It is also interesting that EC-specific KO of integrin-linked kinase led to placenta insufficiency and severe embryonic developmental delay at E9.5 and embryo death and resorption by E11.5 and E12.5 (Friedrich et al., 2004). However, CFKO embryos exhibited defective vascular phenotypes at a later stage and indeed appear normal in E10.5–E12.5. These results also suggested that FAK and integrin-linked kinase, two important mediators of integrin signaling and function, may play differential roles in EC development and embryogenesis.

In conclusion, our establishment and analysis of EC-specific FAK KO mice demonstrate that FAK is required for angiogenesis and vascular development and integrity in late embryogenesis. Together with the previous studies showing a role of FAK in early embryogenesis (Ilic et al., 1995), these studies suggest that FAK signaling plays important roles in at least two stages of embryonic development and perhaps in different cell/tissue populations. These models will allow us to further delineate the role of specific FAK downstream pathways in EC functions and vascular development by rescuing the various phenotypes with FAK mutants lacking specific interactions with its targets. In addition, analysis of the small fraction of EC-specific CFKO mice that survived to adulthood may reveal the function of FAK in adult ECs and its potential role in biological processes such as tumor angiogenesis in mouse models.

Materials and methods

Construction of the targeting vector and generation of floxed FAK mice
A 422-bp EcoRI fragment of chicken FAK cDNA from pBS-FAK (Cary et al., 1998) was used to screen a 129/SvJ BAC Mouse I hybridization library (Genome Systems, Inc.) by Southern hybridization to isolate the genomic clones containing exon 3 of FAK. The genomic clones along with plasmids containing loxP sites, a neomycin-resistant cassette (neo) followed by a loxP site, and HSV-TK were then used to construct the targeting vector (Fig. 1 A). The resulting vector contains the 5′ (left) and 3′ (right) arms of 9- and 1.6-kb FAK genomic sequences, respectively, for homologous recombination.

Electroporation, selection, and blastocyst injection of E14.1 embryonic stem cells were essentially as described previously (Chiang et al., 2000). For Southern blotting, a 510-bp DNA fragment located at 3′ of the right arm of the targeting vector was used (Fig. 1 A). Chimeric mice were identified by coat color and then were bred to C57BL/6J mice. Transmission of the germ line was identified by PCR (see below) and confirmed by Southern blotting.

EIIa-Cre transgenic mice were obtained from Jackson Laboratory and Tie2-Cre transgenic mice have been described previously (Koni et al., 2001). All mice used in this study were bred and maintained at Cornell University Transgenic Animal Core Facility (Ithaca, NY) under specific pathogen-free conditions in accordance with institutional guidelines.

Genotyping by PCR
Mice and embryos were genotyped by PCR analysis of genomic DNA. Isolation of genomic DNA was described previously (Chiang et al., 2000). Primers used to genotype FAK alleles were P1, 5′-GCTGATGTC-CCAAGCTATTCC-3′; P2, 5′-TGGCCTGCTATGGATTTCGC-3′; and P3, 5′-AGGGCTGGTCTGCCTGACAGG-3′, as shown in Fig. 1 A. The combination of the P1 and P3 primers amplifies 1.6-kb, 1.5-kb, and 550-bp fragments from the FAK^{flox}, wild-type, and FAK$^{\Delta}$ alleles, respectively. Use of the P2 and P3 primers amplified a 370-bp fragment from either the FAK^{flox} or wild-type allele, but nothing from the FAK$^{\Delta}$ allele. PCR were performed for 30 cycles of 94°C (3 min), 67°C (2 min), and 72°C (4 min). CreF (5′-CGCAGAACCTGAAGATGTTCGCGATTA-3′) and CreR (5′-TCTCCCACCGTCAGTACGTGAGATATC-3′) primers were used to detect the Cre transgene, which generate a 400-bp fragment after 35 cycles of

95°C (30 s), 60°C (30 s), and 72°C (30 s). Tie2-FAK transgene was detected as described previously (Peng et al., 2004).

Western blotting

Tissue samples, embryos, and cells were homogenized and extracts were used for Western blotting analysis as described previously (Peng et al., 2004). Antibodies used are anti-FAK (C20; Santa Cruz Biotechnology, Inc.), anti-vinculin (Sigma-Aldrich), anti-Pyk2 (Zheng et al., 1998), anti-phospho-tyrosine397-FAK and anti-phospho-tyrosine118-paxillin (Upstate Biotechnology), anti-phospho-JNK (Cell Signaling Technology), or anti-phospho-Erk1/2 (New England Biolabs, Inc.).

Morphological and histological analysis

Timed matings were set up between male FAK$^{\Delta/+}$; Tie2-Cre mice and female FAK$^{flox/flox}$ mice. Embryos, yolk sacs, and placenta were harvested between E8.5 and E17.5, fixed in 4% PFA in PBS at 4°C for 4–16 h, and transferred to 70% ethanol. They were examined and photographed on a dissecting microscope (model S6D; Leica) with a progressive 3CCD camera (Sony) and Image-Pro Plus ver. 3.0.00.00 at RT. The embryos and placenta were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin, nuclear dye Hoechst 33258 (Sigma-Aldrich), or used for immunohistochemistry. They were examined under a microscope (model BX41; Olympus) with UplanF1 ×10/0.3 or UplanF1 ×20/0.5 objective lenses at RT, and the images were captured using a camera (model DP70; Olympus) with DP Controller Ver. 1.2.1.108.

Immunohistochemical analysis

For immunohistochemical analysis, sections were stained after antigen retrieval using primary antibodies against PECAM-1 (M-20, goat anti–mouse, 1:200 dilution; Santa Cruz Biotechnology, Inc.), FAK (C-20, rabbit anti–mouse, 1:200; Santa Cruz Biotechnology, Inc.), or vWF (1:500; DakoCytomation) followed by biotinylated and peroxidase-conjugated secondary antibodies. They were then processed using the DAB Immunostaining assay kit (Santa Cruz Biotechnology, Inc.) according to the instructions. The samples were usually counterstained with hematoxylin before mounting on coverslips. They were then examined under a microscope (model BX41; Olympus) with UplanF1 ×10/0.3 objective lens at RT, and the images were captured using a camera (model DP70; Olympus) with DP Controller ver. 1.2.1.108.

For the whole-mount staining with anti-PECAM-1 antibody, embryos and yolk sacs were fixed in 4% PFA/PBS. After dehydration by a series of methanol, they were treated with 1% H$_2$O$_2$ (diluted in MeOH and DMSO mixed 4:1) to quench endogenous peroxidases. Samples were rehydrated by methanol to PBS, and blocked in 4% BSA with 0.1% Triton X-100 in PBS. They were then incubated with anti-PECAM-1 (rat monoclonal MEC13.3, 1:50 dilution; BD Biosciences) diluted 1:10 in 4% BSA in PBST at 4°C overnight followed by peroxidase-conjugated secondary antibodies. The embryos were developed in 0.25% DAB with H$_2$O$_2$ in PBS. They were examined and photographed on a dissecting microscope (model S6D; Leica) with a progressive 3CCD camera (Sony) and Image-Pro Plus ver. 3.0.00.00 at RT.

Culture of ECs and adenovirus infection

ECs with homozygous FAK floxed alleles were isolated from E12.5 embryos using the magnetic bead (Dyanbead M-450; Dynal Corp.) purification with rat anti–mouse PECAM-1 (BD Biosciences), as described previously (Cattelino et al., 2003; Peng et al., 2004). The endothelial nature of the cells was confirmed by FACS and immunofluorescence microscopy with antibodies to endothelial markers, PECAM-1 (1:100) and VE-Cadherin (1:50). Approximately 90% purity of ECs was routinely obtained in the preparations. Cells were cultured in high glucose DME with 20% FCS (Hyclone), EC growth supplement (5 μg/ml; Worthington), and heparin (100 μg/ml; Sigma-Aldrich) maintenance medium (Peng et al., 2004) on gelatin-coated tissue culture plates. MEFs with floxed FAK alleles were isolated from E12.5 FAK$^{flox/flox}$ embryos as described previously (Sage et al., 2000).

The recombinant adenoviruses encoding Cre recombinase or lacZ control were purchased from Gene Transfer Vector Core (University of Iowa, Iowa City, IA). For most studies, 10^8 plaque-forming units were used for 10-cm dish. To increase efficiency, a second infection was performed after 9–12 h. The recombinant adenoviruses encoding FAK (Ad-FAK), its kinase-defective mutant (Ad-KD), or GPF control (Ad-GFP) were generated using the Adeasy-1 system (Stratagene) according to manufacturer's instruction. For the rescue experiments, cells infected with Ad-Cre were reinfected with Ad-FAK, Ad-KD, or Ad-GFP control at 10^8 plaque-forming units 2 d after infection of Ad-Cre to delete endogenous FAK. No detectable cell toxicity was observed.

Tube formation assay

ECs infected with Ad-LacZ or Ad-Cre were plated on a thin layer of Matrigel (BD Biosciences) at 10^4 cells/well of a 96-well plate in 10% FBS DME and allowed to form a tubular structure for 8 h to overnight. Cells were assessed on their ability to form simple tube structures and their morphology. The samples were examined on a microscope (model IX70; Olympus) with UplanF1 ×10/0.3 objective lens and photographed with a progressive 3CCD camera (Sony) and Image-Pro Plus ver. 3.0.00.00 at RT. The length and branch points were determined as described previously (Haskell et al., 2003).

TUNEL assay

ECs infected with Ad-LacZ or Ad-Cre were assessed for apoptosis by TUNEL assay using the In Situ Cell Death Detection Kit (Roche), according to the manufacturer's recommendations.

BrdU incorporation assay

2 d after infection, ECs were serum starved for 18 h to arrest the cells in G0. BrdU incorporation assay was performed as described previously (Zhao et al., 2003) with the following modifications. In brief, cells were released from G0 by replating the cells in 10% FBS and 150 μM BrdU. After 48 h of growth, cells were fixed, treated with DNase I, and processed for double immunofluorescent staining with anti-BrdU and anti-PECAM-1, as described below. The percentage of BrdU (+)/ECs (PECAM-1+) was determined for ~100 cells in multiple fields in each independent experiment.

Boyden chamber cell migration assay

Cell migration assays were performed using a Neuro Probe (Cabin John) 48-well chemotaxis Boyden chamber as described previously (Cary et al., 1996) with the following modifications. 7.5×10^3 cells were added in each upper well, and the bottom wells contained either 10 ng/ml VEGF or 10 μg/ml fibronectin as chemoattractant, or DME alone as a control. They were then incubated for 4 h in a 37°C humidified CO$_2$ incubator. At the end of the experiment, cells were fixed with methanol for 8 min and stained with modified Giemsa stain (Sigma-Aldrich).

Wound closure cell migration assay

Wound closure assays were performed essentially as described previously (Wu et al., 2004; Rodriguez et al., 2005). Infected ECs or MEFs were plated (10^6 cells) on gelatin (for VEGF-stimulated cell migration) or FN-coated dishes (60 mm), allowed to adhere and spread for 4 h, and then used for assays.

Immunofluorescence staining

ECs infected with Ad-LacZ or Ad-Cre were processed for immunofluorescence staining as described previously (Cary et al., 1996). The primary antibodies used were anti-phosphotyrosine (PY20; 1:100), anti-vinculin (1:50), anti-BrdU (1:50), and anti-PECAM-1 (1:100). FITC-conjugated goat anti–rabbit IgG (1:150) and FITC-conjugated goat anti–mouse IgG (1:150) were used as the secondary antibodies. They were then mounted on Slowfade (Molecular Probes, Inc.) and examined under a microscope (model BX41; Olympus) with UplanF1 ×20/0.5 objective lens at RT. The images were captured using a camera (model DP70; Olympus) with DP Controller ver. 1.2.1.108.

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