

# Endothelial FAK is required for tumour angiogenesis

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Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that plays a fundamental role in integrin and growth factor mediated signalling and is an important player in cell migration and proliferation, processes vital for angiogenesis. However, the role of FAK in adult pathological angiogenesis is unknown. We have generated endothelial-specific tamoxifen-inducible FAK knockout mice by crossing FAK-floxed (FAK<sup>fl/fl</sup>) mice with the platelet derived growth factor b (*Pdgfb*)-iCreER mice. Tamoxifen-treatment of *Pdgfb*-iCreER;FAK<sup>fl/fl</sup> mice results in FAK deletion in adult endothelial cells (ECs) without any adverse effects. Importantly however, endothelial FAK-deletion in adult mice inhibited tumour growth and reduced tumour angiogenesis. Furthermore, in *in vivo* angiogenic assays FAK deletion impairs vascular endothelial growth factor (VEGF)-induced neovascularization. In addition, *in vitro* deletion of FAK in ECs resulted in reduced VEGF-stimulated Akt phosphorylation and correlating reduced cellular proliferation as well as increased cell death. Our data suggest that FAK is required for adult pathological angiogenesis and validates FAK as a possible target for anti-angiogenic therapies.

## INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing vessels, contributes to several pathological conditions including cancer (Carmeliet, 2003; Hicklin & Ellis, 2005). During angiogenesis, endothelial cells (ECs) migrate and proliferate in response to several proangiogenic growth factors (Carmeliet & Tessier-Lavigne, 2005; Gerhardt et al, 2003; Keck et al, 1989; Leung et al, 1989; Yancopoulos et al, 2000). The coordinated downstream signalling via endothelial growth factor receptors

and integrins has been reported to play an important role during tumour angiogenesis (Ramjaun & Hodivala-Dilke, 2009; Silva et al, 2008). One molecule that is common to both signalling pathways is the ubiquitously expressed tyrosine kinase, focal adhesion kinase (FAK) (Mitra et al, 2005). The convergence of these pathways on FAK suggests that this molecule is likely to be important during blood vessel development in the growing tumour.

The requirement for FAK in embryonic angiogenesis has been demonstrated. For example, FAK-deficient mouse embryos are able to implant successfully and initiate gastrulation, but die at embryonic day 8.5 due to gastrulation defects (Ilic et al, 1995). In addition, constitutive deletion of endothelial FAK, in Tie2-cre mice, also resulted in embryonic lethality due to vascular defects and haemorrhaging (Braren et al, 2006; Shen et al, 2005) and *in vitro* deletion of EC-specific FAK affected tubulogenesis, decreased cell survival, proliferation and migration (Shen et al, 2005). These data implied a requirement for FAK in developmental angiogenesis.

In contrast, however, the requirement for FAK in adult angiogenic processes is not so clear (Weis et al, 2008). FAK-floxed (FAK<sup>fl/fl</sup>) mice have been crossed with transgenic mice expressing a tamoxifen-inducible Cre-recombinase under the 5'

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endothelial enhancer of the stem cell leukaemia locus (End-SCL-Cre-ER(T)) in order to induce endothelial-FAK-deletion in adult mice (Weis et al, 2008). Using this mouse model, FAK-deletion in adult End-SCL-positive ECs induces Pyk2 up-regulation resulting in normal blood vessel formation in postnatal angiogenesis assays such as subcutaneous matrigel plugs and normal endothelial sprouting in aortic ring assays. However, the role of endothelial-FAK in tumour angiogenesis was not tested in this study.

In order to investigate further the role of endothelial-FAK in adult angiogenic processes, we have induced FAK deletion in adult ECs using another endothelial-specific Cre model, the *Pdgfb-iCreER;FAKfl/fl* mice. We show that endothelial-FAK-deletion in adult *Pdgfb-iCreER;FAKfl/fl* mice results in reduced tumour growth and reduced tumour angiogenesis. The cellular basis of this phenotype was also studied and showed that adult FAK-null ECs displayed reduced directional migration in response to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), reduced proliferation and increased apoptosis. Our results indicate that FAK, at least in *Pdgfb*-positive ECs, is required for tumour angiogenesis.

The medical relevance of this study is highlighted by the fact that FAK expression is upregulated during the development of several epithelial cancers (McLean et al, 2005; Zhao & Guan, 2009). These observations together with some *in vivo* inhibition data (Mitra et al, 2006; Mitra & Schlaepfer, 2006; van Nimwegen et al, 2005) have led to the development of FAK inhibitors as potential anti-cancer agents. Our data are the first to suggest that efficient inhibition of tumour endothelial FAK function alone may be sufficient to inhibit primary tumour growth.

## RESULTS

### Generation of *Pdgfb-iCreER;FAKfl/fl* mice

Since endothelial-specific deletion of FAK induces lethality during mouse embryonic development (Braren et al, 2006; Cohen & Guan, 2005; Ilic et al, 1995), we have generated a new mouse model that enables us to induce endothelial FAK deletion in adult mice upon tamoxifen treatment. *FAKfl/fl* mice, which have loxP sites flanking the exon that encodes the FAK amino acids 413/444 (McLean et al, 2004), were crossed with *Pdgfb-iCreERT2* mice (Claxton et al, 2008) to generate *Pdgfb-iCreER;FAKfl/fl* mice which were viable and fertile with no obvious defects. *Pdgfb-iCreER;FAKfl/fl* mice were genotyped by PCR analysis (Supplementary Information Fig S1). Tamoxifen treatment of *Pdgfb-iCreER;FAKfl/fl* mice resulted in endothelial-FAK deleted mice (ECFAK<sup>KO</sup>). *FAKfl/fl* littermates or *Pdgfb-iCreER;non-floxed* (ECFAK<sup>WT</sup>) mice, treated with tamoxifen, were used as controls.

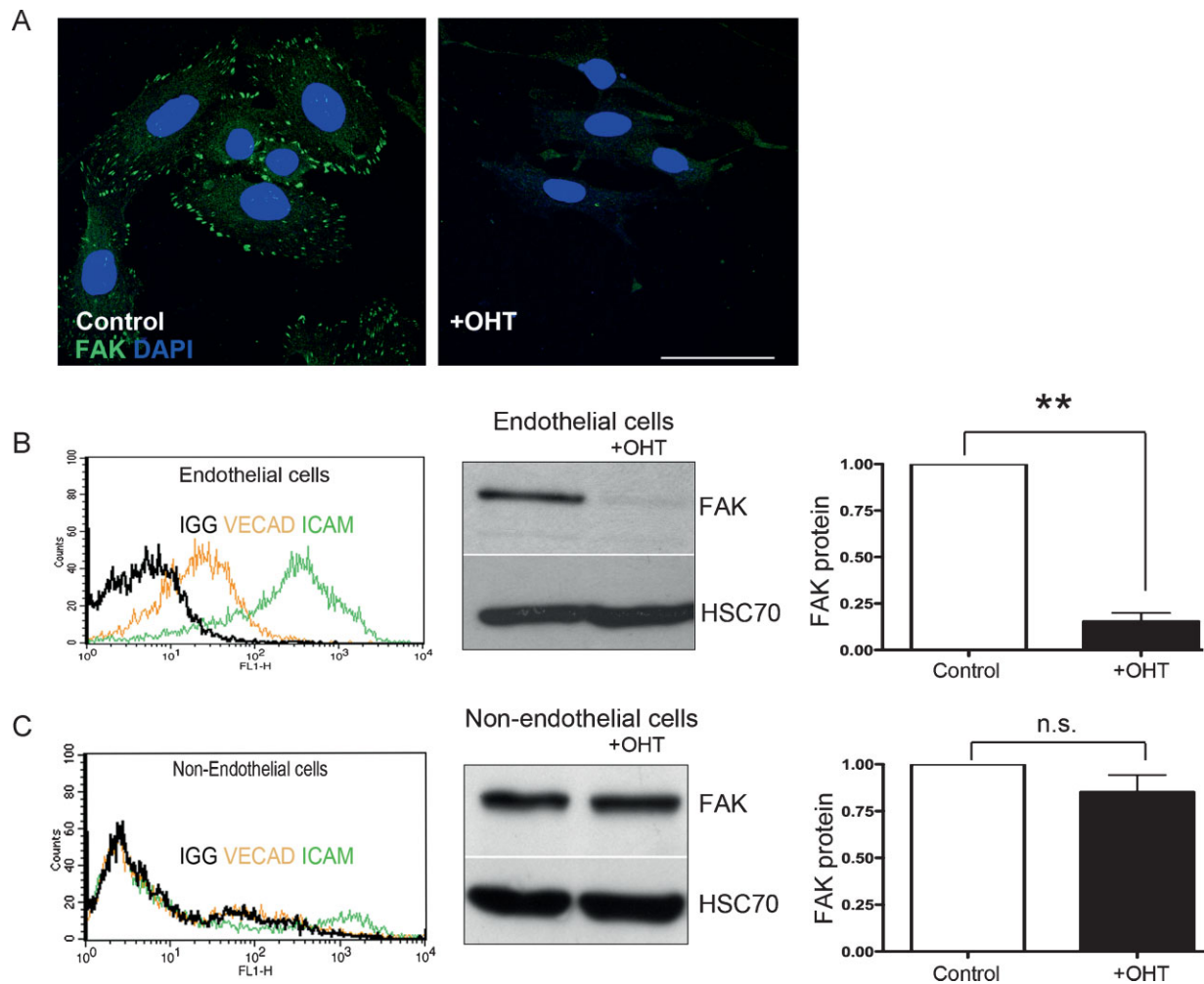
In order to verify efficient FAK deletion, cells isolated from the lungs of *Pdgfb-iCreER;FAKfl/fl* mice were cultured in media supplemented with 4-hydroxytamoxifen (OHT) for 48 h and tested for FAK levels by Western blot analysis and immunofluorescence. Lung ECs (VE-cadherin- and intercellular adhesion molecule, ICAM-positive) isolated from *Pdgfb-iCreER;FAKfl/fl* mice and treated with OHT showed a significant loss of

FAK expression (Fig 1A). These cells also showed an abnormal distribution of F-actin where the cytoskeleton appeared to form dense fibres at the periphery of the cell. However, no apparent defects were detected in either the distribution of the focal contact marker, paxillin, or in overall focal contact appearance between FAK<sup>+/+</sup> and FAK<sup>-/-</sup> ECs (Supplementary Information Fig S2). Western blot analysis confirmed that ECs isolated from *Pdgfb-iCreER;FAKfl/fl* mice and treated with OHT were indeed FAK-deficient (Fig 1B). In contrast, non-ECs, likely including several cell types such as epithelial cells and fibroblasts, isolated from the same animals, were equally positive for FAK expression with or without OHT treatment (Fig 1C). These results indicated that tamoxifen treatment induced efficient FAK-deletion in ECs without apparently affecting other cell types.

### Endothelial-specific deletion of FAK in adult mice inhibits tumour growth and tumour angiogenesis

To test the effect of endothelial-specific FAK deletion on tumour growth and angiogenesis, *Pdgfb-iCreER;FAKfl/fl* mice were either treated or not with tamoxifen (ECFAK<sup>KO</sup> and ECFAK<sup>WT</sup>) and injected subcutaneously with either 10<sup>6</sup> B16F0 melanoma or CMT19T carcinoma cells. At 12 days postinoculation, both B16F0 and CMT19T tumours were significantly smaller in the endothelial FAK-deleted mice, ECFAK<sup>KO</sup>, when compared with controls, ECFAK<sup>WT</sup>, and the same was true for a 16-day time-course of tumour growth (Fig 2A, Supplementary Information Fig S3), suggesting that the loss of FAK in ECs is sufficient to affect tumour growth. To assess whether the inhibition of tumour growth was associated with any change in tumour angiogenesis, midline sections of size-matched, age-matched tumours from both genotypes were analyzed immunohistologically for blood vessel density. Results showed that tumour angiogenesis was reduced significantly in both B16F0 and CMT19T tumours when FAK had been deleted *in vivo* in adult ECs (Fig 2B) ( $p < 0.05$  for B16F0 and  $p < 0.01$  for CMT19T tumours). Endothelial-specific deletion of FAK within the tumour vasculature *in vivo* was confirmed by quantification of the relative expression of FAK in blood vessel endothelium. Results showed that 95% of blood vessels within tumours grown in ECFAK<sup>WT</sup> mice expressed FAK, while only 10% of blood vessels in ECFAK<sup>KO</sup> mice expressed FAK (Fig 2C). These observations suggest that endothelial FAK is required for tumour angiogenesis. Importantly, FAK deletion *in vivo* was endothelial-specific since FAK could be detected in the epithelium and endothelium of ECFAK<sup>WT</sup> mice kidneys, but not in the glomerular endothelium of ECFAK<sup>KO</sup> kidneys (Supplementary Information Fig S4).

To assess whether the defect in tumour angiogenesis in the ECFAK<sup>KO</sup> mice related to changes in blood vessel architecture, we examined various parameters of EC function *in vivo*. Angiogenesis involves various steps including the initial increase in EC proliferation, an increase in migration, tube formation and lastly vessel maturation consisting of recruitment of supporting cells and the deposition of a laminin-rich basement membrane. We showed that: (1) laminin expression was normal and that the ratio of platelet endothelial cell



**Figure 1. Endothelial-specific deletion of FAK in cells isolated from adult mice.**

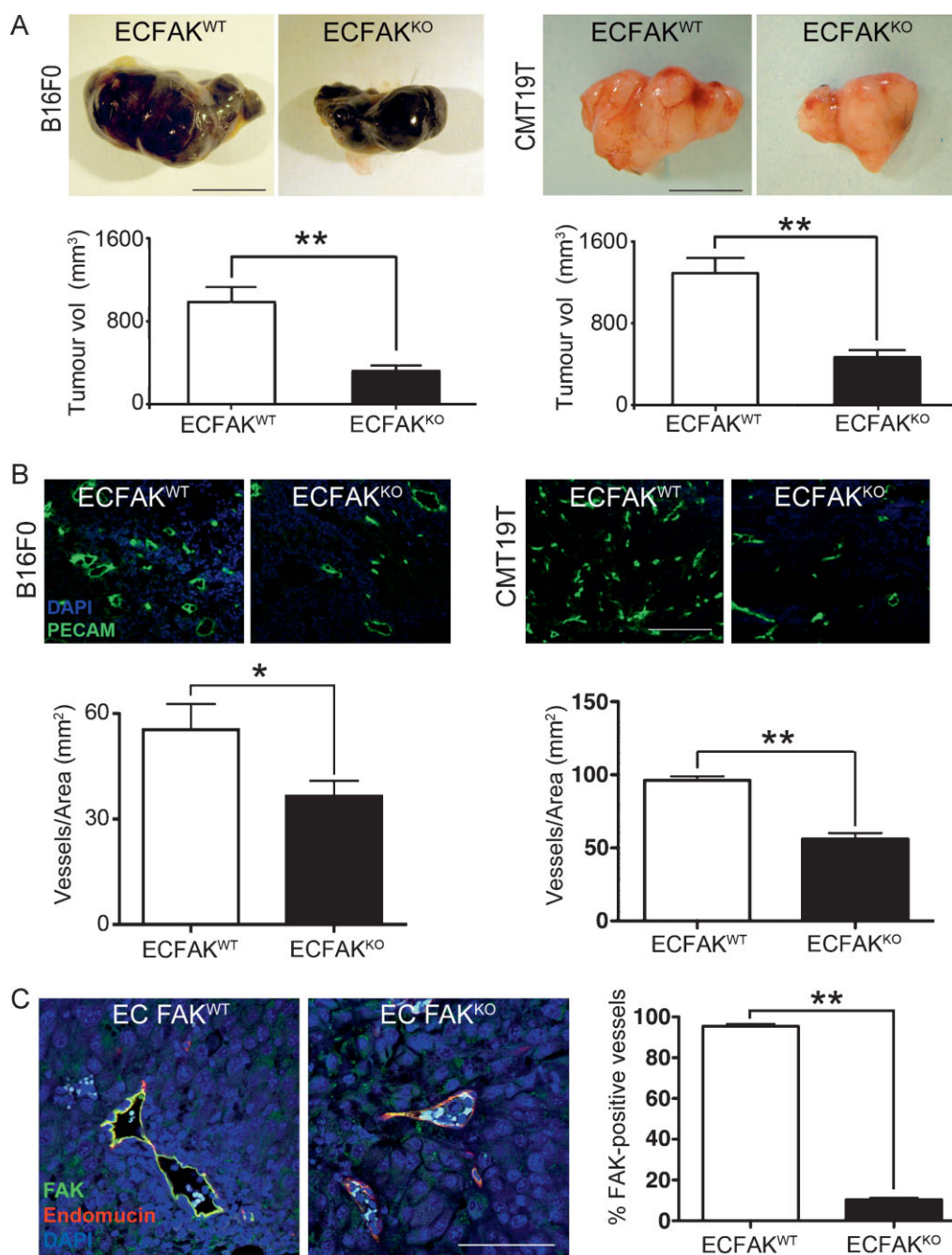
- A.** Immunofluorescence analysis of endothelial cells isolated from *Pdgfb*-iCreER;FAKfl/fl mice shows loss of FAK expression in OHT treated cells (+OHT) but not untreated (Control) cells. FAK (green), DAPI (blue). Scale bar = 50  $\mu$ m.
- B.** Flow cytometric analysis for the endothelial markers intercellular adhesion molecule-2 (ICAM-2) and VE-cadherin (VECAD) was performed to confirm the presence of endothelial enriched populations in cell cultures isolated from the lungs of *Pdgfb*-iCreER;FAKfl/fl mice. Western blot analysis of endothelial cells (EC) isolated from the lungs of *Pdgfb*-iCreER;FAKfl/fl mice shows that FAK is deleted in ECs upon 4-hydroxytamoxifen treatment (OHT). HSC70 acts as loading control. Bar chart represents mean densitometric values  $\pm$ s.e.m.  $**p < 0.01$ .  $n = 3$  independent experiments.
- C.** Flow cytometric analysis for the endothelial markers ICAM-2 and VECAD was performed to confirm the presence of a non-endothelial cell populations in cell cultures isolated from lungs of adult *Pdgfb*-iCreER;FAKfl/fl mice. Western blot analysis confirms that FAK levels do not change in non-endothelial (non-EC) cells isolated from the lungs of *Pdgfb*-iCreER;FAKfl/fl mice. Bar chart represents mean densitometric values  $\pm$ s.e.m. n.s. = not statistically significant.  $n = 3$  independent experiments.

adhesion molecule (PECAM): laminin-positive tumour blood vessels was unchanged between genotypes (Supplementary Information Fig S5); (2) the number of tumour blood vessels with associated  $\alpha$ -SMA-positive supporting cells was the same for the two genotypes (Supplementary Information Fig S6); (3) endothelial 5-bromodeoxyuridine (BrdU) incorporation, as an indicator of cellular proliferation was decreased significantly (Supplementary Information Fig S7A) and (4) tumour cell apoptosis and relative hypoxia tended to increase in ECFAR<sup>KO</sup> mice (Supplementary Information Fig S7B and S8). Taken together, our data suggest that endothelial FAK deletion *in vivo*

does not affect the maturation of neo-blood vessels *per se*, but does induce aberrant proliferative and apoptotic characteristics. This induces an inhibition of angiogenesis which is the likely reason for the increase in tumour cell apoptosis and hypoxia as well as resulting reduction in tumour growth.

**Bone marrow derived cells do not contribute to the impaired angiogenesis in ECFAR<sup>KO</sup> mice**

Since the *Pdgfb* gene is also expressed in megakaryocytes (Gladwin et al, 1990), it is plausible that in ECFAR<sup>KO</sup> mice OHT treatment caused FAK deletion not only in ECs but also in



**Figure 2. Endothelial-specific deletion of FAK in adult mice inhibits tumour growth and angiogenesis.**

- A.** ECFAK<sup>WT</sup> and ECFAK<sup>KO</sup> mice were given subcutaneous injections of syngeneic tumour cell lines, B16F0 melanoma and CMT19T carcinoma. Twelve-day-old tumour size was decreased significantly in ECFAK<sup>KO</sup> mice when compared with ECFAK<sup>WT</sup> controls. Pictures of representative tumours are shown. Bar charts show mean tumour volume +s.e.m. Scale bar = 1 cm;  $n = 11-22$  mice per genotype.  $**p < 0.01$ .
- B.** Tumour blood vessel density is reduced in ECFAK<sup>KO</sup> mice. Representative immunofluorescence micrographs identifying blood vessels (PECAM) in midline sections of tumours. Blood vessel density was assessed by counting the total number of blood vessels per mm<sup>2</sup> across entire midline sections of size-matched tumours. DAPI (blue) was used as a nuclear counterstain. Bar charts represent mean tumour blood vessel density +s.e.m. for both tumour types. Scale bar = 200  $\mu$ m;  $*p < 0.05$ ;  $**p < 0.01$ .
- C.** Double immunofluorescence staining for endomucin (red) and FAK (green) of tumour sections from ECFAK<sup>WT</sup> and ECFAK<sup>KO</sup> mice shows loss of FAK *in vivo* in ECFAK<sup>KO</sup> mice but not ECFAK<sup>WT</sup> controls. DAPI (blue) was used as a nuclear counterstain. Bar chart represents the percentage of FAK-positive vessels. Scale bar = 100  $\mu$ m;  $**p < 0.01$ .

megakaryocytes and resulting platelets. Given that platelets have been implicated in angiogenesis (Sierko & Wojtukiewicz, 2004), we asked whether the potential loss of FAK in platelets and more generally in bone marrow derived cells was sufficient to affect tumour angiogenesis. To address this issue, we first analyzed FAK expression in circulating platelets isolated from ECFAK<sup>WT</sup> and ECFAK<sup>KO</sup> mice. Results showed that platelets isolated from ECFAK<sup>KO</sup> mice had similar levels of FAK protein as platelets isolated from ECFAK<sup>WT</sup> mice, indicating that FAK deletion in platelets is not significant in tamoxifen-treated *Pdgfb-iCreER;FAKfl/fl* mice (Supplementary Information Fig S9A). Furthermore, to confirm further whether bone marrow derived cells contributed somehow to the reduced angiogenesis in ECFAK<sup>KO</sup> mice, wild type mice were transplanted with either *Pdgfb-iCreER;FAKfl/fl* whole bone marrow or, as a negative control, FAK<sup>fl/fl</sup> (Cre-negative) whole bone marrow. Both groups of mice were treated with tamoxifen and were injected subcutaneously with B16F0 melanoma cells. At 12 days postinoculation, tumours were analyzed as described previously and showed no difference in size or blood vessel density (Supplementary Information Fig S9B, C). Taken together, these results demonstrated that the reduced tumour growth and reduced angiogenic responses observed in the ECFAK<sup>KO</sup> mice are likely not due to any alteration in FAK levels in bone marrow derived cells.

#### Endothelial-specific deletion of FAK in adult mice reduces VEGF-induced angiogenic responses *in vivo*

To test the effect of endothelial FAK deletion on VEGF-mediated angiogenesis, sponges were implanted subcutaneously into the flanks of ECFAK<sup>WT</sup> and ECFAK<sup>KO</sup> mice. The sponges were injected with either phosphate buffered saline (PBS) or VEGF every other day for 15 days. Vessel infiltration was assessed immunohistologically by quantification of endomucin-positive vessels per unit area of sponge section. Although treatment with PBS showed no significant difference in blood vessel infiltration, endothelial-FAK-deletion in ECFAK<sup>KO</sup> mice, resulted in a significant reduction in VEGF-stimulated blood vessel infiltration when compared with ECFAK<sup>WT</sup> controls ( $p < 0.05$ ) (Fig 3A). These results suggest that FAK is required for VEGF-mediated angiogenesis *in vivo*.

Given that: (1) FAK is known to be important for the regulation of cell migration; (2) the *Pdgfb* promoter is highly active in retinal endothelial tip cells, specialized ECs at the leading edge of angiogenic sprouts that are highly motile (Gerhardt et al, 2003) and (3) *in situ* hybridization for Cre mRNA in developing retinas from *Pdgfb-iCreER* newborn mice showed high Cre expression levels in endothelial tip cells (Claxton et al, 2008), we hypothesized that part of the reason for the angiogenic impairment in ECFAK<sup>KO</sup> mice could be due to aberrant tip cell migration. To address this hypothesis, we examined the developing retinal vasculature of ECFAK<sup>WT</sup> and ECFAK<sup>KO</sup> mice. We observed that retinal vasculature outgrowth at P5 was delayed significantly in ECFAK<sup>KO</sup> pups (Fig 3B). Interestingly, although endothelial tip sprouts in ECFAK<sup>WT</sup> retinæ were tapered and exhibited filopodia, in ECFAK<sup>KO</sup> retinæ these structures were blunt ended and thickened

(Fig 3B). These data suggest that endothelial FAK deletion results in a change of cellular morphology that is associated with migratory impairment during neovascularization *in vivo*.

Using an *ex vivo* assay to determine the effect of endothelial FAK-deficiency on VEGF-stimulated angiogenesis, aortic rings from ECFAK<sup>WT</sup> and ECFAK<sup>KO</sup> mice were cultured in three-dimensional collagen gels and the numbers of microvessels per ring counted after 6 days of culture. Results show that either *in vivo* tamoxifen administration of the mice prior to aorta dissection, or *in vitro* treatment of the aortic rings directly with tamoxifen was sufficient to inhibit VEGF-mediated microvessel sprouting (Supplementary Information Fig S10).

Given these inhibited responses to VEGF, we next tested the potential effects of FAK deficiency on the major VEGF-receptor, VEGF-receptor 2 (Flk1). Immunostaining of tumour blood vessels from ECFAK<sup>WT</sup> and ECFAK<sup>KO</sup> mice indicated that the expression level of Flk-1 *in vivo* was not altered suggesting that the regulation of angiogenesis by FAK is downstream of Flk-1 (Supplementary Information Fig S11).

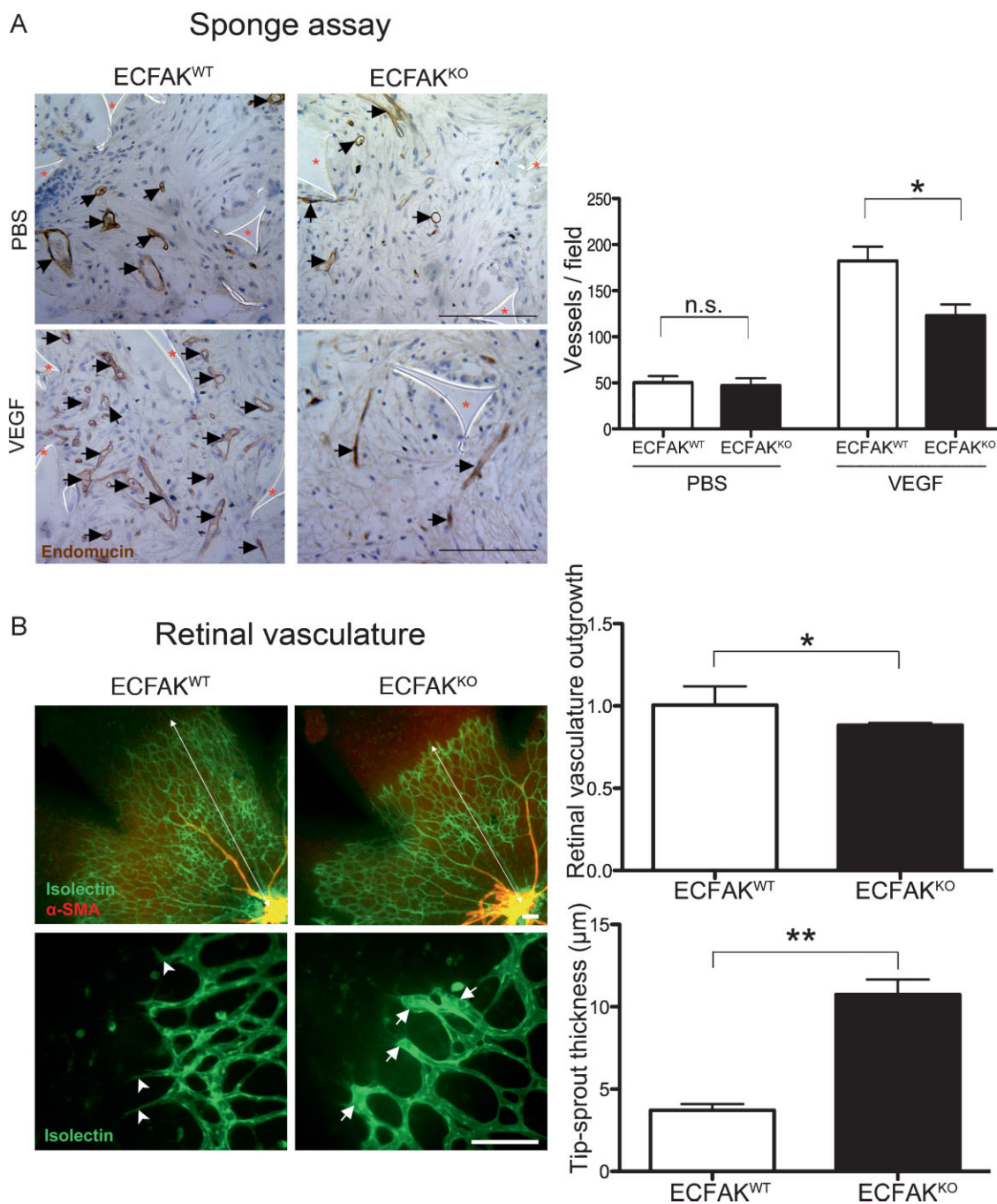
#### Endothelial FAK deficiency reduces VEGF-induced migration and proliferation but increases apoptosis

Given that a critical process during angiogenesis involves the migration of ECs in response to several growth factors, such as VEGF (Gerhardt et al, 2003; Ilic et al, 1995; Shen et al, 2005), we tested the effect of FAK deletion on EC migration *in vitro*. Primary ECs isolated from the lungs of *Pdgfb-iCreER;FAKfl/fl* mice and either OHT-treated or not, to generate FAK<sup>+/+</sup> and FAK<sup>-/-</sup> cells, were exposed to a gradient of VEGF in a Dunn Chamber assay and the migratory behaviour of individual cells assessed. FAK deficiency in ECs inhibited the speed of migration and persistence of cell migration significantly when compared with FAK<sup>+/+</sup> controls (Fig 4A,  $p < 0.01$ ). These data corroborated the migration defect observed in the retinæ of ECFAK<sup>KO</sup> mice.

Furthermore, it has been described that FAK is involved in the proliferation and survival of different cell types including ECs (Mitra & Schlaepfer, 2006; Shen et al, 2005). BrdU incorporation was used to assess proliferation and apoptosis was measured with the terminal deoxynucleotidyl transferase biotinylation (TUNEL) assay. VEGF-stimulated FAK<sup>-/-</sup> cells showed a reduced uptake of BrdU and an increased percentage of TUNEL-positive cells, suggesting that endothelial FAK deletion inhibits cell proliferation and enhances apoptosis (Fig 4B and C). Similar results were obtained for FAK<sup>+/+</sup> and FAK<sup>-/-</sup> ECs grown in full culture medium (Supplementary Information Fig S12).

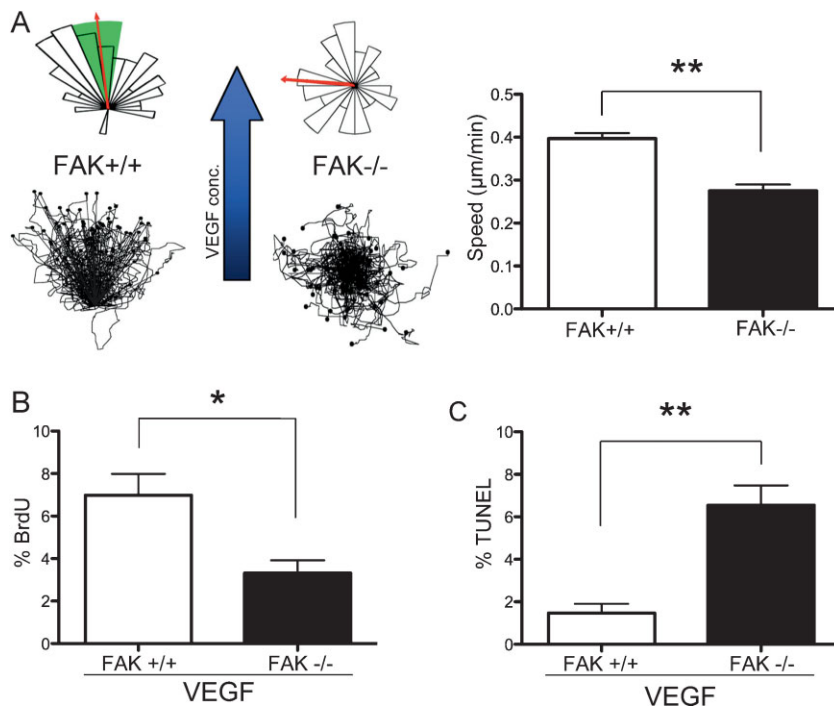
#### bFGF-stimulated angiogenic responses are also reduced after endothelial FAK deletion

Several growth factors are known to be pro-angiogenic including not only VEGF, but also bFGF and others. We show that directional migration, but not speed was inhibited in FAK<sup>-/-</sup> ECs in culture (Supplementary Information Fig S13A), while bFGF-stimulated microvessel sprouting was significantly inhibited in ECFAK<sup>KO</sup> aortic ring assays (Supplementary Information Fig S13B). These data suggest that FAK plays a role not only in VEGF- but also bFGF-stimulated angiogenesis.



**Figure 3. Endothelial-specific deletion of FAK in adult mice reduces VEGF-induced angiogenic responses and migration *in vivo*.**

- A.** In *in vivo* growth factor-induced angiogenic assays, ECF<sup>WT</sup> and ECF<sup>KO</sup> mice were given subcutaneous sponge implants impregnated with either PBS or VEGF. Blood vessel infiltration was examined 14 days postimplantation by immunohistochemical analysis of endomucin-positive blood vessels. Although ECF<sup>WT</sup> and ECF<sup>KO</sup> showed a similar lack of response to PBS treatment, VEGF treatment induced an angiogenic response in ECF<sup>WT</sup> mice but not in ECF<sup>KO</sup> mice. Bar chart represents mean number of infiltrated blood vessels/20× field of view of sponge section + s.e.m.  $n = 7-14$  mice per genotype. \* $p < 0.05$ , n.s. = not statistically significant. Black arrows, endomucin-stained blood vessels; red\*, fragments of synthetic sponge.
- B.** Retinal vasculature at P5 was observed by staining with fluorescein-isolectin B4 (isolectin) and Cy3-coupled antibody against  $\alpha$ -SMA. Retinal vasculature outgrowth (double headed arrow) was reduced in the ECF<sup>KO</sup> retinas when compared with ECF<sup>WT</sup> controls. The fine filopodial extensions (white arrow heads) observed in ECF<sup>WT</sup> tip cells were not present in ECF<sup>KO</sup> retinæ where the 'tip-cells' appeared blunt ended and thick (white arrows). Top bar chart represents the mean vascular network spread \* $p < 0.05$ ; n.s. = not statistically significant;  $n = 8$  retinæ/genotype. Bottom bar chart represents the mean thickness of endothelial tip sprouts. \*\* $p < 0.01$ .  $n = 12$  ECF<sup>KO</sup>;  $n = 11$  ECF<sup>WT</sup> retinæ/genotype; Scale bars = 50  $\mu$ m.



**Figure 4. Endothelial FAK deficiency reduces VEGF-induced migration and proliferation but enhances apoptosis *in vitro*.**

**A.** In Dunn chamber migration assays, directional migration and single cell speed of migration were quantified. VEGF-induced directional migration is reduced in FAK<sup>-/-</sup> endothelial cells. Green area denotes statistically significant directional movement according to Rayleigh testing for clustering in a unidirectional manner. Gradient of the red arrow denotes the trend in direction for movement. Lower track plots represent examples of individual cell migration paths. Bar chart represents the quantification of mean cell migration speed in µm/min + s.e.m. FAK<sup>+/+</sup> ( $n = 74$ ); FAK<sup>-/-</sup> ( $n = 67$ ); \*\* $p < 0.01$ .

**B.** Graph represents the mean % of FAK<sup>+/+</sup> and FAK<sup>-/-</sup> endothelial cells that are BrdU-positive after VEGF stimulation + s.e.m. \* $p < 0.05$ ,  $n = 1001$  FAK<sup>+/+</sup> cells and  $n = 785$  FAK<sup>-/-</sup> cells.

**C.** Graph represents the mean % of FAK<sup>+/+</sup> and FAK<sup>-/-</sup> endothelial cells that are TUNEL-positive after VEGF stimulation + s.e.m. \*\* $p < 0.01$ ,  $n = 639$  FAK<sup>+/+</sup> cells and  $n = 596$  FAK<sup>-/-</sup> cells.

#### Reduced Akt phosphorylation but not ERK1/2 phosphorylation or Pyk2 levels in FAK depleted cells

Our data suggest that FAK deletion in ECs impairs angiogenic responses to VEGF. Since VEGF has been shown to signal via both phospho inositide 3-kinases (PI3K)/Akt and Src/extracellular signal-regulated kinases (ERK1/2), we tested the activation of these pathways in ECs isolated from *Pdgfb-iCreER;FAKfl/fl* mice (Berra et al, 2000; Somanath et al, 2006). Western blot analysis showed that FAK deletion in OHT-treated *Pdgfb-iCreER;FAKfl/fl* ECs resulted in reduced VEGF-induced Akt phosphorylation (at serine 437) (Fig 5A). ECs from *Pdgfb-iCreER;non-floxed* (FAK<sup>+/+</sup>) were also treated with OHT and stimulated with VEGF to test whether OHT-treatment itself interfered with the VEGF-mediated phosphorylation of Akt (Ser 437). Western blot analysis confirmed that the presence of OHT does not affect Akt phosphorylation (Fig 5B) excluding any possible off-target effect that could contribute to a reduction of Akt phosphorylation. In contrast to the suppression of Akt phosphorylation, VEGF-stimulated ERK1/2 phosphorylation was not affected by FAK-deletion when compared with controls (Fig 5C). Additionally, we show that VEGF-stimulated phosphorylation of the p85 subunit of PI3K is inhibited, but that phosphorylation of Src and PDK are not affected by the loss of FAK (Supplementary Information Fig S14). Together these data indicate that the PI3K-Akt, and not the Src-ERK, pathway is affected by FAK deficiency in ECs.

Given that proline rich tyrosine kinase 2 (Pyk2) and FAK are closely related and that Pyk2 has been reported to sometimes compensate for FAK deletion (Weis et al, 2008), Pyk2 levels were analyzed by Western blot. Results showed no difference in Pyk2 levels between FAK<sup>+/+</sup> and FAK<sup>-/-</sup> ECs in culture or

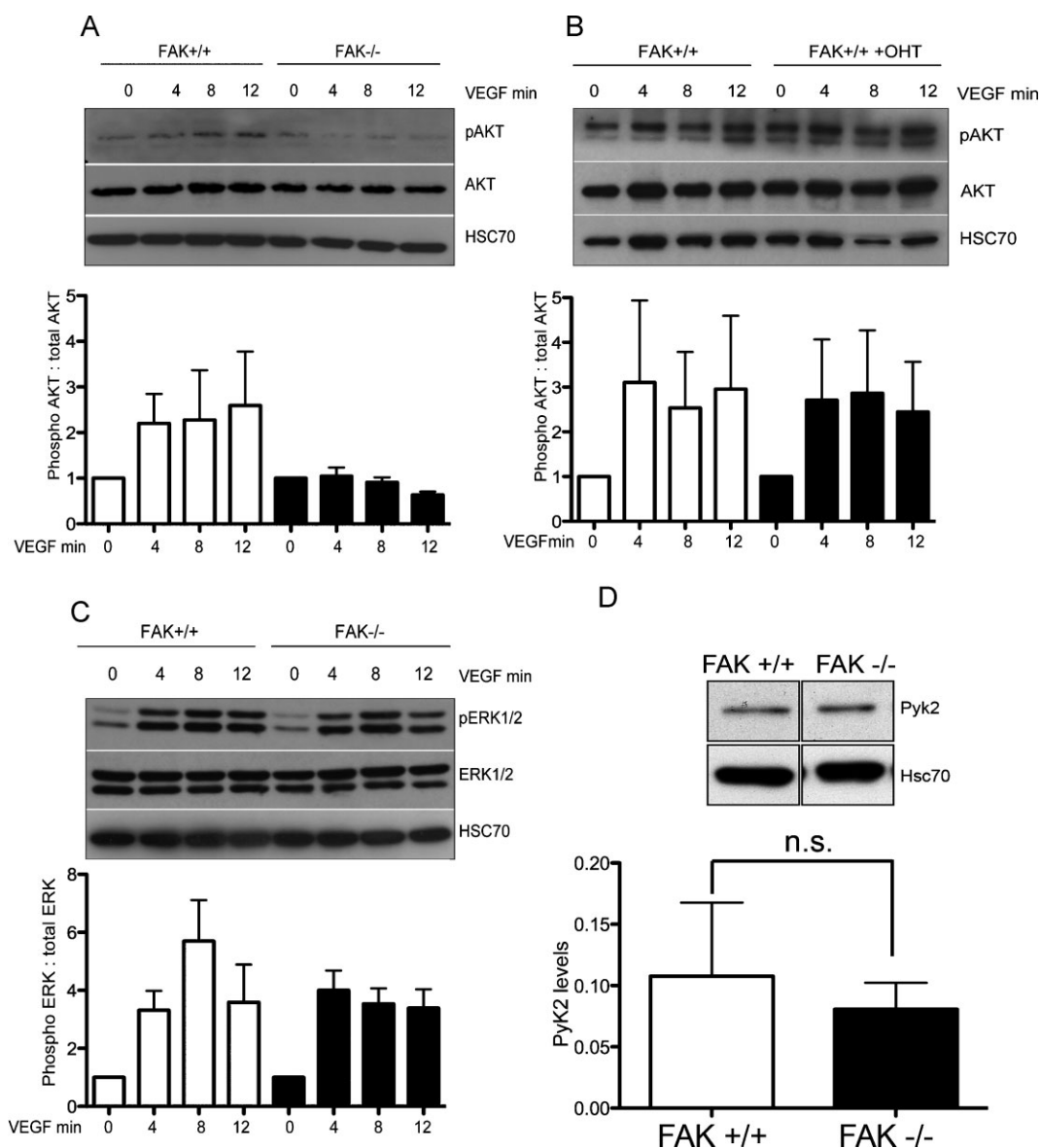
*in vivo* (Fig 5D, Supplementary Information Fig S15), suggesting that in the *Pdgfb-iCreER;FAKfl/fl* model Pyk2 is not compensating for the loss of FAK.

## DISCUSSION

Here, we provide evidence that loss of endothelial FAK in adult mice can inhibit tumour angiogenesis and tumour growth implicating a direct role for FAK in tumour angiogenesis. Indeed, our study suggests that this role for FAK extends to at least VEGF- and bFGF-dependent angiogenic responses.

We have evidence that the basis of the angiogenic defect in the *Pdgfb-iCreER;FAKfl/fl* mice likely involves the effect of endothelial FAK deletion inhibiting EC directional migration and proliferation both *in vivo* and *in vitro*. These changes in cell behaviour correlate with abnormal actin bundling in FAK<sup>-/-</sup> ECs. Similar to findings by Ilic et al (1995) using FAK<sup>-/-</sup> embryo fibroblasts, we observed dense actin fibres around the cell periphery of FAK<sup>-/-</sup> ECs. This abnormal architecture of the actin cytoskeleton is usually found in the early stages of abnormal non-polar cell spreading. In contrast to this abnormal architecture, we provide data demonstrating that focal contact formation and paxillin incorporation is normal in FAK<sup>-/-</sup> ECs. These data imply that endothelial FAK is responsible not for focal contact formation, but rather for the overall organization of the actin cytoskeleton. We speculate that the lack of proper actin organization is likely to be related to the poor proliferation and migration of FAK<sup>-/-</sup> ECs.

Sprouting angiogenesis involves several phases: (1) an initial increase in EC proliferation, migration and tube formation,



**Figure 5. Endothelial FAK deletion impairs Akt phosphorylation but not ERK1/2 phosphorylation.**

- A.** Phosphorylation of Akt (Ser 437) upon VEGF-stimulation was reduced in FAK<sup>-/-</sup> endothelial cells when compared with FAK<sup>+/+</sup> cells. Bar chart represents the mean densitometric quantification of phosphorylated Akt divided by total Akt, normalized to non-VEGF treated samples +s.e.m.  $n = 3$  independent experiments.
- B.** FAK<sup>+/+</sup> cells were either treated or not with OHT to test whether OHT treatment alone could affect Akt phosphorylation. Phosphorylation of Akt (Ser 437) upon VEGF-stimulation was not affected by OHT treatment. Bar chart represents the mean densitometric quantification of phosphorylated Akt divided by total Akt and normalized to non-VEGF treated samples +s.e.m.  $n = 3$  independent experiments.
- C.** Phosphorylation of ERK1/2 upon VEGF-stimulation was similar for FAK<sup>+/+</sup> and FAK<sup>-/-</sup> cells. Bar chart represents the mean densitometric quantification of phosphorylated ERK1/2 divided by total ERK1/2 and normalized to non-VEGF treated samples +s.e.m.  $n = 3$  independent experiments.
- D.** Western blot analysis for Pyk2 levels in FAK<sup>+/+</sup> and FAK<sup>-/-</sup> endothelial cells reveal no difference in the amount of FAK protein levels. Bar chart represents the mean densitometric reading divided by HSC70 +s.e.m.  $n = 3$  independent experiments.

followed by (2) maturation of vessels which includes both the recruitment of supporting cells and the deposition of an intact basement membrane. We show that tumour blood vessels in ECFAK<sup>KO</sup> mice *in vivo* have reduced proliferation *in vivo* and that FAK<sup>-/-</sup> ECs also show reduced proliferation and enhanced

apoptosis. In contrast however, the deposition of a laminin-rich basement membrane and the numbers of tumour vessels with associated  $\alpha$ -SMA-positive supporting cells is normal in ECFAK<sup>KO</sup>. Our results have thus dissected the role of FAK in the phases of angiogenesis and suggest that the *in vivo* role for



FAK in pathological angiogenesis is to support the initial phase of neovascularization but not maturation of the vessels.

Our observations that postnatal retinal blood vessel outgrowth is impaired *in vivo* provides a second example of how the deletion of endothelial FAK can inhibit angiogenesis. Endothelial tip cell filopodia detect proangiogenic growth factor gradients and these are thought to guide tip cell migration during angiogenesis in the retina (Gerhardt et al, 2003). FAK is also known to be important in filopodia formation (Braren et al, 2006; Hsia et al, 2003). We observed blunted tip cell formation in ECFAK<sup>KO</sup> retinæ, and thus provide *in vivo* evidence that endothelial FAK deletion may cause dysfunctional EC migration during angiogenesis in the whole organism and thus inhibit angiogenesis.

How does FAK regulate endothelial proliferation? Previous studies, using fibroblasts, have shown a dominant role for FAK upstream of the PI3K/Akt signalling pathway (Xia et al, 2004). In addition, this pathway has been implicated in EC survival (Ackah et al, 2005; Chen et al, 2005; Gerber et al, 1998; Phung et al, 2006; Somanath et al, 2006; Sun et al, 2005; Yang et al, 2003). We observed poor VEGF-stimulated phosphorylation of p85 and Akt in FAK-deleted ECs with no apparent effect on Src, ERK or PDK phosphorylation. This decrease in p85 and Akt phosphorylation provides a possible explanation for the increased cellular apoptosis and decreased proliferation in FAK-deleted ECs.

Our results in adult mice corroborate previous data, which show that deletion of FAK can inhibit developmental angiogenesis (Braren et al, 2006; Shen et al, 2005). In contrast however, the inducible deletion of endothelial-specific FAK using OHT treated FAK fl/fl;End-SCL-Cre-ER(T)/+ mice was reported to not affect adult angiogenic responses (Weis et al, 2008). How can we explain the apparent discrepancies between these data and our own?

Firstly, OHT treatment of FAK fl/fl;End-SCL-Cre-ER(T)/+ mice results in molecular compensation by the FAK-related molecule Pyk2. In our study, using *Pdgfb*-iCreER;FAKfl/fl mice, we saw no overexpression of Pyk2, either *in vitro* or *in vivo*, or compensation by Pyk2 and others have also shown that this molecule does not always compensate for FAK (Park et al, 2009). Secondly, since in our study *Pdgfb* drives CreERT, but in the Weis study *End-SCL* drives CreERT, it is possible that the two different promoters have different spatial and temporal activities in ECs and possibly generate different off-target effects. Our results suggest that off-target effects in the *Pdgfb*-iCreER;FAKfl/fl mice are unlikely since FAK levels in platelets isolated from *Pdgfb*-iCreER;FAKfl/fl mice appear normal and that bone marrow transplants from *Pdgfb*-iCreER;FAKfl/fl do not affect tumour growth or angiogenesis. Rather, it is tempting to speculate that the deletion of FAK in FAK fl/fl;End-SCL-Cre-ER(T)/+ mice versus *Pdgfb*-iCreER;FAKfl/fl mice could highlight the different requirements for FAK in different subpopulations of ECs during adult pathological angiogenesis. Lastly, the difference in duration of induction required for activation of Cre in End-SCL-Cre-ER(T);FAKfl/fl versus *Pdgfb*-iCreER;FAKfl/fl mice might actually contribute to the lack of an angiogenic phenotype in the former (Weis et al, 2008). The Cre recombinase (CreER<sup>T2</sup>) used in the *Pdgfb*-iCreER mice has been reported to be

ten-times more sensitive to OHT than the Cre recombinase used in the (CreER<sup>T</sup>) End-SCL-Cre-ER(T) mice (Indra et al, 1999; Shimshek et al, 2002). This is illustrated by the fact that efficient FAK deletion requires over 20 days of tamoxifen treatment in End-SCL-Cre-ER(T);FAKfl/fl mice but only 2 days in *Pdgfb*-iCreER;FAKfl/fl mice. Thus taken together, detailed reading indicates that the two systems used are sufficiently different to give apparently opposing effects of endothelial FAK deletion.

Our study has clinical relevance. The role of FAK in tumourigenesis has been based mainly on studies of FAK in tumour cells (Gabarra-Niecko et al, 2003; Luo et al, 2009; McLean et al, 2004; Provenzano et al, 2008). The majority of these studies have shown that FAK is required for tumour cell proliferation, migration, survival, and invasion. However, tumour ECs, together with other tumour stromal cells, interact with transformed cells to generate a microenvironment that supports tumour development. Importantly, the stromal compartment recently has been recognized as playing an important role in overall tumour behaviour (Anton & Glod, 2009). Given the recent development of FAK inhibitors as potential anti-cancer agents (Brunton & Frame, 2008), our results additionally identify endothelial FAK as a possible anti-angiogenic target thus highlighting further the importance of the anti-cancer efficacy of such drugs.

## MATERIALS AND METHODS

### Mice

FAKfl/fl allele was generated by gene targeting of embryonic stem (ES) cells that resulted in the insertion of loxP sites flanking the exon that encodes the FAK amino acids 413/444 (McLean et al, 2004). *Pdgfb*-iCreER mice were provided by Marcus Fruttiger (Claxton et al, 2008). FAKfl/fl mice were bred with the *Pdgfb*-iCreER mice in order to generate FAKfl/fl mice that express CreERT2 under the *Pdgfb* promoter (*Pdgfb*-iCreER;FAKfl/fl) resulting in mice with a mixed C57BL6/129 background.

### Tumour growth assays

Syngeneic mouse tumour cell lines, B16F0 (melanoma, derived from C57black6) and CMT19T (carcinoma, derived from C57black6) were used in subcutaneous tumour growth experiments. *Pdgfb*-iCreER;FAKfl/fl mice and wild type control mice (*Pdgfb*-iCreER;non-floxed or FAKfl/fl) were anaesthetized and slow release (25 mg/pellet, 21-day release) tamoxifen pellets (Innovative Research America, Sarasota, Florida, USA) were implanted subcutaneously into the flank via trocar. After 2 days,  $1 \times 10^6$  cells (B16F0 or CMT19T) resuspended in 100  $\mu$ l of PBS were injected subcutaneously into the scruff of the neck. After allowing the tumours to grow for 12 days, animals were culled and the tumours were excised. The tumour volume was measured using a digital calliper and photographed. Tumours were either fixed in 4% formaldehyde in PBS or snap-frozen in isopentane (cooled in liquid nitrogen) for subsequent immunohistochemical analysis (see below).

### Endothelial cell isolation and culture

Primary mouse lung endothelial cells (MLECs) were isolated from lungs of *Pdgfb*-iCreER;FAKfl/fl or *Pdgfb*-iCreER;non-floxed adult mice

(2 months or older) as described previously (Reynolds & Hodivala-Dilke, 2006). Anti-ICAM-2 and anti-VECAD antibodies (BD Biosciences) were used to assess the EC purity by flow cytometric analysis using a Becton Dickinson FACSCalibur flow cytometer. As a negative control, IgG-matched isotypes were used.

### **In vivo growth factor induced angiogenic assay**

Two sterile polyether sponges (approximately  $1 \times 0.5 \times 0.8 \text{ cm}^3$ ) (Caligen Foam) were inserted subcutaneously in the flanks of *Pdgfb-iCreER;FAKfl/fl* (ECFAK<sup>KO</sup>) or *FAKfl/fl* (ECFAK<sup>WT</sup>) adult mice which had been previously implanted with slow-release tamoxifen pellets 2 days before. The sponges were implanted using a trocar under sterile conditions and the wound was sealed with vet-bond and a wound clip. The sponges were injected every other day with 100  $\mu\text{l}$  of 10 ng/ml VEGF or 100  $\mu\text{l}$  of PBS as a negative control. After 15 days, the mice were culled and sponges removed and fixed in 4% formalin overnight at 4°C. The next day, sponges were transferred to 70% ethanol and paraffin embedded. In order to evaluate the blood vessel infiltration within the sponges, 7  $\mu\text{m}$  sponge sections were immunostained for endomucin. The number of endomucin positive vessels was counted in multiple optical fields.

### **Western blot analysis**

Mouse lung endothelial cells isolated from *Pdgfb-iCreER;FAKfl/fl* or *Pdgfb-iCreER;non-floxed* adult mice were grown until 50% confluent. MLEC media was replaced with fresh media or media supplemented with 500 nM of OHT. After 48 h the cells were rinsed twice with PBS and incubated for 4 h with serum free media (OPTI-MEM I + Glutmax; Gibco) and incubated at 37°C. After 4 h of serum starvation VEGF was added at a final concentration of 30 ng/ml and the cells were lysed with RIPA buffer at 0, 4, 8 and 12 min. Protein concentration was determined using the Bio-Rad Dc Protein Assay Kit (Bio-Rad Laboratories). 15–30  $\mu\text{g}$  of protein from each sample was loaded onto 8–10% polyacrylamide gels. The protein was transferred to a nitrocellulose membrane and incubated for 1 h in 5% milk Tris-buffered saline with 0.1% Tween-20 (TBS-T), followed by an overnight incubation of primary antibody diluted 1:1000 in 5% bovine serum albumin (BSA)–TBS-T at 4°C. The blots were then washed three times with TBS-T and incubated with the relevant horseradish peroxidase (HRP)-conjugated antibody diluted 1:1000 in 5% milk in TBS-T, for 1 h at room temperature. Chemiluminescence was detected by exposing the membrane to high performance Super XR film (Fujifilm, PYSER-SGI limited). Densitometry readings were calculated with the Lab Images software. HSC-70 and Akt were used as loading controls. The following antibodies were used: mouse monoclonal anti-FAK antibody (clone 77/FAK—BD Biosciences); anti-phospho-Akt (Ser473 clone 193H12—Cell Signaling); anti-phospho-ERK1/2 (Thr202/Tyr204 for ERK1 and Thr185/Tyr187 of ERK2—Cell Signaling); anti-phospho-PI3K p85 (Tyr458—Cell Signaling); anti-phospho-PDK1 (Ser241—Cell Signaling); anti-phospho-Src (Tyr416—Cell Signaling); Akt (clone 11E7—Cell Signaling); ERK1/2 (Cell Signaling); anti-PI3K p85 (Cell Signaling); anti-PDK1 (Cell Signaling); anti-Src (Cell Signaling); anti-PYK2/CAK $\beta$  (clone 11—BD Biosciences) and anti-HSC70 (clone B-6—Santa Cruz Biotechnology).

### **Immunohistochemical analysis**

After treating *Pdgfb-iCreER;FAKfl/fl* mouse lung ECs with OHT for 48 h,  $4 \times 10^4$  cells, either treated or not-treated with OHT, were plated on 10 cm glass coverslips in 6-well tissue culture plates. The cells were

then fixed with acetone for 5 min at 20°C and washed three times in PBS. After blocking for 30 min at room temperature in 5% normal goat serum (NGS) and washing once with PBS, the cells were incubated with either mouse anti-FAK antibody (clone 77/FAK, BD—BD Biosciences) or mouse anti-Paxillin (clone 177/paxillin, BD Biosciences) diluted 1:100 in PBS. For F-actin staining, Phalloidin Rhodamine (Molecular Probes) diluted 1:300 in PBS was used. After another three washes in PBS, the cells were incubated with anti-mouse Alexa 488 antibody (Molecular Probes, Eugene, OR, USA) and Phalloidin Rhodamine (Molecular Probes) diluted 1:100 and 1:300 in PBS, respectively. After three final PBS washes, the coverslips were mounted on microscope slides with Prolong antifade reagent and 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Paisley, UK).

Immunostaining of frozen mouse tumour sections to quantify blood vessel density was done using anti-PECAM antibody (BD Biosciences, San Jose, CA, USA) as described previously (Reynolds et al, 2002). Blood vessel density was presented as the number of blood vessels per  $\text{mm}^2$  of midline sections from age-matched, size-matched tumours.

Blood vessel density in subcutaneous sponges was determined after immunostaining formalin fixed and paraffin embedded sponge sections with anti-endomucin antibody (clone V.7C7, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The number of endomucin positive vessels in multiple 40 $\times$  fields was determined in a double-blinded fashion.

In order to stain FAK in tumour blood vessels and endomucin in subcutaneous sponges, formaldehyde fixed sections were de-waxed by immersing in xylene for 4 and 3 min, followed by re-hydration in a gradient of ethanol diluted in distilled water (100, 90, 60 and 50%) for 5 min in each solution. After washing once in PBS, antigen retrieval was performed by heating the samples in 10 mM Na citrate buffer (trisodium citrate diluted in distilled water, pH 6.0) for 20 min in a microwave. The samples were then incubated for 15 min at room temperature in 3% hydrogen peroxide diluted in methanol. Anti-FAK and endomucin antibodies, diluted 1:200 in 1% NGS and PBS, were incubated on the tissue sections overnight at 4°C. Mouse IgG1 (IgG1 clone Ci4, Millipore) was used as a negative control. After incubation with the primary antibodies, tissue sections were washed three times in PBS followed by a 1 h incubation at room temperature with anti-mouse biotinylated (E354, Dako) and anti-rat Alexa 546 (Molecular Probes) antibodies, diluted 1:200 in 1% NGS PBS. After washing with PBS the tissue sections were stained with a streptavidin-HRP/fluorescein kit (TSA Fluorescence Systems). DAPI (Invitrogen, Paisley, UK) was used to identify cell nuclei.

All microscopy was carried out on a confocal microscope LSM510META (Carl Zeiss).

### **BrdU incorporation assay**

Mouse lung endothelial cells were isolated from *Pdgfb-iCreER;FAKfl/fl* (ECFAK<sup>KO</sup>) or *Pdgfb-iCreER;non-floxed* (ECFAK<sup>WT</sup>). Pre-confluent cells were plated at a density of  $4 \times 10^4$  cells on 10 cm glass coverslips in 6-well tissue culture plates. After allowing the cells to attach for 24 h, MLEC medium was replaced by MLEC medium with and without 500 nM of OHT. After 48 h, new MLEC medium, supplemented with 50  $\mu\text{M}$  BrdU, was added to each well of the 6-well plates for 1 h. Cells were then fixed in 4% paraformaldehyde (Sigma) for 10 min at room temperature and subsequently washed with PBS three times for 5 min. Cells were permeabilized with 0.2% Triton X-100 (Sigma) for 5 min, followed by three washes in PBS. For antigen retrieval, cells were

## The paper explained

### PROBLEM:

Tumour angiogenesis is essential for primary tumour growth. Understanding the molecular mechanisms that regulate this process is necessary to develop new anti-angiogenic drugs that inhibit tumour growth. The signalling molecule, focal adhesion kinase (FAK) has recently been implicated in the development of several epithelial cancers but its role in tumour angiogenesis has not been studied. Furthermore, FAK plays an important role in many of the biological processes vital for angiogenesis. We set out to test the hypothesis that FAK regulates tumour angiogenesis and understand the molecular basis for this regulation.

### RESULTS:

Using an inducible mouse model in which FAK is deleted specifically on endothelial cells, we were able to inhibit tumour

growth and angiogenesis *in vivo*. At the cellular level, we show that FAK deletion causes reduced endothelial cell proliferation, migration and alterations in cell signalling, all biological processes that are likely responsible for the observed decrease in tumour angiogenesis.

### IMPACT:

Our results show for the first time that endothelial FAK is crucial for tumour growth and angiogenesis. These results implicate endothelial FAK as a potential target for anti-angiogenic therapy in the treatment of cancer.

incubated in 2 M HCl for 30 min at room temperature. The acid was inactivated by washing twice with 0.1 M sodium tetraborate, pH 6.5. The cells were then blocked in 3% NGS and 0.1% BSA in PBS for 1 h at room temperature followed by incubation with the primary antibody, mouse monoclonal anti-BrdU (Dako) diluted 1:200 in 1.5% NGS 0.05% BSA in PBS, for 2 h at room temperature. After washing three times for 5 min in PBS, the cells were incubated with the secondary antibody anti-mouse Alexa 546. After three final PBS washes, the coverslips were mounted on slides containing Prolong antifade reagent with DAPI (Invitrogen). The epifluorescence microscope (Carl Zeiss) equipped with a digital camera (Hamamatsu Photonics) and the Open Lab v4.4 software (Improvision) was used to capture immunofluorescence images. The percentage of BrdU positive cells was determined by counting the number of BrdU positive cells as a percentage of DAPI-positive cells in multiple fields in each experiment.

### TUNEL assay

The TUNEL method was used to evaluate apoptosis, using the TUNEL Apoptosis Detection Kit (Millipore). *Pdgfb-iCreER;FAKfl/fl* (ECFAK<sup>KO</sup>) or *Pdgfb-iCreER;non-floxed* (ECFAK<sup>WT</sup>) cells were cultured and starved under the same conditions as described in the BrdU assay. The percentage of TUNEL positive cells was determined by counting the TUNEL positive cells and the total number of propidium iodide positive cells in multiple fields in each experiment.

Terminal deoxynucleotidyl transferase biotinylation nick end labelling positive cells were detected in tumour sections from ECFAK<sup>WT</sup> and ECFAK<sup>KO</sup> mice with the TUNEL Apoptosis Detection Kit (Millipore) according to the manufacturer instructions and counterstained with nuclear DAPI staining. The area of TUNEL positive cells over the total tumour section area was quantified using microscope fluorescence images.

### Dunn chamber chemotaxis migration assay

Chemotaxis was studied using Dunn chambers (Zicha et al, 1997). Dunn Chambers were stored in 7× detergent, washed in water to

remove any wax, rinsed in distilled water and sterilized in ethanol before use. Chambers were rinsed in media and both outer and inner wells were filled with OPTI-MEM. MLECs isolated from *Pdgfb-iCreER;FAKfl/fl* mice, were plated at a density of 5000 cells/cm<sup>2</sup> on coated coverslips and cultured overnight in normal MLEC medium supplemented or not with 500 nM of OHT. The following day cells were washed with PBS and serum starved, for at least 5 h, by replacing the MLEC media with serum free medium (OPTI-MEM I + GlutaMAX, Gibco, Invitrogen, Paisley, UK) with or without 500 nM OHT. Coverslips were inverted onto the Dunn Chamber leaving a gap in the outer well and sealed on three sides with hot wax mixture (Vaseline:paraffin:beeswax—1:1:1). The media was removed from the outer well by capillary action and was rinsed with OPTI-MEM before filling with OPTI-MEM containing 30 ng/ml of either VEGF or bFGF. The chamber was then sealed with wax and mounted on a Zeiss Axio100 inverted microscope. Images were acquired by phase contrast imaging using a 10× N-Achroplan Phase contrast objective (NA 0.25). Cell images were collected using a Sencam (PCO Cook) CCD camera, taking a frame every 10 min for 16 h using AQM acquisition software (Kinetic Imaging Ltd., Andor Belfast, UK). Subsequently all the acquired time-lapse sequences were displayed as an AVI file and cells from the time-lapse sequence were tracked using Andor Bioimaging Tracking. Tracking resulted in the generation of a sequence of position coordinates relating to each cell in each frame, motion analysis was then performed on these sequences using Wolfram Mathematica 6. Rose plots show the proportion of cells with migratory direction lying within each 20° interval. The red arrow represents the mean direction of migration and the green segment represents the 95% confidence interval determined by Rayleigh test.

### Retinal angiogenesis

Newborn litters from *Pdgfb-iCreER;FAKfl/fl* × *FAKfl/fl* breeding pairs were intraperitoneally injected, at P1 and P2, with 20 µl/g of 4 mg/ml of OHT (Sigma). OHT was prepared with two successive dilutions: first diluted to 20 mg/ml in ethanol; subsequently diluted to 4 mg/ml in

peanut oil. At P5 animals were culled and the eyes dissected and fixed in 4% PFA in PBS for 1 h at room temperature and then transferred to PBS (Puri et al, 1995). After fixation, retinae were incubated in 1% BSA and 0.3% Triton, washed three times in Pblec buffer (1% Triton X-100, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub> in PBS [pH 6.8]) and incubated with fluorescein-isolectin B4 (Vector labs) and Cy3 coupled antibody against  $\alpha$ -smooth muscle actin.

### Ethical regulations

All animals were used according with the United Kingdom Home Office regulations.

### Author contributions

BT and KMHD designed the experiments. BT performed the experiments. SB assisted with immunostaining, tumour harvesting and genotyping. LER and SR assisted with Western blots. SJ and MF performed the retinal angiogenesis experiments. VK assisted with mouse colony maintenance and performed the initial experiments. MP performed the Dunn Chamber experiments. BT and KMHD wrote the paper with substantial input from IH.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that there is no conflict of interest.

### References

Ackah E, Yu J, Zoellner S, Iwakiri Y, Skurk C, Shibata R, Ouchi N, Easton RM, Galasso G, Birnbaum MJ, et al (2005) Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. *J Clin Invest* 115: 2119-2127

Anton K, Glod J (2009) Targeting the tumor stroma in cancer therapy. *Curr Pharm Biotechnol* 10: 185-191

Berra E, Milanini J, Richard DE, Le Gall M, Vinals F, Gothie E, Roux D, Pages G, Pouyssegur J (2000) Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. *Biochem Pharmacol* 60: 1171-1178

Braren R, Hu H, Kim YH, Beggs HE, Reichardt LF, Wang R (2006) Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. *J Cell Biol* 172: 151-162

Brunton VG, Frame MC (2008) Src and focal adhesion kinase as therapeutic targets in cancer. *Curr Opin Pharmacol* 8: 427-432

Carmeliet P (2003) Angiogenesis in health and disease. *Nat Med* 9: 653-660

Carmeliet P, Tessier-Lavigne M (2005) Common mechanisms of nerve and blood vessel wiring. *Nature* 436: 193-200

Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV (2005) Akt1 regulates pathological angiogenesis, vascular maturation and permeability *in vivo*. *Nat Med* 11: 1188-1196

Claxton S, Kostourou V, Jadeja S, Chambon P, Hovidala-Dilke K, Fruttiger M (2008) Efficient, inducible Cre-recombinase activation in vascular endothelium. *Genesis* 46: 74-80

Cohen LA, Guan JL (2005) Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. *J Biol Chem* 280: 8197-8207

Gabarra-Niecko V, Schaller MD, Dunty JM (2003) FAK regulates biological processes important for the pathogenesis of cancer. *Cancer Metastasis Rev* 22: 359-374

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273: 30336-30343

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, et al (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 161: 1163-1177

Gladwin AM, Carrier MJ, Beesley JE, Lelchuk R, Hancock V, Martin JF (1990) Identification of mRNA for PDGF B-chain in human megakaryocytes isolated using a novel immunomagnetic separation method. *Br J Haematol* 76: 333-339

Hicklin DJ, Ellis LM (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 23: 1011-1027

Hsia DA, Mitra SK, Hauck CR, Strebblow DN, Nelson JA, Ilic D, Huang S, Li E, Nemerow GR, Leng J, et al (2003) Differential regulation of cell motility and invasion by FAK. *J Cell Biol* 160: 753-767

Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 377: 539-544

Indra AK, Warot X, Brocard J, Bornert JM, Xiao JH, Chambon P, Metzger D (1999) Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: Comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res* 27: 4324-4327

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 246: 1309-1312

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246: 1306-1309

Luo M, Fan H, Nagy T, Wei H, Wang C, Liu S, Wicha MS, Guan JL (2009) Mammary epithelial-specific ablation of the focal adhesion kinase suppresses mammary tumorigenesis by affecting mammary cancer stem/progenitor cells. *Cancer Res* 69: 466-474

McLean GW, Komiya NH, Serrels B, Asano H, Reynolds L, Conti F, Hovidala-Dilke K, Metzger D, Chambon P, Grant SG, et al (2004) Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression. *Genes Dev* 18: 2998-3003

McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC (2005) The role of focal-adhesion kinase in cancer—A new therapeutic opportunity. *Nat Rev Cancer* 5: 505-515

Mitra SK, Schlaepfer DD (2006) Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol* 18: 516-523

Mitra SK, Hanson DA, Schlaepfer DD (2005) Focal adhesion kinase: In command and control of cell motility. *Nat Rev Mol Cell Biol* 6: 56-68

- Mitra SK, Mikolon D, Molina JE, Hsia DA, Hanson DA, Chi A, Lim ST, Bernard-Trifilo JA, Ilic D, Stupack DG, *et al* (2006) Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. *Oncogene* 25: 5969-5984
- Park AY, Shen TL, Chien S, Guan JL (2009) Role of focal adhesion kinase Ser-732 phosphorylation in centrosome function during mitosis. *J Biol Chem* 284: 9418-9425
- Phung TL, Ziv K, Dabydeen D, Eyah-Mensah G, Riveros M, Perruzzi C, Sun J, Monahan-Earley RA, Shiojima I, Nagy JA, *et al* (2006) Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell* 10: 159-170
- Provenzano PP, Inman DR, Eliceiri KW, Beggs HE, Keely PJ (2008) Mammary epithelial-specific disruption of focal adhesion kinase retards tumor formation and metastasis in a transgenic mouse model of human breast cancer. *Am J Pathol* 173: 1551-1565
- Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J (1995) The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J* 14: 5884-5891
- Ramjaun AR, Hoidalva-Dilke K (2009) The role of cell adhesion pathways in angiogenesis. *Int J Biochem Cell Biol* 41: 521-530
- Reynolds LE, Hoidalva-Dilke KM (2006) Primary mouse endothelial cell culture for assays of angiogenesis. *Methods Mol Med* 120: 503-509
- Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, Sheppard D, Hynes RO, Hoidalva-Dilke KM (2002) Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. *Nat Med* 8: 27-34
- Shen TL, Park AY, Alcaraz A, Peng X, Jang I, Koni P, Flavell RA, Gu H, Guan JL (2005) Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. *J Cell Biol* 169: 941-952
- Shimshek DR, Kim J, Hubner MR, Spergel DJ, Buchholz F, Casanova E, Stewart AF, Seeburg PH, Sprengel R (2002) Codon-improved Cre recombinase (iCre) expression in the mouse. *Genesis* 32: 19-26
- Sierko E, Wojtukiewicz MZ (2004) Platelets and angiogenesis in malignancy. *Semin Thromb Hemost* 30: 95-108
- Silva R, D'Amico G, Hoidalva-Dilke KM, Reynolds LE (2008) Integrins: The keys to unlocking angiogenesis. *Arterioscler Thromb Vasc Biol* 28: 1703-1713
- Somanath PR, Razorenova OV, Chen J, Byzova TV (2006) Akt1 in endothelial cell and angiogenesis. *Cell Cycle* 5: 512-518
- Sun JF, Phung T, Shiojima I, Felske T, Upalakalin JN, Feng D, Kornaga T, Dor T, Dvorak AM, Walsh K, *et al* (2005) Microvascular patterning is controlled by fine-tuning the Akt signal. *Proc Natl Acad Sci USA* 102: 128-133
- van Nimwegen MJ, Verkoeyen S, van Buren L, Burg D, van de Water B (2005) Requirement for focal adhesion kinase in the early phase of mammary adenocarcinoma lung metastasis formation. *Cancer Res* 65: 4698-4706
- Weis SM, Lim ST, Lutu-Fuga KM, Barnes LA, Chen XL, Gothert JR, Shen TL, Guan JL, Schlaepfer DD, Cheresh DA (2008) Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. *J Cell Biol* 181: 43-50
- Xia H, Nho RS, Kahm J, Kleidon J, Henke CA (2004) Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta 1 integrin viability signaling pathway. *J Biol Chem* 279: 33024-33034
- Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. *Nature* 407: 242-248
- Yang ZZ, Tschopp O, Hemmings-Mieszczyk M, Feng J, Brodbeck D, Perentes E, Hemmings BA (2003) Protein kinase B alpha/Akt1 regulates placental development and fetal growth. *J Biol Chem* 278: 32124-32131
- Zhao J, Guan JL (2009) Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev* 28: 35-49
- Zicha D, Dunn G, Jones G (1997) Analyzing chemotaxis using the Dunn direct-viewing chamber. *Methods Mol Biol* 75: 449-457