**Tumour angiogenesis is differentially regulated by endothelial cell Focal Adhesion
Kinase tyrosine-397 and -861 phosphorylation**

Short running title: Differential tumour angiogenesis control by FAK-Y397 & Y861

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Abstract

Expression of Focal Adhesion Kinase (FAK) in endothelial cells (EC) is essential for angiogenesis, but how FAK-phosphorylation at tyrosine-(Y)397 and Y861 regulate tumour angiogenesis *in vivo* is unknown. Here, we show that tumour growth and angiogenesis are constitutively reduced in inducible, *ECCre+;FAK^{Y397F/Y397F}* mutant mice. Conversely, *ECCre+;FAK^{Y861F/Y861F}* mice have normal tumour growth with an initial reduction in angiogenesis that recovered in end stage tumours. Mechanistically, FAK-Y397F ECs exhibit increased Tie-2 expression, reduced Vegfr-2 expression, decreased β 1-integrin activation, and disrupted downstream FAK/Src/PI3K(p55)/Akt signalling. In contrast, FAK-Y861F ECs showed decreased Vegfr-2 and Tie-2 expression with an enhancement in β 1-integrin activation. This corresponds with a decrease in Vegf-a-stimulated response, but an increase in Vegf-a+Ang-2- or conditioned medium from tumour cells-stimulated cellular/angiogenic responses, mimicking responses in end stage tumours with elevated Ang-2 levels. Mechanistically, FAK-Y861F, but not FAK-Y397F, ECs showed enhanced p190RhoGEF/P130Cas-dependent signalling that is required for the elevated responses to Vegf-a+Ang-2. This study establishes the differential requirements of EC-FAK-Y397 and EC-FAK-Y861-phosphorylation in the regulation of EC-signalling and tumour angiogenesis *in vivo*.

Introduction

Deciphering the molecular mechanisms that control tumour angiogenesis is fundamental to understanding its regulation (1, 2). Vascular endothelial growth factor (Vegf-a) is essential in triggering angiogenesis, whilst angiopoietin 2 (Ang-2) controls vessel maintenance and is up-regulated in response to Vegf-a in both endothelial (ECs) and tumour cells (2-4), during tumour progression (5-9). Despite this, how essential signalling mediators, such as Focal Adhesion Kinase (FAK), control these regulatory responses is poorly understood.

FAK is a ubiquitously expressed non-receptor tyrosine kinase that regulates several EC processes important for angiogenesis, including motility and proliferation (10). FAK is composed of an N-terminal FERM domain, a kinase domain, preceded by a tyrosine (Y)397 residue, and a C-terminal FAT (focal adhesion-targeting) domain, preceded by Y861 residue. The C-terminal FAT domain indirectly links FAK to integrins via other focal adhesion signalling complex proteins, such as p190RhoGEF, a RhoA-specific GEF (guanine exchange factor) (10-13). FAK-kinase activity results in Y397-phosphorylation, which, in turn, allows proteins containing the Src-homology (SH2) domain to bind to FAK, eg., Src and PI3K. FAK-Src complexing potentiates further FAK phosphorylation at other FAK domains including FAK-Y861 (10, 14). In addition, other FAK binding partners, such as p130Cas and phospholipase C-gamma (Plc-γ), enable FAK to function as a transducer of integrin- and growth-factor-stimulation leading to phosphorylation of Erk and Akt (15-17).

Previous work indicates that constitutive FAK genetic ablation or pharmacological inhibition of FAK-kinase activity results in embryonically lethal vascular defects (18-21). Additionally, deletion of exon 5, which encodes FAK-Y397, also leads to an embryonic lethal phenotype with vascular permeability defects (20). In adult mice however, data resulting from EC loss of FAK are not consistent and depend on the model used: *FAK* deletion in adult *Tie2-Cre*-positive ECs reduced human glioma burden, with increased blood vessel density and decreased vascular permeability (22); in contrast, tumour growth is inhibited in *Pdgfrb-Cre^{ERT};FAK^{fl/fl}* mice, with reduced blood vessel density but with no changes in vascular maturation (23). Notwithstanding these data, the *in vivo* requirement for phosphorylation of FAK-Y397 and Y861 in controlling tumour angiogenesis has been unknown until this report.

Here, using inducible endothelial cell-specific *FAK^{Y397F/Y397F}* and *FAK^{Y861F/Y861F}* mice (24), we have identified the differential *in vivo* requirements for these motifs in tumour angiogenesis.

Materials and Methods

Mice

To study the effect of inducible EC-specific FAK mutations *in vivo*, a Cre-inducible system for mutant FAK expression was generated by designing mutant chicken-FAK constructs (preceded by a STOP sequence flanked by loxP sites) that were targeted to the ubiquitous Rosa26 locus (24). R26FAK^{KI/KI} mice were bred with endothelial cell-type specific *Pdgfb-Cre^{ert};FAK^{fl/fl}* mice to generate mice with tamoxifen-inducible mutant FAK-knockin and endogenous FAK-knockout in endothelial cells (24), *Pdgfb-iCre^{ert};FAK^{fl/fl};R26FAK^{Y397F/Y397F}* (ECCre; *FAK^{Y397F/Y397F}*) and *Pdgfb-iCre^{ert};FAK^{fl/fl};R26FAK^{Y861F/Y8617F}* (ECCre; *FAK^{Y861F/Y8617F}*) mice, in which tyrosines 397 and 861 were mutated to phenylalanine, respectively. *Pdgfb-iCre^{ert};FAK^{fl/fl};R26FAK^{WT/WT}* mice expressed wild type FAK, as controls.

All animals were used in accord with United Kingdom Home Office regulations (Home Office license number 70/7449). The in-house Ethics Committee at Queen Mary University of London has approved all experiments, using mice under the project license.

B16F0 and CMT19T cell culture

B16F0 melanoma cells (ATCC CRL-6322) and CMT19T lung carcinoma cells (CR-UK Cell Production) were grown in Dulbecco's Modified Medium (DMEM, Invitrogen, Paisley, UK), supplemented with 10% foetal calf serum (FCS, PAA, UK). Cells were tested for Mycoplasma by PCR in 2015. Cells were cultured on uncoated T175 flasks at 37°C 5% CO₂ and passaged 2-5 times until used in mouse injections. Tumour cells were 70-80% confluent when trypsinized, washed 3 times in 50mL PBS, passed through a 70µm cell strainer (BD Falcon, Bedford, MA, USA) and counted using the Automated Cell Counter (Scepter 2.0, Millipore). The cell suspension concentration was adjusted to 10⁷ cells/mL in PBS prior to 100µL tumour cell suspension subcutaneous injection.

Subcutaneous Tumour Growth

12- to 16-week old mice were given tamoxifen (150 µl of 10 mg/ml solution) (Sigma) via intraperitoneal (ip) injection on two consecutive days; mice were fed with tamoxifen-containing diet (Tamoxifen400; Harlan Laboratories) starting from the day of the second tamoxifen injection until the end of the experiment. Five to seven days after initial tamoxifen injection, mice were anesthetized with Isoflurane (Abbot) and subcutaneously injected into the flank with 100µl of 1 million B16F0 mouse melanoma cells or CMT19T lung carcinoma cells. Once tumours became palpable (usually at day 5-7 for B16F0 or at day 8-10 for CMT19T), the tumour size was measured with digital calipers every other day until the experimental endpoint.

When required, *ante-mortem* procedures were performed as described in the next section, and dissected tumours were snap-frozen in liquid nitrogen or fixed in 10% formalin.

Blood vessel density analysis

Snap frozen sections were fixed with cold acetone for 10 minutes, blocked with 5% BSA in PBS, incubated with a rat anti-endomucin antibody (dilution 1/400 clone V.7c7, Santa Cruz Biotechnology) overnight at 4 °C, washed in PBS 0.02% Triton X-100, and then incubated for 60 min at room temperature with an Alexa Fluor-labelled anti-rat antibody (dilution 1/300 Invitrogen, Paisley, UK). Tumour blood vessels were counted across entire midline sections, and the numbers expressed as vessels per mm² of tumour section.

Hypoxia analysis

Pimonidazole-HCl (Hypoxyprobe, Burlington, Massachusetts, USA) at 60 mg/kg body weight was intraperitoneally injected one hour before culling mice. Snap frozen tumour sections were prepared as described previously and incubated overnight at 4 °C with a FITC-conjugated mouse anti-pimonidazole antibody (Hypoxyprobe HPI Burlington; MA01803), and then washed and mounted. The hypoxic index was calculated as the area of pimonidazole staining/area of tumour section.

Subcutaneous Sponge Assay

Two sterile polyether sponges (approximately 1 x 0.8 x 0.8 cm) (Caligen Foam) were inserted subcutaneously in the flanks of adult mice, previously injected with tamoxifen as described for subcutaneous tumour experiments. The sponges were injected every other day with 100µl of 10ng/ml Vegf (Peprotech, 450-32), 10ng/ml of PIGF (R&D Systems, 465-PL- 010), 5ng/ml of bFGF (Peprotech, 450-33), 10ng/ml of Ang-2 (R&D Systems, 7186-AN-025), 10ng/ml of both Vegf-a and Ang-2 or 100µl of PBS as a negative control.

After 15 days, mice were culled and sponges removed and fixed in 10% formalin overnight. The next day, sponges were transferred to 70% ethanol, paraffin-embedded and 5µm sections were cut for endomucin staining.

Blood vessel immunohistochemistry and quantitation

Paraffin-embedded sections of sponges and matrigel plugs were dewaxed, rehydrated and fixed with Methanol+H₂O₂. Sections were processed as follows: antigen-retrieval using 10mM Tri-Sodium Citrate pH6 in the microwave; slides washed in distilled water; incubated with blocking buffer containing 2% Goat serum / 1% BSA / 0.1% TritonX100 / PBS; incubated with anti-endomucin (Endomucin clone v.7C7 Santa Cruz; 1/200 in blocking buffer) overnight at 4C; washed three times for 5min at RT in PBS 1X; incubated with Elite ABC reagent (Vector kit PK6100 or 6102) according to manufacturer's instructions, for 30min at RT; washed twice in PBS; incubated with DAB substrate (Vector kit SK4100) until the colour of the reaction was evident (approx. 5 mins); slides counterstained with hematoxylin; dehydrated, cleared and mounted with DPX (Sigma, Dorset, UK).

Slides were scanned using the Pannoramic 250 Scanner. For sponges: Infiltrated

microvessels were quantified across the width of the sponge at 30X magnification. The number of microvessels was quantified for the fields that presented with infiltrated cells and the average number of microvessels per field was determined for each sponge. For Matrigel plugs: Infiltrated microvessels were counted across each individual plug and divided by the area of the plug (No. $\times 10^{-5}/\mu\text{m}^2$).

Cell Treatment

Primary non-immortalized and immortalized endothelial cells were starved overnight in Opti-mem and then treated with Vegf-a (30 ng/ml) and/or Ang-2 (30 ng/ml) for the indicated times. For generation of mouse lung endothelial cell lines (MLECs) and immunofluorescence of cells see supplementary material and methods.

Spheroid assay

One thousand MLECs were incubated overnight on a hanging drop in order to form a spheroid, and transferred to a collagen type I gel (1mg/ml). After polymerization, 1% FBS EBM2 medium with the indicated growth factors at the same concentration as cell treatment were added on top of the gels, and sprout formation was quantified 24 hours later.

Embedded spheroids were fixed in 4% PFA for 30 min, washed in PBS, permeabilised in 0.3%Triton X-100 PBS for 1 hour, and stained overnight with Rhodamine Phalloidin (Invitrogen). The next day, spheroids were washed for 30 minutes three times with 0.1%Triton X-100 PBS, and mounted with FluoromontG (Thermo Fisher). Superimposed brightfield and fluorescent images were acquired using a Confocal Spinning disk microscope with a 20x Objective and sCMOS confocal camera (Nikon).

Migration assays

For details on preparation of Dunn Chambers see supplementary methods.

45,000 mouse endothelial cells were plated on coated 22mm glass coverslips and cultured overnight. The following day cells were washed with PBS and serum-starved for 5 hours in serum free medium (OPTI-MEM I + GlutaMAX, Gibco, Invitrogen, Paisley, UK). Coverslips were inverted onto the Dunn Chambers leaving a gap in the outer well and sealed on three sides with hot wax mixture (Vaseline : paraffin : beeswax - 1:1:1). The medium was removed from the outer well by capillary action and was rinsed with OPTI-MEM before filling with OPTI-MEM containing 30ng/ml of Vegf (Peprotech, 450-32) or Ang-2 (R&D Systems, 7186-AN-025) or both together. The chamber was then sealed with wax and mounted on an Olympus IX71 inverted microscope. Images were acquired by phase contrast imaging using a 10x phase contrast objective (NA 0.25). Cell images were collected using a Retiga R6 CCD camera (Photometrics/Qimaging), taking a frame every 10 min for 16 hours using Micro Manager acquisition software (NIH, open source). Subsequently all the acquired time-lapse sequences were displayed as an AVI file and cells from the time-lapse sequence were tracked using ImageJ. 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 7 software. Data is depicted as pooled example tracks of cells or rose plots that 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.

Western blotting

Endothelial cells were lysed in ice-cold RIPA Buffer supplemented with 50mM NaF, 60mM β -glycerol phosphate, 10mM Na pyrophosphate, 2mM Na_3VO_4 and a dilution 1/100 of proteases inhibitors cocktail III (Roche) in distilled water. Lysates were then centrifuged at 10,000g for 10 min. The clear supernatants were transferred into a clean tube and frozen at -80°C until analysis. Before electrophoresis, lysates were thawed quickly at 37°C and transferred to wet ice. Protein concentrations were assessed using DC Biorad assay and adjusted between lysates using RIPA Buffer. Samples were prepared for western blotting by supplementing with NuPage 4X loading buffer and Beta-Mercaptoethanol 1/100 volume and heat-treating for 10min at 70 degrees.

Total protein lysates were separated by reducing SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 30 min at RT in 5% milk in Tris buffered saline + 0.1% Tween-20 (TBST) then incubated overnight at 4 °C in 4% BSA in TBST with the following antibodies: myc-tag clone 9E10 (#600-302) from Novus Biologicals, pY397-FAK (#3283), Phospho - FAK (Tyr925) (3284S), pY410-p130Cas (#4011), pY416-Src (#2101), p-Akt (#4058), p-Erk (#9102), PLC γ 1 (2822), pY783-PLC γ 1(2821), total Vegf-r2 (#2479), and phospho- PI3 Kinase p85 (Tyr458)/ p55 (Tyr199) (#4228) from Cell Signaling Technology; pY861-FAK (#PS1008), GAPDH (#MAB374) and total β 1 Integrin (#MAB1997) from Millipore; Tie-2 (AF762) from R&D Technologies, and Ang-2 (#PA5-27297), phospho-paxillin (44722g) from Invitrogen, pY576-FAK (44652G) from Biosource/Thermofisher and with HRP-conjugated sheep anti-mouse or goat anti-rabbit, anti-rat (Jackson Immunoresearch). Membranes were stripped and reprobed for total protein levels: FAK (#610088) from BD Transduction Laboratories, p130Cas (#05-469, Millipore), Src (#2110), Akt (#2966), and Erk (#9102) from Cell Signaling Technology and paxillin (610052) from BD. p190RhoGEF was a kind gift of David Schlaepfer (Moores Cancer Center, University of California, San Diego, La Jolla) (8E8E5 mAb, Mouse IgG1, 82% purity, Protein G-purified antibody, directed against aa 1502-1519 of the Rgnef protein).

Stress fibre alignment analysis

Endothelial cells were prepared and stained as described (see IF of endothelial cell lines). Phalloidin Z-stack images were acquired using a Confocal Spinning disk microscope with a 40x objective and sCMOS camera (Nikon). For further details of analysis see supplementary materials and methods.

Co-immunoprecipitation assay

Endothelial cells were lysed in ice-cold RIPA Buffer supplemented with 50mM NaF, 60mM β -glycerol phosphate, 10mM Na pyrophosphate, 2mM Na₃VO₄ and a dilution 1/100 of proteases inhibitors cocktail III (Roche) in distilled water. 1 μ g of anti-myc tag antibody (Novus Biologicals, #600-302) was bound to 50 μ l of protein G magnetic Dynabeads (Life technologies) prior to overnight incubation with 300 μ g of lysate at 4°C. Beads were subsequently washed prior to the addition of sample buffer containing 0.1 M DTT. Following this, immunoprecipitates were analysed on SDS page gels as outlined above.

RhoA activation G-LISA

65,000 endothelial cell lines were plated in pre-coated 6 wells plates and allowed to attach overnight; washed twice in PBS; serum-starved for 3 days in serum free medium (OPTI-MEM I + GlutaMAX, Gibco, Invitrogen, Paisley, UK); media was removed and replaced with OPTI-MEM containing 30ng/ml Vegf-a (Peprotech, 450-32) or Ang-2 (R&D Systems, 7186-AN-025) or the combination, for 10 and 40 minutes. G-LISA was then performed according to the manufacturer's instructions (BK 124 from Cytoskeleton, Inc.).

Small interfering RNA transfection

Endothelial cells were plated the day before transfection and left in Opti-MEM overnight. Lipofectamin transfection reagent was used according to manufacturer's instructions (Lipofectaminb RNAi/MAX- 13778- Invitrogen) with 20 μ M of either scrambled (Scr) (ON-TARGETplus Non-targeting Pool - D-001810-10-20- Dharmacon), or p190RhoGEF siRNA (Smartpool ON-TARGETplus Arhgef28 siRNA- L-040493-01-0005- Dharmacon) or Ang-2 (Smartpool ON-TARGETplus Angpt2 siRNA- L-040375-01-0005- Dharmacon) siRNAs. Cells were left in Opti-MEM for 5 hours post transfection and then supplemented with FBS (Sigma). The day after transfection cells were detached and plated for the essays (Proliferation and Spheroids). For conditioned medium experiments after transfection B16F0s cells were washed and medium changed for OPTI-MEM and left for 48h to produce conditioned medium that was then used in stimulation essays on endothelial cells.

Analysis of statistical significance

Prism software was used to statistically analyze data. Student t-test and Mann-Whitney test were employed when two individual groups were compared. One-way ANOVA was used when comparing with a control group (or selected columns) and Two-way ANOVA was used when comparing between different conditions or groups. Selected columns were compared with Sidak's post-test or Dunnet's post-test used when comparing to a control. Bonferroni's multiple comparison test was used when multiple comparisons were performed. Comparison of variance was performed, confirming no difference in variance between two samples nor interaction between mean and variance.

Results

Characterisation of $\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ and $\text{ECCre}^{+};\text{FAK}^{\text{Y861F/Y861F}}$ mice.

To investigate the *in vivo* effects of endothelial cell (EC)-specific homozygous non-phosphorylatable FAK-Y397F and FAK-Y861F, we developed $\text{Pdgfb-iCre}^{\text{ert}};\text{FAK}^{\text{fl/fl}};\text{R26FAK}^{\text{WT/WT}}$, $\text{Pdgfb-iCre}^{\text{ert}};\text{FAK}^{\text{fl/fl}};\text{R26FAK}^{\text{Y397F/Y397F}}$ or $\text{Pdgfb-iCre}^{\text{ert}};\text{FAK}^{\text{fl/fl}};\text{R26FAK}^{\text{Y861F/Y861F}}$ mice (**Supplementary Figure 1a and Supplementary Methods**). Tamoxifen treatment of $\text{Pdgfb-iCre}^{\text{ert}};\text{FAK}^{\text{fl/fl}};\text{R26FAK}^{\text{WT/WT}}$, $\text{Pdgfb-iCre}^{\text{ert}};\text{FAK}^{\text{fl/fl}};\text{R26FAK}^{\text{Y397F/Y397F}}$ or $\text{Pdgfb-iCre}^{\text{ert}};\text{FAK}^{\text{fl/fl}};\text{R26FAK}^{\text{Y861F/Y861F}}$ mice (24), generated $\text{ECCre}^{+};\text{FAK}^{\text{WT/WT}}$, $\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ and $\text{ECCre}^{+};\text{FAK}^{\text{Y861F/Y861F}}$ mice respectively (**Supplementary Figure 1b-d**). DNA sequencing confirmed mutation specificity in mouse genomic DNA (**Supplementary Figure 2a**). Tamoxifen-treatment induced endogenous endothelial cell mouse-FAK deletion and simultaneous myc-tagged knockin chicken-FAK RNA expression (**Supplementary Figure 2b**); and myc-tagged FAK-knockin protein expression in Cre-positive but not Cre-negative mouse lung ECs (**Supplementary Figure 2c and d**).

Tumour growth is inhibited in $\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ but not $\text{ECCre}^{+};\text{FAK}^{\text{Y861F/Y861F}}$ mice.

$\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ and $\text{ECCre}^{+};\text{FAK}^{\text{Y861F/Y861F}}$ and respective ECCre^{-} control mice were injected subcutaneously with melanoma (B16F0) or lung carcinoma cells (CMT19T). B16F0 and CMT19T tumour growth were reduced in $\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ mice (**Figure 1a**) but not $\text{ECCre}^{+};\text{FAK}^{\text{Y861F/Y861F}}$ mice (**Figure 1b**). Knockin efficiency was confirmed in: freshly isolated tumour endothelial cells that showed significant down-regulation of mouse FAK, and expression of mutant chicken FAK, (**Supplementary Figure 2e, f**) and myc-Tag expression in ECCre^{+} but not ECCre^{-} tumour blood vessels *in vivo* (**Supplementary Figure 2 g**). Endpoint tumour hypoxia was elevated significantly in tumours grown in $\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ mice (**Figure 1c**) but not affected in $\text{ECCre}^{-};\text{FAK}^{\text{Y861F/Y861F}}$ mice (**Figure 1d and Supplementary Figure 3a**) (33).

Tumour angiogenesis is significantly decreased in $\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ mice but only transiently in $\text{ECCre}^{+};\text{FAK}^{\text{Y861F/Y861F}}$ mice.

Given that tumour angiogenesis involves distinct molecular processes at initiation and maintenance we examined angiogenesis in early and end stage tumours. Blood vessel density was significantly reduced in early stage tumours from $\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ (**Figure 1e**) and $\text{ECCre}^{+};\text{FAK}^{\text{Y861F/Y861F}}$ (**Figure 1f and Supplementary Figure 3b**). In contrast, although reduced blood vessel density was still apparent in end stage B16F0 and CMT19T tumours grown in $\text{ECCre}^{+};\text{FAK}^{\text{Y397F/Y397F}}$ mice (**Figure 1g**), no significant differences in blood vessel density was observed in $\text{ECCre}^{+};\text{FAK}^{\text{Y861F/Y861F}}$ mice when compared with respective controls (**Figure 1h**).

Furthermore, in both early and end-stage tumours in $ECCre+;FAK^{Y397F/Y397F}$ or $ECCre+;FAK^{Y861F/Y861F}$ mice no significant differences in blood vessel perfusion, size, relative blood vessel leakage, or NG2-positive pericyte association were observed in these mutant mice (**Supplementary Figure 4 a-d**).

Overall, the loss of phosphorylation of either FAK-Y397 or FAK-Y861 reduces the initiation of tumour angiogenesis and appears to recover in $ECCre+;FAK^{Y861F/Y861F}$ mice only, suggesting that phosphorylation of FAK-Y861 or FAK-Y397 are involved in distinct molecular mechanisms in the control of sprouting angiogenesis.

Differential responses of $ECCre+;FAK^{Y397F/Y397F}$ and $ECCre+;FAK^{Y861F/Y861F}$ mice to Vegf-a +Ang-2.

Tumour angiogenesis is controlled by several different growth factors including Vegf-a, Ang-2, bFGF and PlGF (2). Angiogenic responses to Ang-2 involve essential co-stimulation with Vegf-a (3, 25-29). We hypothesised that the restoration of angiogenesis observed in end stage tumours in $ECCre+;FAK^{Y861F/Y861F}$, but not in $ECCre+;FAK^{Y397F/Y397F}$, mice could be a consequence of differential responses to tumour angiogenic growth factors. To test this, synthetic sponges were implanted subcutaneously into $ECCre+;FAK^{Y397F/Y397F}$, $ECCre+;FAK^{Y861F/Y861F}$ and respective $ECCre-$ control mice, and subsequently impregnated with growth factors to induce angiogenesis. Vegf-a, PlGF, bFGF and Vegf-a+Ang-2 stimulated angiogenesis were all reduced in $ECCre+;FAK^{Y397F/Y397F}$ when compared with $ECCre-;FAK^{Y397F/Y397F}$ mice (**Figure 2a and c, Supplementary Figure 5a**). In contrast, Vegf-a was reduced and PlGF- or bFGF-stimulated angiogenesis was not affected in $ECCre+;FAK^{Y861F/Y861F}$ mice. However, the combination of Vegf-a+Ang-2 significantly increased angiogenesis in $ECCre+;FAK^{Y861F/Y861F}$ mice, relative to control mice (**Figure 2 b and d, and Supplementary Figure 5b**).

In vitro we confirmed that Vegf-a and Vegf-a+Ang-2 treatment stimulated FAK-WT and FAK-Y861F but not FAK-Y397F EC sprout formation. Stimulation with Vegf-a alone, reduced sprouting response in FAK-Y861F EC spheroids compared with FAK-WT spheroids. Importantly, Vegf-a+Ang-2 stimulation enhanced FAK-Y861F spheroid sprouting compared with stimulation with Vegf-a alone, but no significant difference in sprouting was observed in WT-spheroids stimulated with Vegf-a vs. Vegf-a+Ang-2 (**Figure 2e**).

Ang-2-stimulated angiogenic responses have been described to be autocrine in ECs (3, 25-29). However, Ang-2 expression levels were similar between FAK-WT, FAK-Y397F and FAK-Y861F ECs (**Supplementary Figure 5c**), suggesting that EC-FAK mutations did not regulate autocrine EC Ang-2 levels. In contrast, B16F0 cells secrete high levels of Ang-2 compared with Vegf-a (**Figure 2f**). Importantly, a significant increase in Ang-2 transcript levels was detected in end- vs early-stage tumours (**Figure 2g**).

Together endothelial specific mutations in FAK-Y397F and FAK-Y861F trigger distinct angiogenic responses depending on the angiogenic stimuli, particularly to combined Vegf-a + Ang-2.

Non-phosphorylatable mutations in FAK-Y397 and FAK-Y861 differentially affect cell proliferation and migration responses to Vegf-a, Ang-2 and combined Vegf-a+Ang-2.

To further dissect the differential angiogenic responses that we observed, particularly to Vegf-a and combined Vegf-a+Ang-2, FAK-WT, FAK-Y397F and FAK-Y861F mutant EC lines were used in *in vitro* assays of proliferation and migration (30). FAK-WT ECs showed increased cell proliferation upon Vegf-a stimulation, but not Ang-2 alone or Vegf-a+Ang-2. FAK-Y397F ECs did not respond to Vegf-a, Ang-2 or Vegf-a+Ang-2. In contrast, FAK-Y861F ECs, while not responding to Vegf-a or Ang-2 alone, exhibited increased proliferation upon combination Vegf-a+Ang-2 stimulation (**Figure 3a**).

We next examined whether phosphorylation of FAK-Y397 or FAK-Y861 played a role in EC chemotactic migration towards Vegf-a, Ang-2 or combined Vegf-a+Ang-2 using the Dunn Chamber assay. Vegf-a-induced directional migration was apparent in WT ECs, but not in FAK-Y397F or FAK-Y861F ECs, whereas Ang-2 or Vegf-a+Ang-2 combination treatment did not promote directional migration in any of the cell lines tested (**Figure 3 b-d and Supplementary movies**). Migration speed was decreased in FAK-WT and FAK-Y397F cells stimulated with Ang-2 or Vegf-a+Ang-2 compared with Vegf-a alone treatment (**Figure 3e**). In contrast, FAK-Y861F mutant ECs cells, despite exhibiting reduced migration speed in Vegf-a, Ang-2 or combined Vegf-a+Ang-2 compared to both FAK-WT and FAK-Y397F cells, increased their migratory speed in Vegf-a+Ang-2 compared with Vegf-a alone (**Figure 3e**).

We next tested the effect of Ang-2 depletion in B16F0 cells on angiogenic responses *in vitro* and *in vivo*. FAK-Y861F vs FAK-WT endothelial cell spheroids cultures were exposed to conditioned media (CM) from Ang2-siRNA and Scr-siRNA transfected B16F0s *in vitro*. Ang-2 expression levels were reduced partially in Ang-2 siRNA depleted B16F0 cells 48h after transfection when CM was collected (**Supplementary Figure 6a**). FAK-Y861F endothelial sprouting was enhanced compared with FAK-WT endothelial cell sprouting when exposed to B16F0-scr siRNA CM (**Figure 3 f and g**). In contrast, CM from Ang-2 siRNA depleted B16F0s inhibited the enhanced sprouting of FAK-Y861F endothelial cells, but had no significant on FAK-WT endothelial cell sprouting (**Figure 3 f and g**).

In an *in vivo* matrigel plug angiogenic assay, matrigel containing either Ang-2-siRNA depleted B16F0 cell CM, Scr-siRNA transfected B16F0 CM or PBS was injected into *ECCre-;FAK^{Y861F/Y861F}* and *ECCre+;FAK^{Y861F/Y861F}* mice and blood vessel infiltration assessed 6 days post inoculation. Matrigel plugs were immunostained to detect endomucin positive vessel infiltration. CM from Scr-siRNA transfected B16F0 cells resulted in enhanced microvessel infiltration in matrigel plugs from *ECCre+;FAK^{Y861F/Y861F}* mice compared with *ECCre-*

;FAK^{Y861F/Y861F} mice. In contrast, CM from Ang-2-siRNA transfected B16F0 cells reduced the enhanced microvessel infiltration in ECCre+;FAK^{Y861F/Y861F} mice down to that observed in PBS controls (**Supplementary figure 6 b and c**).

Endothelial cell FAK-Y397F and FAK-Y861F mutations differentially regulate Vegfr-2, Tie-2 expression and β 1-integrin activation.

Ang-2 has dual roles during angiogenesis: in the absence of Vegf-a, Ang-2 binds Tie-2 promoting EC death and vessel regression (31); whilst in the presence of Vegf-a, Tie-2 is downregulated and the pro-angiogenic function of Ang-2 is mediated mainly through β 1-integrin signalling (25, 26, 28, 29). Thus, the balance between these receptors can influence the effect of Ang-2 on angiogenesis. We therefore examined the levels of Vegf-a and Ang-2 receptors (Vegfr-2 and Tie-2, respectively) in FAK-Y397F and FAK-Y861F ECs. Vegfr-2 expression levels were significantly decreased in FAK-Y397F, and more modestly decreased in FAK-Y861F ECs. In contrast, Tie-2 levels were increased in FAK-Y397F but decreased in FAK-Y861F ECs relative to FAK-WT ECs controls (**Figure 4a**). Although total β 1-integrin levels appeared unchanged (**Supplementary Figure 7a and b**), immunofluorescence analysis indicated that the localisation of activated β 1-integrin to focal contact sites at the end of stress fibres, was severely disrupted in FAK-Y397F ECs when cells were either unstimulated, or stimulated with Vegf-a or a combination with Vegf-a and Ang-2. In contrast, elevated levels of activated β 1-integrin were observed in focal contact sites of FAK-Y861F ECs, in both unstimulated and combined stimulation with Vegf-a + Ang-2 (**Figure 4b and c**). Notably, in FAK-WT ECs, only Vegf-a induced increased activation of β 1-integrin compared with unstimulated levels, whereas in FAK-Y861F ECs only combined Vegf-a+Ang-2 induced an increase activated β 1-integrin (**Figure 4b and c**). This observation correlated with a significant disruption in F-actin organisation and fibre alignment, assessed by image analysis of phalloidin staining, in FAK-Y397F ECs but not FAK-Y861F ECs under all the conditions tested (**Figure 4 c and d**).

The *in vivo* relevance of these observations was then investigated. End-stage B16F0 tumour sections from ECCre- controls, ECCre+;FAK^{Y397F/Y397F} and ECCre+;FAK^{Y861F/Y861F} mice were double-immunostained for the endothelial marker endomucin together with either Vegfr-2 or Tie-2. Corroborating our *in vitro* data, Vegfr-2 expression levels were reduced significantly in both ECCre+;FAK^{Y397F/Y397F} and ECCre+;FAK^{Y861F/Y861F} murine tumour blood vessels (**Figure 4e**). In contrast, Tie-2 expression was significantly enhanced in ECCre+;FAK^{Y397F/Y397F} mice, but significantly reduced in ECCre+;FAK^{Y861F/Y861F} mutant mice tumour blood vessels (**Figure 4f**).

This indicates that non-phosphorylatable mutations in FAK-Y397 or FAK-Y861 control an inside-out signal that regulates the expression of Vegfr-2, Tie-2 and β 1-integrin activation,

and that these changes correlate with distinct angiogenic responses to Vegf-a+Ang-2 or tumour cell derived conditioned medium.

Non-phosphorylatable mutations in FAK-Y397 and FAK-Y861 residues trigger distinct downstream signals.

We next sought to investigate how FAK-Y397F and FAK-Y861F mutations differentially affect downstream signalling and compare these responses with our previously published FAK-Kinase Dead (FAK-KD) mutant ECs (32). Western blot analysis of FAK-Y397F and FAK-KD ECs showed reduced FAK phosphorylation at Y397, Y577, Y861, and Y925. In contrast, the Y861F mutants showed reduced Y861 and Y925 phosphorylation, but no appreciable change in upstream Y397 or Y577 phosphorylation when compared with controls (**Figure 5a**). Moreover, double immunofluorescence analysis detection of the knock-in-FAK (FAK-Myc-tag) and p-Src showed reduced colocalization in FAK-Y397F and FAK-KD ECs, but not in FAK-Y861F ECs (**Figure 5 b and c**). Accordingly, immunoprecipitation of Myc-tagged FAK followed by western blotting revealed markedly less co-precipitation of FAK with Src, or p-Src, in FAK-Y397F and FAK-KD ECs compared with FAK-WT ECs. In contrast, FAK-Y861F mutant ECs, showed evidence of co-precipitating well with p-Src and Src (**Figure 5d**). Src phosphorylation dynamics were then analysed in FAK mutant primary ECs in response to Vegf-a + Ang-2. Stimulation with Vegf-a+Ang-2 tended to induced an increase in p-Src/Src levels in FAK-WT ECs. In contrast, Src phosphorylation kinetics showed no significant increase over time in FAK-Y397F or FAK-Y861F, possibly due to constitutively high basal p-Src levels and/or other FAK independent mechanisms (**Supplementary figure 8a**). Additionally, p-Src association with paxillin (focal adhesion marker) was disrupted in FAK-Y397F ECs but not in FAK-Y861F ECs (**Supplementary figure 8b**) despite no changes in paxillin-phosphorylation across the mutants in whole cell lysates (**Supplementary figure 8c**).

To further investigate other downstream signalling pathways that could be affected by Y397F and Y861F FAK mutations in endothelial cells, we performed western blot analysis using both immortalized and primary ECs. Phospho-p130Cas was reduced in FAK-Y397F ECs and increased in FAK-Y861F ECs (**Figure 5e and Supplementary Figure 8d**). Both phosphorylated-Plc γ , and total Plc γ were elevated in FAK-Y397F ECs when compared with WT and FAK-Y861F ECs (**Figure 5e**). These data suggest a possible compensation by Plc γ . PI3K p85-phosphorylation was also increased in the FAK-Y397F ECs, and not changed in FAK-Y861F while p55- phosphorylation was decreased in both FAK-Y397F and FAK-Y861F mutants when compared with WT (**Figure 5e and Supplementary Figure 8d**); Akt-phosphorylation was significantly decreased in FAK-Y397F ECs but not in FAK-Y861F ECs; and Erk-phosphorylation remained unchanged in FAK-Y397F and FAK-Y861F ECs (**Figure 5e**). Reverse Phase Protein Array (RPPA) analysis, designed for FAK signalling, additionally

identified other known FAK targets/signalling partners, such as PKC, GSK-3, AMPK, Smad1/5 and IRS that were downregulated in FAK-Y397F ECs to a greater extent than in FAK-Y861F ECs (**Supplementary Figure 8e**).

Together, these data suggest that the loss of Y397-phosphorylation, observed also in KD ECs, constitutively disrupts FAK-p-Src co-association and causes downregulation of other FAK-dependent downstream signalling pathways. In contrast, non-phosphorylatable Y861F mutation does not disrupt FAK-p-Src interaction but Vegf-a+Ang-2 stimulation is not sufficient to induce an increase in p-Src kinetic response above the constitutively elevated levels of p-Src, suggesting that the increased angiogenesis observed in FAK-Y861F ECs is independent of FAK/Src signaling.

Loss of endothelial cell FAK-Y397 and -Y861 phosphorylation affect RhoA activation and p190RhoGEF binding.

In order to further examine how Vegf-a and Ang-2 affect EC migration and endothelial sprouting differently, and since FAK has been implicated in the regulation of cell migration through RhoA activity (33-35), we investigated possible changes in RhoA activity. FAK-WT, FAK-Y397F and FAK-Y861F ECs were serum-starved, and either unstimulated or stimulated with Vegf-a+Ang-2 for 10 or 40 mins, and RhoA activity measured using an ELISA-based assay. RhoA activity was increased in FAK-WT cells following 10 mins of Vegf-a+Ang-2 stimulation, which reduced back to unstimulated levels at 40 minutes. This was in line with previous work demonstrating that growth factor stimulated migration is associated with transient RhoA activity (36). FAK-Y397F ECs showed no change in RhoA activity at either 10 or 40 mins of Vegf-a+Ang-2 stimulation. In contrast, FAK-Y861F ECs exhibited a time-dependent increase in active RhoA following Vegf-a+Ang-2 stimulation, and levels were significantly higher at 40 mins than in FAK-WT or FAK-Y397F cells (**Figure 6a**).

Moreover, since p190RhoGEF is a known RhoA activator and a specific C-terminal FAK binding partner (13, 37), we investigated p190RhoGEF binding to FAK. Immunoprecipitation of myc-tagged-FAK followed by western blotting for p190RhoGEF from protein lysates isolated from FAK-WT, FAK-Y397F and FAK-Y861F endothelial cells showed that FAK-Y861F ECs had higher levels of p190RhoGEF/FAK association when compared with FAK-Y397F and WT ECs (**Figure 6b**). This was further validated by proximity ligation assays where FAK/p190RhoGEF association was enhanced in FAK-Y861F, but not in FAK-Y397F compared with FAK-WT ECs (**Figure 6c**). The functional relevance of the enhanced FAK-Y861F/p190RhoGEF interaction was tested by siRNA depletion of p190RhoGEF in FAK-Y861F endothelial cells. In FAK-Y861F ECs, depletion of p190RhoGEF reversed the increase in phosphorylation of p130Cas, compared with WT ECs (**Figure 6d**). Furthermore, knock-down of p190RhoGEF also reverted Vegf-a+Ang-2 stimulated enhancement in cell

proliferation (**Figure 6e**) and sprouting in endothelial spheroid analysis (**Figure 6f**) in FAK-Y861F but not WT ECs.

Together, these data suggest that non-phosphorylatable FAK-Y861 mutation in ECs causes constitutively increased binding to p190RhoGEF, sustained RhoA activation and downstream p130Cas phosphorylation that is required for the enhanced endothelial cell proliferation and sprouting after Vegf-a+Ang-2 stimulation.

Discussion

Our data demonstrate that phosphorylation of EC-FAK-Y397 and Y861 act as critical regulators of tumour angiogenesis through distinct molecular mechanisms where FAK lies upstream of growth factor receptor regulation, signalling and responses (**Figure 7**).

Our results show that in $ECCre+;FAK^{Y397F/Y397F}$ mice, angiogenesis is constitutively disrupted, corresponding with reduced tumour growth. In contrast, although angiogenesis is reduced in early stage $ECCre+;FAK^{Y861F/Y861F}$ mice tumours, this defect is resolved in end stage tumours, resulting in no overall effect on tumour growth. Reduced levels of tumour angiogenesis have been previously shown in EC-FAK^{KO} mice (23). EC-FAK-kinase dead mice ($ECCre+;FAK^{KD/KD}$) (32) have a similar phenotype to ECFAK^{KO} mice in inhibiting tumour angiogenesis, enhancing tumour hypoxia and reducing tumour growth, all effects that we also observe in $ECCre+;FAK^{Y397F/Y397F}$ mice. However, unlike the $ECCre+;FAK^{KD/KD}$ mice, which presented a vascular leakage defect (32), the $ECCre+;FAK^{Y397F/Y397F}$ mutants did not. This could be explained by the fact that the kinase domain in $ECCre+;FAK^{Y397F/Y397F}$ mice is intact and thus may affect other downstream responses apart from the phosphorylation of FAK-Y397 in the control of vessel leakage (10). Importantly, although previous studies have begun to address the effect of FAK-Y397 deletion (exon 15) or FAK-kinase inactivation (20, 32, 38), ours is the first study to investigate the requirements of phosphorylation of ECFAK-Y397 and -Y861 in tumour angiogenesis *in vivo*.

We hypothesized that differential phosphorylation of FAK could regulate an inside-out signalling cascade affecting sensitivity to angiogenic growth factors. In fact, since the balance of tumour-derived angiogenic growth factor expression is known to temporally regulate tumour angiogenesis (2) we explored the effect of different pro-angiogenic stimuli that represent the angiogenic growth factor profile of early and later stage tumours. For example, we and others have shown that Ang-2 is upregulated in advanced stage tumours (5, 8, 9) providing a rationale to investigate how angiogenesis is apparently rescued in later stage tumours of $ECCre+;FAK^{Y861F/Y861F}$ mice.

Previous work has shown that in a Vegf-a/Ang-2-rich environment, Vegf-a causes down-regulation of Tie-2, the canonical Ang-2 receptor. This leaves Ang-2 free to bind and activate β 1-integrins and in turn activate downstream phosphorylation of FAK-Y397 to induce sprouting (26, 29). Our data indicate that loss of phosphorylation of FAK-Y397 is associated with a constitutive increase in Tie-2 expression levels but decreased Vegfr-2 levels with disrupted activation and clustering of β 1-integrins (27). This alteration in the profile of these surface receptors correlates with a constitutive loss of angiogenic responses in FAK-Y397F ECs *in vitro* and *in vivo*, which is in line with previous *in vitro* reports showing that deletion of FAK or FAK-Y397- leads to loss of integrin- and growth factor-stimulated downstream signalling, proliferation, migration and RhoA activation (20, 39-41). FAK-Y397F ECs have

drastically impaired FAK/Src interactions. In these cells Src is predominantly observed in intracellular puncta, potentially reflecting an increase in Src degradation, as reported previously in FAK-deficient cells (50). Although loss of FAK-Y397-phosphorylation is responsible for reducing FAK-Src complex formation, levels of p-Src are not reduced, possibly due to compensatory mechanisms independent of FAK (18). Indeed, phosphorylation of the p85 subunit of PI3 Kinase, which has been shown to be Src dependent and FAK-independent is also constitutively increased in FAK-Y397F (42). However, as expected other SH2 binding proteins that directly bind to p-Y397 domain such as PI3K p55 and subsequent downstream signalling via Akt are reduced (42). The loss of FAK-Src complex formation with the decrease in p-p130Cas may be responsible for the disorganised F-actin bundling observed in FAK-Y397F ECs (43).

In contrast, FAK-Y861F ECs showed constitutively reduced Vegfr-2, which may account for the reduced tumour angiogenesis in early stage tumours where tumour derived Ang-2 levels are lower. Additionally FAK-Y861F ECs have constitutively decreased Tie-2 expression and enhanced activation of β 1-integrin. The role of β 1-integrins in tumour angiogenesis has been extensively studied (44) and a better understanding of the co-operative roles of these integrins with other growth factor receptors are emerging (45). In fact, the reduction in Tie-2 is relevant in end stage tumours where enhanced Ang-2- β 1-integrin signalling leads to increased angiogenesis, in line with previous findings (26, 29). It has been shown that FAK binds to p190RhoGEF via its C-terminal FAT domain (13), and this interaction is critical for the GEF-dependent RhoA activation (33). Moreover, p190RhoGEF has also been shown to function as a scaffold, via a GEF-independent mechanism, to enhance FAK activation upon integrin clustering (46). Indeed our data suggests that upon Vegf+Ang-2 stimulation while FAK-Y861F mutant cells depend on p190RhoGEF for increased sprouting, FAK-WT cells do not. This suggests the increased complexing of p190RhoGEF with non-phosphorylatable FAK-Y861F is required for the enhanced responses to Vegf-a + Ang-2, consistent with published data showing that p190RhoGEF links FAK to integrins (13). Furthermore, we speculate that the GEF independent function of p190RhoGEF, acting upstream of FAK (37, 46) results in constitutively increased activation of β 1-integrin in FAK-Y861F cells, making them more responsive to Vegf-a+ Ang-2 or tumour cell derived conditioned medium. In FAK-Y861F ECs we also observed constitutively increased phosphorylation of p130Cas and sustained RhoA activation after Vegf-a + Ang-2 stimulation. Therefore, we postulate that, upon Vegf-a + Ang-2 stimulation, mimicking the growth factor environment in end stage tumours, FAK-Y861F binding to p190RhoGEF enhances p130Cas phosphorylation and sustains RhoA activation in maintaining F-actin binding. Previous studies have shown that p190RhoGEF-FAK complexing enhances β 1-integrin mediated downstream signalling (46). Together these molecular pathways lead to recovery of angiogenesis in an Ang-2 enriched tumour microenvironment.

This differential expression of surface receptors between FAK-Y397F and FAK-Y861F ECs indicate that the altered phosphorylation status of these mutants is sufficient to differentially control inside-out signalling. In the same way that FAK activity is required for integrin activation, regulating its recycling, degradation and surface expression (27, 47-49), it could be speculated that it also regulates Vegf-a and Ang-2 receptors. On the other hand, it has been demonstrated that adhesion mediated through integrins and FAK can also influence the expression of surface proteins (50), so the effect on Tie-2 and Vegfr-2 may also be a consequence of constitutively altered β 1-integrin activation.

Although beyond the scope of this study, it is of interest to consider that inhibitors of FAK kinase activity, which lead to a reduction in FAK-Y397-phosphorylation, are currently in clinical trials for the treatment of cancer (51). These inhibitors are reported to have effects on targeting both tumour cells and stromal cells such as ECs (52), but as long-term resistance to kinases in malignant cells is likely, the effects of inhibitors in affecting the tumour stroma become important. Allosteric FAK inhibitors that bind to distinct domain sites are also being developed, but are not specific only to FAK (53, 54). Although inhibitors and genetic manipulation may have different biological effects, our work predicts that inhibitors that target FAK-Y397-phosphorylation are likely to be effective anti-angiogenic drugs.

Author contributions

RP and NB contributed equally to the paper. RP acquired images representative for blood vessel quantitation in early and end stage B16F0 tumours; performed the early blood vessel analysis in CMTs tumours and the combined stimulation with Vegf-a+ Ang-2 in the sponge assays; Matrigel plug assays; Vegf-a and Ang-2 ELISAs and qPCRs for Ang-2 in early and end stage tumours; quantified the growth factor receptors in cells and tumours; carried out endothelial immunofluorescence and analysis; proliferation assays; receptors and Ang-2 western blots; prepared cells for migration and RhoA activation essays and samples of RPPA analysis; isolation and analysis of TECs; generation of lung endothelial primary preparations; developed the molecular mechanism with EC and JGE; generated the figures and led the manuscript writing with help from co-authors. NB characterised the mutant mice, did the majority of the *in vivo* tumour experiments (excepted for those listed elsewhere), performed and analysed sponge assays stimulated with Vegf-a, PlGF and bFGF, developed and characterised the mutant immortalised endothelial cells and carried out much of the *in vivo* histological blood vessel analysis, including BVD, perfusion, extravasation, pericyte coverage and hypoxia analysis. JGE did *in vitro* spheroid assays, acquired cell and Glut1 immunofluorescent images, performed PLA analysis, and analysed and filtered RPPA data. EC performed the co-immunoprecipitation and signalling western blot experiments. LR and PNG did some of the blood vessel quantitation. IF, AA and DL helped with the generation of the immortalised endothelial cells; VK, helped to characterise the knock-in system and AA, SB and BT generated the knockin mice. BS performed RPPA assay. MP carried out the Dunn chamber assays, RhoA assays and helped to design the mechanistic study. TI carried out the actin quantitation. KH-D initiated and supervised the project. RP, NB, JGE, EC and KH-D designed and wrote the manuscript.

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Figure legends

Figure 1. Constitutively reduced tumour angiogenesis in $ECCre+;FAK^{Y397F/Y397F}$, but transient reduced tumour angiogenesis in $ECCre+;FAK^{Y861F/Y861F}$ mice.

(a-b) Subcutaneous B16F0 or CMT19T tumour growth. **(c-d)** End-stage tumours immunostained to identify hypoxia (hypoxiprobe). **(e-f)** Early stage (day 7-8) B16F0 tumour blood vessel density in tumours from (a) and (b). **(g-h)** End stage B16F0 (days 17-21) and CMT19T (day 28) tumour blood vessel density. Each sample on scatter plots shows hypoxia levels (a and b) or blood vessel density (e-h) for an individual mouse. In (a) and (b) a representative experiment of a total of 3 independent experiments is shown. Means $\pm$ s.e.m across mice are given. *n*, 6-12 mice per cohort. **p*<0.05, ****p*<0.001, ns, no significant difference.

Figure 2. Loss of EC-FAK-397-phosphorylation reduces both Vegf-a and Vegf-a+Ang2-stimulated angiogenic responses whilst loss of EC-FAK-861-phosphorylation reduces Vegf-a but enhances Vegf-a+Ang2 responses.

(a-b) In vivo blood vessel density in synthetic sponges impregnated with PBS, Vegf-a or Vegf-a+Ang-2 implanted subcutaneously in mice. Each sample on scatter plots shows value from an individual sponge with means $\pm$ s.e.m across sponges. (*n*, 5-18 sponges per cohort). **(c-d)** Representative images of endomucin-stained sponge sections from mice treated with Vegf-a+Ang-2. **(e)** In vitro vessel sprouting from FAK-WT, FAK-Y397F and FAK-Y861F ECs spheroids treated with Vegf-a or Vegf-a+Ang2. Representative bright field and Phalloidin fluorescent (red) images of spheroids sprouts (*arrowheads*). *Bar Chart*, Cumulative sprout length (in pixels) was quantified. *n*, 10 spheroids were analysed per condition in 3 independent experiments. **(f)** Vegf-a and Ang-2 protein levels in B16F0 conditioned medium (CM) (pg/ml) assessed by ELISA; Positive control, mouse/rat Vegf-a and Ang-2 protein solution, respectively. *n*, 2 independent experiments. **(g)** Ang-2 mRNA levels from early (days 7-8) and end (days 17-21) stage B16F0 tumours (*n*, 3 tumours per stage). **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. ns, no significant difference.

Figure 3. FAK-Y397F and FAK-Y861F mutations reduce Vegf-a-stimulated proliferation and migration speed, whilst only FAK861F mutation enhances responses to combined Vegf-a+Ang2 or tumour-derived conditioned medium.

(a) FAK-WT, FAK-Y397F and FAK-Y861F immortalised MLECs were stimulated with either Vegf-a, Ang-2 or Vegf-a+Ang-2. Proliferation was measured (MTS assay). Bar charts show mean cell proliferation $\pm$ s.e.m; *n*, 6-8 technical replicates per condition of two independent experiments. **(b-d)** Directional migration of FAK-WT, FAK-Y397F and FAK-Y861F ECs towards a gradient of Vegf-a **(b)**, Ang-2 **(c)** and Vegf-a+Ang-2 **(d)** in Dunn chamber analyses. Rose plots represent directional chemotaxis. Green area denotes statistically significant directional movement according to Rayleigh statistical testing for clustering in a unidirectional

manner. Trackplots represent examples of tracks of multiple cells from each genotype normalised to the same origin. **(e)** Migration speed from b-d (mm/min +s.e.m.). FAK-WT n=70 cells; FAK-Y397F n=60 cells and FAK-Y861F n=55 cells pooled from 3 independent experiments. **(f)** FAK-WT and FAK-Y861F ECs spheroids were stimulated with conditioned medium from B16F0 cells either transfected with scrambled siRNA or Ang-2 siRNA. Representative bright field and Phalloidin fluorescent (red) images of spheroids sprouts (*arrowheads*). **(g)** Cumulative sprout length (in pixels) was quantified. n, 10 spheroids per condition in 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ns, no significant difference.

Figure 4. Loss of endothelial cell FAK-Y397-phosphorylation reduces Vegfr-2 and β 1-integrin activation with an increase in Tie-2 levels, whilst loss of FAK-Y861 phosphorylation results in reduced Vegfr-2 and Tie-2 expression but enhanced β 1-integrin-activation .

(a) Representative Western blot analysis of Vegfr-2 and Tie-2 in FAK-WT, FAK-Y397F and FAK-Y861F immortalized MLEC lysates. Densitometric quantification is shown with bars representing mean values +s.e.m; n, 1 or 2 technical replicates of 2 independent cell lysates of each genotype, to have at least 3-4 replicates. **(b)** Activated β 1-integrin in the cell periphery was quantified by mean pixel intensity using Image J. n, 20 cells per genotype. Box-and-whisker represent maximum and minimum values. **(c)** Immunodetection of activated β 1-integrin (using Ab clone 9EG7) (green) and phalloidin (pink) in FAK-WT, FAK-Y397F and FAK-Y861F ECs with *arrowheads* showing positive colocalization. **(d)** Actin stress fiber alignment analysis (average coherence in main alignment direction of the F-actin). n, 30-40 cells per genotype. Box-and-whisker represent maximum and minimum values. **(e)** Representative images of B16F0 end stage tumour sections double-immunostained for endomucin and either Vegfr-2 or Tie-2. **(f)** Vegfr-2 and Tie-2 expression levels were quantified and normalized to the endomucin expression and expressed as endothelial Vegfr-2 and Tie-2 positive areas, relative to the respective controls. White arrows represent positive double endomucin- Vegfr-2/Tie-2 areas. n, 5 tumours per cohort. Error bars represent +/- sem. *p<0.05, ***p<0.001, ****p<0.0001 and ns, no significant difference.

Figure 5. Characterization of FAK phosphorylation and downstream signalling changes in FAK-Y397F and FAK-Y861F ECs.

(a) Representative Western blot analysis of p-Y397F-FAK, p-Y577F-FAK, p-Y861-FAK, p-Y925F-FAK, total FAK and GAPDH as loading control in FAK-WT, FAK-Y397F, FAK-Y861F and FAK-KD immortalized MLEC lysates. *Bar chart*, Densitometric quantification from two independent experiments. **(b)** Representative confocal images of FAK-WT, FAK-Y397F, FAK-Y861F and FAK-KD ECs immunostained for Myc-Tag (identifying FAK) (red) and p-Src (green). Black and white, single channel images. **(c)** Cell peripheral Myc-tag- p-Src

colocalization was quantified using pixel correlation and calculated as a Pearson's correlation coefficient (R value) using colocalization plugin in Image J. A value of 1 corresponds to 100% colocalization. n, 20 cells per genotype. Box-and-whisker represent maximum and minimum values. **(d)** Pulldown of Myc-Tag (identifying knockin chicken FAK) in immortalized MLEC lysates subjected to analysis by western blot where total FAK and p-Src interaction was analysed. **(e)** Representative WB analysis of p-p130Cas and total p130Cas, pY783-Plc γ , total Plc- γ , p-Akt and total Akt, p-Erk and total Erk, phospho-PI3K subunits p85 and p55 in FAK-WT, FAK-Y397F, FAK-Y861F immortalized MLEC lysates. Bar charts show mean densitometric analysis $\pm$ s.e.m from 3 independent experiments. * $p < 0.05$, **** $p < 0.0001$. ns, no significant difference.

Figure 6. Increased Vegf-a+Ang-2-stimulated angiogenic responses of FAK-Y861F endothelial cells are regulated by elevated FAK-p190RhoGEF binding.

(a) Relative RhoA activation in FAK-WT, FAK-Y397F and FAK-Y861F MLECs, either unstimulated, or stimulated for 10 and 40 minutes with Vegf-a+Ang-2. n, 6 technical replicates of 2 independent experiments. **(b)** Pulldown of Myc-Tag (FAK) in immortalized MLEC lysates were subjected to western blot analysis, where total FAK and p190RhoGEF interaction was analysed. **(c)** Representative confocal sections of proximity ligation assay (PLA) for Myc-Tag and p190RhoGEF. Red dots are indicative of proximity between the two proteins. Quantification of the number of PLA dots/ cell. Error bars represent $\pm$ sem. **(d)** Representative western blot of scrambled (siScr) or siRhoGEF p190RhoGEF in FAK-WT and FAK-Y861F ECs showing reduced p-P130Cas after knockdown of p190RhoGEF in Y861F cells but not WT. **(e)** FAK-WT and FAK-Y861F immortalised MLECs were transfected with either siScr and siRhoGEF and stimulated with Vegf-a+ Ang-2. Proliferation was measured (MTS assay). Bar charts show mean cell proliferation $\pm$ s.e.m, normalized to unstimulated levels (1). n, 16-24 technical replicates per condition of 2 independent experiments. **(f)** FAK-WT and FAK-Y861F ECs spheroids were stimulated with Vegf-a + Ang-2 stimulation. Representative bright field and Phalloidin fluorescent (red) images of spheroids sprouts (arrowheads). Fold change cumulative sprout length. n, 10 spheroids per condition of 2 independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ns, no significant difference.

Figure 7. Schematic representation of the effect of loss of EC FAK phosphorylation at Y397 and Y861 on angiogenic processes.

(a) Schematic representation of changes described in early versus end stage tumour development. In early stage tumours, mimicking an environment with Vegf-a + low Ang-2 levels resulted in reduced tumour angiogenesis in both $ECCre^{+};FAK^{Y397F/Y397F}$ and $ECCre^{+};FAK^{Y861F/Y861F}$ mice. In end stage tumours, where tumour cells secretion of Ang-2 increases, mimicking an environment where Vegf-a + Ang-2 are present we have observed reduced tumour angiogenic responses in $ECCre^{+};FAK^{Y397F/Y397F}$ mice. In contrast, in a Vegf-a + high Ang-2 environment, $ECCre^{+};FAK^{Y861F/Y861F}$ showed rescued tumour angiogenic

responses. **(b)** Schematic representation of constitutive molecular changes observed in FAK-Y397F and FAK-Y861F ECs. Loss of phosphorylation at FAK-Y397 is sufficient to decrease phosphorylation at FAK-Y577, -Y861 and -Y925. This mutation causes disrupted FAK-Src interactions and localization to focal adhesion sites, decreases downstream signalling (p. e PI3K p55/Akt and p-P130Cas), reduces RhoA activation, and causes F-actin disorganization. This reduced actin organisation correlates with reduced β 1-integrin activation, reduction in Vegfr-2 levels with an increase in Tie-2 levels. Together this results in constitutively reduced tumour angiogenesis. In contrast, loss of phosphorylation at FAK-Y861 does not affect phosphorylation of FAK-Y397 or -Y577, but does reduce FAK-Y925 phosphorylation. It is also sufficient to maintain FAK-Src association, sustain binding of p190RhoGEF and thus p-p130Cas and RhoA activation. Reduced Vegfr-2 levels, and particularly Tie-2 levels, allow for enhanced activation of β 1-integrin. Overall this reduces angiogenesis in early stage tumour, where Ang-2 levels are relatively low and restores angiogenesis, in end stage tumours, where tumour-derived Ang-2 levels increase. *Blunted lines* indicate inhibition; *arrows* indicate activation; *dotted line* indicates disrupted interaction and *triple line* indicates increased binding.

Figure 2

a

b

FAK-Y397F

Reduced pro-angiogenic effects of Vegf-a-Vegfr-2 and enhanced vascular destabilization effects of Ang2-Tie-2 causes constitutively reduced tumour angiogenesis

FAK-Y861F

Reduced pro-angiogenic effects of Vegf-a-Vegfr-2 causes reduced early stage tumour angiogenesis

Enhanced pro-angiogenic effects of Ang2-integrin β 1 and reduced vascular destabilization via Ang2-Tie-2 drives restored end stage angiogenesis