Supplementary Information

Differential Expression of VEGFA Isoforms Regulates Metastasis and Response to Anti-VEGFA Therapy in Sarcoma

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Supplementary Materials and Methods

Development of the vector pCLIIP for transposase generation of stable cell lines. The basis of the piggyBac transposon vector pCLIIP was pCYL50 (kindly provided by Allan Bradley, Sanger Institute, UK) and was constructed in four steps. In an attempt to reduce variability in transgene expression levels following the stable integration of the final construct in transfected cells, we positioned two HS4 insulator elements (each element comprising two 250bp HS4 core elements, Felsenfeld lab) either side of a custom polylinker. This was achieved by cloning the first HS4 element as a EcoRI-BamHI fragment into EcoRI and BamHI sites in the pCYL50 polylinker. We then inserted our own polylinker into the EcoRI site of pCYL50 by annealing the following complimentary oligos.

Sense; $^{5'}$AATTGAATTCAGGAGATCTACGCGTCAATTGGGCCGGCC$^{3'}$

Antisense; $^{5'}$AATTGGCCGGCCCAATTGACGCGTAGATCTCCTGAATTC$^{3'}$

This linker destroyed the recipient EcoRI site used in the cloning step, but introduced a new EcoRI site in a new position. The new sites introduced by this polylinker, 5' to 3', were EcoRI, BglII, MluI, MfeI and FseI. The second HS4 element was then introduced as an EcoRI-BamHI fragment as before, but this time introduced into the new EcoRI-BglII sites. We next cloned a puromycin expression cassette (PGK-puro-pA) into a unique NheI site of our vector (from the original pCYL50 polylinker) by PCR amplifying the cassette from another vector (pBS.DAT.LSL, kindly provided by the Tuveson lab, Cold Spring Harbour,

US) using primers with NheI sites on their 5' ends. (Note: all PCR cloning steps performed with Phusion Taq polymerase, New England Biolabs).

prPGK-puro-pA 5'; TGAGCTAGCCTCGAAATTCTACCGGGT

prPGK-puro-pA 3'; TGAGCTAGCGACCAGCTTCTGATGGAA

This completed the pCLIIP backbone (p**C**yl50, **L**inker, **I**nsulator, **I**nsulator, **P**uro). To make pCLIIP-C-LS we cloned a CAGGS promoter as a 1.7kb PCR product with EcoRI ends into the unique MfeI site in the polylinker of pCLIIP.

prCAGGS 5'; $^{5'}$GATGAATTCCGACATTGATTATTGACT$^{3'}$

prCAGGS 3'; $^{5'}$GCCGAATTCCTTTGCCAAAATGATGAG$^{3'}$

Finally, we cloned a Luciferase2-E2A-mStrawberry-pA fragment (previously constructed by S Lyons, unpublished) following PCR amplification using primers with FseI ends into the unique FseI site in the polylinker of our vector.

prLuc2-FP 5'; $^{5'}$AATTAAGGCCGGCCACCATGGAAGATGCCA$^{3'}$

prLuc2-FP 3'; $^{5'}$AATTAAGGCCGGCCTCAGAAGCCATAGAGCC$^{3'}$

All steps were verified by sequencing.

Preparation of Luciferase2 and mStrawberry (LS) expressing fibrosarcomas. SV40 and hRAS transformed embryonic fibroblasts (1) expressing single VEGFA isoforms through genetic alteration of the endogenous *vegfa* gene were prepared

as follows: 1 x 10^5 of each fibrosarcoma cell line were seeded overnight in 6 well plates before the medium was changed. 1 μg of pmPB (kindly provided by Allan Bradley, Sanger Institute, UK) and pCLIIP-C-LS were transfected using Lipofectamine 2000 (Invitrogen) overnight before the cells were expanded into T175 flasks. mStrawberry positive cells were put through 3 rounds of FACS using a BD FACS ARIA. The resulting cell lines were > 95% positive for mStrawberry. Bioluminescence output per cell (p/s/cell) was measured by plating serial dilutions of the fs-LS cell lines in black 96 well plates in 100 μl complete medium and adding 50 μl D-Luciferin (0.6 mg/ml) immediately before measurement on an IVIS Lumina II (Perkin Elmer). Bioluminescence output was 2.8, 3.6, 3.2 and 4.6 x 10^3 p s^{-1} $cell^{-1}$ for fs120-LS, fs164-LS, fs188-LS and fsWT-LS respectively. No significant difference in tumor growth was observed between the parental lines and the LS derivatives (data not shown). Cell lines were validated in house at passage 8 and then used to a maximum of passage 30 (typically 2-3 months). Correct VEGFA isoform expression, SV40 and hRAS expression levels, maintenance of cell morphology at low density and population doubling times were confirmed as we described before (Kanthou et al, PloS). The cells were also free of mycoplasma.

VEGFR1-ΔTKD mice. C57Bl6/J VEGFR1-ΔTKD mice, where exon 17 containing amino acids 787-830 of the tyrosine kinase domain were replaced by a *neo* gene cassette, resulting in generation of a membrane-associated form of VEGFR1 with deletion (Δ) of the tyrosine kinase domain (TKD) (2) were generously provided by Dr KH Plate (Johan Goethe University Medical School, Germany) and Prof. M Shibuya (Tokyo Medical and Dental University, Japan). These were crossed to

generate C57Bl6/SCID mice (from Charles River; see main text) homozygous for wild type *vegfr1* or *vegfr1-ΔTKD* (VEGFR1-WT or VEGFR1-ΔTKD mice respectively).

In vivo models of tumor growth, metastasis and treatment. The majority of experiments were conducted in C57Bl6/SCID mice (VEGFR1-WT or VEGFR1-ΔTKD mice). Experiments examining the effect of B20-4.1.1 and cediranib were conducted in CB-17-SCID mice (from Charles River, see main text). Levels of metastasis from subcutaneous tumors or on intravenous injection were not found to be significantly different between the two strains of mice (unpublished data). Subcutaneous tumors were established by injection of 1×10^6 cells in 50 μl PBS. Tumor volume (*V*) was measured using calipers where $V = 0.52 \times d1 \times d2 \times d3$ and *d1*, *d2* and *d3* are the three orthogonal tumor diameters in mm. For treatment with control IgG (BE5) or the anti-VEGF antibody B20-4.1.1 (kindly provided by Genentech Inc. San Francisco, USA), tumors were grown to 200 mm^3 in volume and mice were then treated with 5 mg/kg antibody in saline twice weekly by intraperitoneal injection. For experimental models of lung metastasis, mice were injected iv via the tail vein with 5×10^4 cells in 100 μl PBS. In experimental models of metastasis where initial survival of cells in the lung was examined, mice were treated with B20-4.1.1 at 5 mg/kg in saline ip 48h before injection of cells and then on the same day as cells were injected iv. For treatment with cediranib (Seleck Chem) in the same model mice were dosed at 48h by gavage at 6 mg/kg in 1% w/v Tween80 in H_2O before injection of cells iv and again on the day of cell injection.

Analysis of luciferase activity in lung homogenates. Mice were killed and lungs excised and snap frozen before storage at -80°C until needed. Frozen lungs were thawed on ice before cutting into small fragments and adding to ice-cold luciferase activity cell lysis buffer (Promega). Lungs were then homogenized using an IKA Ultra-Turrax before centrifugation at 300 x g to remove tissue debris. 50 μl of supernatant containing lung homogenate was added to each well of a 96 well black microtitre plate. Immediately before reading, 50 μl of luciferase activity buffer containing D-luciferase (Promega) was added and luminescence (p/s) measured on an IVIS Lumina II (Perkin Elmer). To correct for variability between homogenate protein content, protein concentration was estimated using a BCA assay (Thermofisher) and luciferase activity expressed as p/s/μg protein.

Bioluminescence measurements in live mice and of excised lung lobes. Mice were anaesthetized with 2-2.5% isofluorane in O_2 and injected ip with 15 mg/kg D-luciferin (Perkin Elmer) in PBS that had been sterilized through a 0.2 μm filter. Bioluminescence measurement of the thoracic region of mice was made 10 min after luciferase injection using an IVIS Lumina II (Perkin Elmer). Bioluminescence output (p/s/cm^2/sr) of the thoracic region was measured using the Living Image™ software (Perkin Elmer) after drawing an appropriate region of interest (ROI). For imaging of excised lung lobes on necropsy, mice were anaesthetized and injected with D-luciferin as described above, before being killed. Lungs were excised and dissected into individual lobes, placed on a square of black plastic and imaged in the IVIS Lumina II for analysis of bioluminescent output. Bioluminescence output (p/s/cm^2/sr) of the individual lobes was

measured after drawing an ROI around each lobe with the final measurement the sum of all five lobes.

Preparation of cell conditioned medium for injection into mice. Fs120 cells were seeded into T175 flasks and grown to 70-80% confluence before the cells were washed 3 x with antibiotic and serum free medium. As fs120 tumors have significant levels of necrosis that could indicate significant areas of hypoxia (3) and this is a major regulator of VEGFA and PlGF2 expression, cells were incubated in 10 ml of antibiotic and serum free medium in separate flasks in either atmospheric O_2 or 1% O_2 for 24 h before the medium was combined. The cell-conditioned medium was centrifuged at 10,000 x g in a micro-centrifuge before the supernatant was filtered through a 0.2 μm membrane filter and stored at -80°C. cell conditioned medium or control medium was injected ip once per day for 6 days at 200μl/20g mouse before iv injection of 5 x 10^4 fs120-LS cells to examine cell survival in the lungs as described previously.

Immunohistochemistry and analysis of CD31 mean vascular density. Tumors were formalin fixed and paraffin wax embedded (FFPE) and sectioned to 5 μm thickness before mounting on slides. Staining for CD31 (Rat anti-mouse DIA 310, Dianova) was performed as previously described by Akerman et al (3). Mean vascular density per high powered field of view was calculated by counting $CD31^{+VE}$ vessels in viable tumor regions at 20 x magnification, with the mean taken of 10-20 fields of view per tumor, in a blinded study. Images were taken with a Nikon Optiphot-2 microscope equipped with a Nikon DS-Fi1 camera.

Immunofluorescence staining and analysis. For immunofluorescence staining of SV40, CD11b, Ki67, pS139-γH2AX, CD31, Laminin or Collagen-I, tumors or lungs were embedded in OCT and frozen before 7-10 μm frozen sections cut using a cryostat. Sections were thawed, dried and fixed in methanol/acetone at -20°C for 20 min before re-hydrating in PBS. Sections were blocked in PBS, 10% v/v goat serum for 1 h before incubating in PBS with 10% v/v goat serum and anti-CD31 (BD Biosciences BD557355, 1:100), anti-SV40 (SantaCruz SC147 or SC20800 1:400), Ki67 (Abcam AB15580 1:400), anti- pS139 γH2AX (Cell Signaling Technologies 2577, 1:800), anti-laminin (Abcam AB11575, 1:100) or anti-collagen-I (Millipore AB765P, 1:200). Sections were then washed 3 x 15 min in PBS before incubation with the appropriate secondary antibody conjugated with Alexa488 or Alexa555 dyes at 1:500 (Invitrogen) in PBS and 10% v/v goat serum for 1 h before washing in PBS and mounting in Vectorshield + DAPI (Vector Labs).

For CD11b staining, lungs sections were dried and fixed as described before, then blocked in PBS, 10% v/v horse serum, 0.1% w/v triton X-100 (Thermofisher) for 1 h. After blocking sections were incubated with anti-CD11b directly conjugated to Alexa488 (Biolegend 101217 1:100) for 1 h before washing 3 x 15 min in PBS and mounting in Vectorshield + DAPI. Images were taken with an Olympus BX61 at 20 x magnification from 5 random fields of view of alveolar regions. $CD11b^{+VE}$ cells were counted blind, and corrected for area of tissue present using the tissue auto-fluorescence detected with the FITC filter in the field of view using the threshold and measure functions of Image J. Where the relative location of CD11b cells to fibrosarcoma cells was to be determined lung sections were stained for both CD11b and SV40. The number of CD11b positive

(+VE) cells within a 20 μm distance from the center of an SV40^{+VE} cell were counted for between 7 and 25 cells per biological replicate. For normal lung tissue, the clustering of CD11b positive cells around points of a chalky grid was measured as a randomized point control.

Perivascular cell coverage was analysed using FFPE embedded material as this improved their visualization. Vessels were detected using CD34 as the anti-CD31 antibodies we had previously characterized by IHC did not work using immunofluorescence. CD31^{+VE} vessels showed a very high degree of overlap with CD34 on immunofluorescence staining in frozen sections and virtually the same vessel number when counted per high-powered field of view (Supplementary Fig S1). For co-staining of CD34^{+VE} blood vessels and αSMA^{+VE} perivascular cells, FFPE sections were cut as described above followed antigen recovery after de-waxing in citrate buffer, pH6 by heating in a 900 W microwave at full power for 5 min followed by 5 min at 50% power. Sections were then treated with 100 mM glycine, 0.1% w/v Triton X-100, pH 7.4 for 5 min before blocking in 10% v/v horse serum in PBS for 30 min. Rat anti-CD34 (Serotech, MCA1825GA) and rabbit anti-αSMA (Abcam, Ab5694) were used at 1:200 in PBS overnight at 4°C. Sections were washed in PBS before incubating with Alexafluor-555 anti-rat and Alexafluor-488 anti-rabbit at 1:500 in PBS for 1 h at room temperature. Slides were then washed and mounted in Prolong Gold with DAPI. Images were taken with a 20 x objective on an Olympus BX61 microscope equipped with appropriate filters from 5-6 fields of view containing at least 1 CD34^{+VE} vessel, with the αSMA channel image taken blind. CD34^{+VE} vessels were counted as pericyte positive if they had flattened, elongated cells positive for αSMA staining in close proximity to the CD34^{+VE} vessel.

For confocal imaging of fibrosarcoma cells and CD31 within lung tissues, frozen lung sections were stained with the antibodies described above and the appropriate Alexa488/555 conjugated secondary antibodies before mounting. Images were captured using a laser scanning Zeiss LSM510 NLO Inverted confocal microscope with a 40 x 1.3 NA oil immersion lens with an optimal pinhole setting for capturing optical slices.

Transwell migration assays. 8 μm pore size cell culture transwell inserts for 24 well plates (BD biosciences) were coated with 100 μl of 200 μg/ml laminin or 100 μg/ml collagen or in sodium carbonate buffer (Sigma) pH 9.8 for 16 h at 37°C on the upper surface. Cells were serum starved for 6 h in serum free growth medium before seeding 2.5 x 10^4 cells in 500 μl in the transwell insert. 800 μl DMEM containing 10% v/v FBS was added to the well of the plate as a chemo-attractant before incubating for 24 h at 37°C. 10 μg/ml B20-4.1.1 or control IgG BE5 (Genentech Inc. San Francisco, USA) were added in both the upper and lower chambers. Cells remaining in the upper chamber of the transwell were removed with a cotton bud before the inserts were fixed in methanol for 5 min at -20°C and left to dry. The membranes from each insert were removed and mounted on Menzel-Gläser Superfrost® glass slides (Thermo-fisher Scientific) in Vectashield® mounting medium (Vector labs) containing DAPI and sealed with a coverglass. Slides were stored in -2°C to -8°C in the dark. Images of 4 separate fields of view per membrane for 4 transwells were taken with an inverted microscope (DMI4000b, Leica) using a 10 x 0.3 NA objective (Nikon) and the LASAF software for data capture. Image analysis and cell counting was

performed using Image J. The experiment was performed 3 times to ensure data obtained were representative.

Single cell migration using live video microscopy. Optimal coating concentrations of tissue culture plastic was first determined by measuring adhesion of cells on increasing concentrations of collagen-I and laminin in 96 well plates to determine the point at which maximal adhesion was reached. For live video experiments, wells of 12 well plates were coated with 1 ml per well of 20 μg/ml of collagen-1 in 0.02N acetic acid or 5μg/ml of laminin-1 in PBS at room temperature for 2 h. Coated wells were then washed with 1 ml of PBS before rinsing with culture medium. 5 x 10^3 of either fs120 or fs188 were seeded in 1 ml of culture medium per well of a 12-well plate overnight in culture medium as described previously. Prior to transfer to the microscope for live image capture, the medium was exchanged with fresh medium containing either 20 μg/ml of the anti-VEGFA antibody B20-4.1.1 or the control IgG BE5. The cells were allowed to equilibrate within the humidified chamber of the AF6000 time-lapse microscope (Leica, Milton Keynes) at 37°C, 5% CO_2 for 2 h before commencing image capture using a 20 x objective and the LAS AF software (Leica, Milton Keynes). 2 positions per well were used across three separate well and images for each position were taken every 15 mins. The duration of imaging was 5 h. The Manual Tracking Tool, produced by Image J, was used to track the path of cell migration. The migration distance and speed were then calculated with the ImageJ Chemotaxis Tool (Ibidi) using the coordinates generated by the manual-tracking tool. A minimum of 30 cells was tracked within each experimental condition. Tracks from cells undergoing mitosis during imaging were excluded.

Western blotting of tumor lysates. 10 mm^3 pieces of tumors were cut into smaller pieces before homogenisation in an IKA Ultraturrax homogeniser using ice cold radioimmunoprecipitation assay (RIPA) buffer (Thermofisher) containing Complete™ proteinase inhibitor cocktail (Roche) and phosphatase inhibitor cocktail II at 2 x the recommended concentration. Homogenates were passed sequentially through 19 – 30 gauge needles on ice before centrifugation at 10,000 x g for 5 min at 4°C to remove debris. Protein concentration of the supernatant (lysate) was measured using a micro BCA assay kit (Thermofisher). 5 μg of each tumor lysates was resolved on 8% w/v reducing (laminin) or non-reducing (collagen-I) Tris-Glycine SDS page gels before transferring to nitroceullulose membranes using a semi-dry transfer system (Invitrogen). Membranes were blocked with 5% w/v fat free milk powder (Marvel) in Tris-buffered Saline (TBS) with 0.1% Tween-20 v/v for 30 min. Antibodies were used according to the manufacturers' instructions. Anti-collagen-I was purchased from Millipore (AB765P, 1:500) and pan-laminin and GAPDH antibodies from Abcam (AB11575, and AB9484 both 1:5000). Detection was with the appropriate HRP-conjugated secondary antibody (Dako) and enhanced chemiluminescence substrate (ECL) and film. Densitometry of collagen-I (aggregates, dimers and monomers) and laminin for each tumor lysates was performed using the Image studio Lite software (LiCor, Cambridge). This was normalised for loading after the membrane was stripped using Restore western blot stripping solution (Thermofisher, UK), re-probed with anti-GAPDH. Data was also normalised against a common reference sample included with each

separate gel made from multiple pooled tumor lysates to correct for small differences in ECL intensity across separate Western Blots.

Angiogenesis array analysis of 3D multicellular spheroids. Cells were seeded at 1 x 10^4 cells/well in 150 µl growth medium containing 5% v/v methocellulose solution in u-bottom 96 well suspension culture plates (Greiner). After 5 days, a further 100 µl of spheroid culture medium was added to each well. Plates were incubated for an additional 2 days. Spheroids were harvested by pipetting cells from the 96 well plate followed by centrifugation for 5 min at 800 x g and washed in ice cold PBS. The spheroid pellet was then incubated in 250 µl RIPA buffer containing the proteinase and phosphatase inhibitor cocktail described above for 10 min on ice. The spheroid pellet was then lysed and dissociated by syringing sequentially through 21 to 30 gauge needles and debris removed by centrifugation at 10,000 x g in a microfuge at 4°C before protein concentration was measured using a micro BCA assay kit (Thermofisher). 200 µg lysate was incubated on the Proteome Profiler Mouse Angiogenesis Array kit following the manufacturers' instructions (R&D Systems). Western Sure® Premium Chemiluminescent (LiCor, Cambridge) substrate was used to detect spots on a C-digit® blot scanner (LiCor, Cambridge) with the images being acquired using Image Studio software (LiCor, Cambridge). The signal and background was adjusted using Image studio software (LiCor, Cambridge) and normalised using the control spots on each membrane.

Statistical analysis. Data was plotted and analysed using GraphPad Prism 6.0e (GraphPad Prism Software Inc). Pair-wise analysis of data with a normal

distribution was made using an unpaired Student's t-test with a Welch's correction. Pair-wise comparisons for data that did not have a normal distribution were made using a Mann-Whitney test. For analysis of three or more groups, data having a normal distribution was analyzed using ANOVA followed by Sidak's multiple comparison test for key pair-wise comparisons. For data that did not follow a normal distribution, a Kruskal-Wallis test followed by a Dunn's multiple comparison test for key pair-wise comparisons was used. Kaplan Meier analysis was used to analyze differences in time to reach a pre-determined humane end point as a surrogate for survival. For subcutaneous tumor growth this was set at ≥1000 mm^3 and for live bioluminescence imaging this was set to ≥1x10^7 p/s within the thoracic region. In some experiments, data were combined from more than one cohort of mice to reach statistical power and to ensure similar data was obtained from separate litters. Where data were obtained from more than one cohort, data was normalized against the mean of a common parameter before statistical analysis, as indicated in the figure legends. Experiments comparing VEGFR1-WT and VEGFR1-ΔTKD mice were conducted using littermates from the same cohort.

References

1. Tozer GM, Akerman S, Cross NA, et al. Blood vessel maturation and response to vascular-disrupting therapy in single vascular endothelial growth factor-A isoform-producing tumors. *Cancer Res.* 2008;68(7):2301-11.

2. Hiratsuka S, Minowa O, Kuno J, Noda T, and Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad of Sci* 1998;95(16):9349-54.
3. Akerman S, Fisher M, Daniel RA, et al. Influence of soluble or matrix-bound isoforms of vascular endothelial growth factor-A on tumor response to vascular-targeted strategies. *Int J Cancer* 2013:113(11):2563-76.