Materials and Methods

Genetic Reduction of VEGFR2 Rescues Aberrant Angiogenesis Caused by Epsins Deficiency

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Running Title: Reducing VEGFR2 Corrects Abnormal Angiogenesis

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Materials and Methods

Antibodies and reagents:
Mouse anti-EEA1 and anti-GAPDH were from Santa Cruz (sc-6415; sc-166545). Rat anti-CD31 was from BD Pharmingen (550274). Rabbit anti-VEGFR2, anti-phospho-VEGFR2 (pY1175), rabbit anti-PLCγ, rabbit anti-phospho-PLCγ, rabbit anti-Akt, rabbit anti-phospho-Akt, mouse anti-ERK and mouse anti-phospho-ERK antibodies were from Cell Signaling Technology. Rabbit anti-epsin 1 was obtained as previously described.[1, 2] Secondary antibodies were all obtained from Invitrogen. VEGF-A was from R&D systems (293-VE/CF). EdU was from Invitrogen (A10044). Alexa Fluor 594 Azide was from Invitrogen (C10339). BrdU and 4-hydroxytamoxifen were from Sigma (B5002; H6278). Matrigel was from BD Biosciences (354230). Basic laboratory reagents were all from Sigma.

Generation of the constitutive EC-DKO and conditional EC-iDKO mice:
Epn2$^{-/-}$ mice were obtained as described.[3] Conditional *Epn1*$^{fl/fl}$ mice were obtained as described (Figure IA in the online Data Supplement).[4] *Epn1*$^{fl/fl}$ mice were mated with *Epn2*$^{-/-}$ mice to generate *Epn1*$^{fl/fl}$*; Epn2*$^{-/-}$ mice. Constitutive endothelial cell-specific DKO (EC-DKO) mice were obtained by crossing *Epn1*$^{fl/fl}$*; Epn2*$^{-/-}$ mice with *Tie2-Cre* deleter mice obtained from Jackson Laboratories (8863) (Figure IB in the online Data Supplement).[5] Tamoxifen-inducible endothelial cell-specific DKO mice (EC-iDKO) were obtained by crossing *Epn1*$^{fl/fl}$*; Epn2*$^{-/-}$ mice with *iCDH5 Cre* deleter mice obtained as described (Figure IC in the online Data Supplement).[6, 7] To induce postnatal deletion, we administered 4-hydroxytamoxifen (150 µg per 30 g of body weight) by i.p. injection into ten-week-old mice. Injections were performed once per day for 5-7 consecutive days, followed by a 5-7 day resting period. All mice were bred on C57BL/6J background.

Generation of the EC-iDKO-Flk$^{fl/+}$ mice:
Flk$^{fl/+}$ mice were obtained from Jackson Laboratories (18977).[8] Tamoxifen-inducible endothelial cell-specific epsin DKO-Flk$^{fl/+}$ mice (EC-iDKO- Flk$^{fl/+}$) were obtained by crossing *Epn1*$^{fl/fl}$*; Epn2*$^{-/-}$ mice with the *Flk*$^{fl/+}$ mice generating *Epn1*$^{fl/fl}$*;Epn2*$^{-/-}$*; Flk*$^{fl/+}$ mice (Figure 1D in the online Data Supplement), then subsequently crossing with *iCDH5 deleter Cre* mice (Figure 1E in the online Data Supplement). Tamoxifen-induced deletion was done as described above. All mice were bred on C57BL/6J background.

Generation of the Streptozotocin (STZ)-induced Diabetic mice:
Male mice, aged 2-3 months, were injected i.p. with 50 mg/kg STZ in citrate buffer, pH 4.5 for 5 consecutive days. One week post-injection, blood glucose was measured by applying tail blood to a glucometer as described previously.[9] Mice with blood glucose levels >300 mg/dL were considered diabetic and used for the wound healing assays.

Primary Mouse Endothelial Cell Isolation and Culture:
Primary mouse endothelial cells (MEC) isolation from brains was performed as described previously.[10] Specifically, meninges of three week-old brains isolated from WT, EC-iDKO *or* EC-iDKO-Flk$^{fl/+}$ mice were carefully removed and the gray matter minced and then digested with 1 mg/mL collagenase (Gibco; 17100-017) in DMEM (Mediatech; 10-013-CM) for 1 h at 37°C. Microvessels were separated by centrifugation through 20% BSA/DMEM (1000 x g, 20 min). Microvessels from the pellet were further digested with 1 mg/mL collagenase-dispase (Roche) in DMEM for 15 min at 37°C. Microvessel MEC clusters were washed twice with DMEM before planting on 0.2% gelatin-coated plates. Cultures were maintained in DMEM supplemented with 20% FBS (Biowest; S01520HI) and 1 ng/mL bFGF (Roche). MECs were treated for 48 h at 37°C with 5 μM of 4-hydroxytamoxifen (dissolved in ethanol) diluted culture medium followed by incubation for an additional two days without 4-hydroxytamoxifen. Freshly isolated primary MEC were used for all experiments without further passages.

Immunohistochemistry and immunofluorescence:

Embryos, tissue samples, and ECs were processed for immunostaining as described below. All samples were imaged using an Olympus IX81 Spinning Disc Confocal Microscope and Hamamatsu Orca-R^2 Monochrome Digital Camera C1D600. Immunostaining was quantified using the companion Slidebook 5.0 software.[4, 11]

Embryo Whole Mount Staining: Embryos were harvested at E10 and fixed overnight in cold 4% paraformaldehyde in PBS. After washing with ice-cold PBS, embryos were whole-mounted or paraffin embedded, sectioned, and processed for staining.[12] For blocking and permeabilization, sections were incubated in PBS containing 1% BSA, 0.5% Triton X-100 overnight at 4°C. Samples were stained with anti-CD31 overnight at 4°C. Biotinylated goat anti-rat IgG was absorbed to remove anti-murine IgG, and then streptavidin-conjugated to fluorescein isothiocyanate was used for secondary staining. Samples were mounted with Vectashield and analyzed as described above.

E10 Hind Brain: Embryos were harvested at E10 and fixed in cold 4% paraformaldehyde. Hindbrain was harvested and processed for immunofluorescent staining. For blocking and permeabilization, sections were incubated in PBS containing 1% BSA, 0.5% Triton X-100 overnight at 4°C. Samples were stained with anti-CD31 overnight at 4°C. After washing, donkey anti-rat Alex Fluor 488 secondary antibody was used for secondary staining. Samples were mounted and analyzed as described above.

Embryonic Skin: Embryos were harvested at E10 and fixed in 4% paraformaldehyde. Skin was harvested and processed for staining with anti-CD31 and donkey anti-rat Alex Fluor 488 secondary antibody as described above.

Embryonic Intestine: Embryos were harvested at E10 and fixed in 4% paraformaldehyde. Small intestines was harvested and processed for staining with anti-CD31 and donkey anti-rat Alex Fluor 488 secondary antibody as described above.

In vitro EC staining: ECs cultured on gelatin-coated coverslips were stimulated with 50 ng/mL of VEGF-A at 37°C for 0, 1, or 5 min. Cells were fixed in 4% paraformaldehyde then permeabilized and blocked in PBS containing 5% donkey serum, 3% BSA and 0.3% Triton X-100. Cells were immunostained with rabbit anti-VEGFR2, rabbit anti-phosphorylated VEGFR2, and mouse anti-EEA1 for 2 h at room temperature. Cells were washed then incubated with respective fluorescent secondary antibodies for 1 h at room temperature. Cells were mounted using PermaFluor (ThermoScientific; TA-030-FM) and analyzed as described above.

***In vitro* EdU staining:**

Primary ECs were grown on gelatin-coated coverslips until they reached 50% confluence. EdU was added to the culture media at 10 µM for 16 h.[13] After labeling, cells were washed with PBS, fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. EdU incorporation was detected by incubating cells with 100 µM fluorescent azide diluted in 100 mM Tris, 0.5 mM $CuSO_4$ and 50 mM ascorbic acid. Cells were counterstained with 0.2 µg/mL DAPI then processed for imaging as described above.

Scratch and network/tube formation assays:

Monolayer EC scratch assay. Monolayer EC wound assays were performed as described.[10] ECs were cultured in 0.5% FBS/DMEM overnight and subjected to “wound injury” assay with a plastic pipette tip. Cells were then plated with fresh media supplemented with or without 50 ng/mL VEGF-A and further cultured for 12 h. Quantification of wound distance at 12 h was performed using NIH Image J software.

EC network/tube formation. EC network/tube formation in Matrigel was performed as described.[10] First, 0.2 mL matrigel was added to each well of 24-well plate and incubated at 37°C for 1 h. ECs were serum starved overnight then plated on Matrigel (2 x 10^3 cells). After 30 min, fresh medium supplemented with or without 50 ng/mL VEGF-A was added and cells were incubated for additional 16 h. Quantification of tube formation at 16 h was performed using NIH ImageJ software.

Plasma Membrane isolation from MECs:
Isolated and cultured MECs were stimulated with 50 ng/mL of VEGF-A at 37°C for the time indicated followed by homogenization in TES Buffer (255 mM TES, 10 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM Na^3VO^4) and centrifuged at 16,000 x g for 15 min. Pellets were further homogenized in TES Buffer, layered on a 1.15 M sucrose cushion, and centrifuged at 100,000 x g for 70 min. Plasma membrane interface was isolated and pelleted by centrifugation at 48,000 x g for 45 min. Plasma membrane pellets were resuspended and subjected to SDS-PAGE and immunoblot according to standard protocols.

***In vivo* BrdU staining:**
WT, EC-iDKO or EC-iDKO-Flk$^{fl/+}$ pups were injected i.p. with 5 mg/kg (body weight) of 4-hydroxytamoxifen (10 mg/ml of 4-hydroxytamoxifen resuspended in 20% of ethanol and 80% of DMSO) per day from postnatal day 1 (P1) to P3. Pups were euthanized at P6 after i.p. injection of BrdU (100 mg/kg body weight) for 3 hr. Small intestines were harvested. Small intestines were embedded and paraffin sections were stained with anti-CD31 and anti-BrdU antibodies (Invitrogen).

***In vivo* Wound Healing Assay:**
Under anesthesia, mice were wounded using a 4-mm-diameter full-thickness dorsal skin punch biopsy. Wounds were then photographed daily over 9 days after injury, and wound area was measured using NIH ImageJ software. The wounds were biopsied to include the margin of normal surrounding skin and snap-frozen in Tissue-Tek OCT (Sakura) and fixed in cold 4% paraformaldehyde overnight. Cryosections were processed for immunofluorescent staining as described above.

***In vivo* Matrigel Angiogenesis Assay:**
Matrigel plug assay was performed as described previously.[14] Briefly, mice were injected s.c. with 500 µL of unpolymerized growth factor-reduced Matrigel mixed with either PBS or VEGF (200 ng/mL). Mice were euthanized after 5 days and the plugs removed. Plugs were photographed, formalin-fixed, paraffin embedded and processed for immunostaining with anti-CD31 as described above.

***In vivo* Tumor Xenograft Assay:**
Lewis Lung Carcinoma (LLC) cells obtained from ATCC were injected s.c. (1x10^6 cells/tumor) in twelve-week-old WT, EC-iDKO, or EC-iDKO-FLK$^{fl/+}$ mice.[4] Time of tumor appearance was estimated and tumor growth was monitored in three groups of mice by measuring tumor size with digital calipers. Tumors more than 2 mm in diameter were recognized as positive. Tumor volumes were calculated based on the formula: 0.5326 (length [mm] x width [mm]^2). Tumors were harvested, photographed, paraffin embedded and processed for immunofluorescence.

Miscellaneous Procedures:
SDS/PAGE and immunoblotting were performed according to standard procedures.

Study Approval:
All animal studies were performed in compliance with institutional guidelines and were approved by Institutional Animal Care and Use Committee (IACUC), Oklahoma Medical Research Foundation, Oklahoma City, OK.

Statistical Analysis:

Data was shown as ± S.E.M. Data were analyzed by the two-tailed student's t test or ANOVA, where appropriate. The Wilcoxon signed-rank test was used to compare data that did not satisfy the student's t test or ANOVA. P value ≤ 0.05 was considered significant.

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