Journal of Visualized Experiments

A patient-derived Xenograft Model for Venous Malformation

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61501R1
Full Title:	A patient-derived Xenograft Model for Venous Malformation
Section/Category:	JoVE Biology
Keywords:	Venous malformation; Xenograft; Endothelial cells; TIE2; PIK3CA; Vascular Lesion
Corresponding Author:	Elisa Boscolo University of Cincinnati Medical Center Cincinnati, Ohio UNITED STATES
Corresponding Author's Institution:	University of Cincinnati Medical Center
Corresponding Author E-Mail:	Elisa.Boscolo@cchmc.org
Order of Authors:	Sandra Schrenk
	Jillian Goines
	Elisa Boscolo
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	cincinnati, ohio, us

TITLE:

A Patient-Derived Xenograft Model for Venous Malformation

AUTHORS AND AFFILIATIONS:

Sandra Schrenk[1]*, Jillian Goines[1]* and Elisa Boscolo[1,2]

*These authors contributed equally

[1]Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center and [2]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA

Corresponding author:

Elisa Boscolo (elisa.boscolo@cchmc.org)

Email addresses of co-authors:

Sandra Schrenk (sandra.schrenk@cchmc.org)

Jillian Goines (jillian.goines@cchmc.org)

KEYWORDS:

Venous malformation, xenograft, endothelial cells, TIE2, PIK3CA, vascular lesion

SUMMARY:

We present a detailed protocol to generate a murine xenograft model of venous malformation. This model is based on the subcutaneous injection of patient-derived endothelial cells containing hyper-activating TIE2 and/or PIK3CA gene mutations. Xenograft lesions closely recapitulate the histopathological features of VM patient tissue.

ABSTRACT:

Venous malformation (VM) is a vascular anomaly that arises from impaired development of the venous network resulting in dilated and often dysfunctional veins. The purpose of this article is to carefully describe the establishment of a murine xenograft model that mimics human VM and is able to reflect patient heterogeneity. Hyper-activating non-inherited (somatic) *TEK* (TIE2) and *PIK3CA* mutations in endothelial cells (EC) have been identified as the main drivers of pathological vessel enlargement in VM. The following protocol describes the isolation, purification and expansion of patient-derived EC expressing mutant TIE2 and/or PIK3CA. These EC are injected subcutaneously into the back of immunodeficient athymic mice to generate ectatic vascular channels. Lesions generated with TIE2 or PIK3CA-mutant EC are visibly vascularized within 7–9 days of injection and recapitulate histopathological features of VM patient tissue. This VM xenograft model provides a reliable platform to investigate the cellular and molecular mechanisms driving VM formation and expansion. In addition, this model will be instrumental for translational studies testing the efficacy of novel drug candidates in preventing the abnormal vessel enlargement seen in human VM.

INTRODUCTION:

Defects in the development of the vasculature are the underlying cause of many diseases including venous malformation (VM). VM is a congenital disease characterized by abnormal morphogenesis and expansion of veins[1]. Important studies on VM tissue and endothelial cells (EC) have identified gain-of-function mutations in two genes: *TEK*, which encodes the tyrosine kinase receptor TIE2, and *PIK3CA*, which encodes the p110α (catalytic subunit) isoform of PI3-kinase (PI3K)[2–5]. These somatic mutations result in ligand-independent hyper-activation of key angiogenic/growth signaling pathways, including PI3K/AKT, thereby resulting in dilated ectatic veins[3]. Despite these important genetic discoveries, the subsequent cellular and molecular mechanisms triggering abnormal angiogenesis and the formation of enlarged vascular channels are still not fully understood.

During normal and pathological angiogenesis, new vessels sprout from a pre-existing vascular network and EC undergo a sequence of important cellular processes including proliferation, migration, extracellular matrix (ECM) remodeling and lumen formation[6]. Two- and three-dimensional (2D/3D) in vitro cultures of EC are important tools to investigate each of these cellular properties individually. Nevertheless, there is a clear demand for a mouse model recapitulating pathological vessel enlargement within the host microenvironment while providing an efficient platform for preclinical evaluation of targeted drugs for translational research.

Up to date, a transgenic murine model of VM associated with TIE2 gain-of-function mutations has not been reported. Current transgenic VM mouse models rely on the ubiquitous or tissue-restricted expression of the activating mutation PIK3CA p.H1047R[3, 5]. These transgenic animals provide significant insight into whole-body or tissue-specific effects of this hotspot PIK3CA mutation. The limitation of these models is the formation of a highly pathological vascular network resulting in early lethality. Thus, these mouse models do not fully reflect the sporadic occurrence of mutational events and localized nature of VM pathology.

On the contrary, patient-derived xenograft models are based on the transplantation or injection of pathological tissue or cells derived from patients into immunodeficient mice[7]. Xenograft models are a powerful tool to broaden knowledge about disease development and discovery of novel therapeutic agents[8]. In addition, using patient-derived cells allows scientists to recapitulate mutation heterogeneity to study the spectrum of patient phenotypes.

Here, we describe a protocol where patient-derived VM EC which express a mutant constitutively-active form of TIE2 and/or PIK3CA are injected subcutaneously in the back of athymic nude mice. Injected vascular cells are suspended in an ECM framework in order to promote angiogenesis as described in previous vascular xenograft models[9–11]. These VM EC undergo significant morphogenesis and generate enlarged, perfused pathological vessels in the absence of supporting cells. The described xenograft model of VM provides an efficient platform for preclinical evaluation of targeted drugs for their ability to inhibit uncontrolled lumen expansion.

PROTOCOL:

Patient tissue samples were obtained from participants after informed consent from the Collection and Repository of Tissue Samples and Data from Patients with Tumors and Vascular Anomalies under an approved Institutional Review Board (IRB) per institutional policies at Cincinnati Children's Hospital Medical Center (CCHMC), Cancer and Blood Disease Institute and with approval of the Committee on Clinical Investigation. All animal procedures described below have been reviewed and approved by the CCHMC Institutional Animal Care and Use Committee.

1. Preparation of materials and stock solutions

1.1. Preparation of complete endothelial cell growth medium (EGM)

1.1.1. Supplement endothelial basal medium (EBM) with the following growth factors present in the kit (see **Table of Materials**): human Fibroblast Growth Factor-Beta (hFGF-β), vascular endothelial growth factor (VEGF), Long Arg3 Insulin-Like Growth Factor- I (R^3-IGF-I), ascorbic acid, epidermal growth factor (EGF), gentamycin sulphate and amphotericin-B, and heparin. Add 1% Penicillin/Streptomycin/L-Glutamine (PSG) solution and 20% fetal bovine serum (FBS).

NOTE: We do not recommend adding hydrocortisone.

1.1.2. Sterile filter the solution under a laminar flow hood through a 0.2 µm bottle top filter into an autoclaved glass bottle and aliquot media into 50 mL conical tubes and store at 4 °C up to a week or at -20 °C for up to one year.

1.2. Prepare collagenase A stock solution

1.2.1. Prepare 50 mg/mL collagenase A stock solution in 1x phosphate buffer saline (PBS).

1.2.2. Sterile filter the solution under a laminar flow hood with 0.2 µm filter and syringe, and store 100 µL aliquots at -20 °C.

1.3. Prepare tissue collection medium (Buffer A)

1.3.1. Prepare a Ca^{2+}/Mg^{2+} stock solution by adding 0.927 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) and 1 g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) to 500 mL of distilled water. Sterile filter the solution through a 0.2 µm bottle top filter into an autoclaved glass bottle and store at room temperature (RT).

1.3.2. Supplement Dulbecco's Modified Eagle Medium (DMEM) with 10% Ca^{2+}/Mg^{2+} solution and 2% FBS.

1.3.3. Sterile filter the solution under a laminar flow hood with 0.2 µm filter and syringe and store aliquots of 5 mL at -20 °C.

1.4. Fibronectin coating of tissue culture plates

1.4.1. Prepare coating buffer (Buffer B) by dissolving 5.3 g of sodium carbonate (Na_2CO_3; 0.1 M) in 500 mL of deionized water. Adjust the pH of the buffer to 9.4 using 1 M hydrochloric acid (HCl). Filter under a laminar flow hood through a 0.2 µm bottle top filter into an autoclaved glass bottle and store at RT.

1.4.2. For coating, pipette 2 mL/5 mL/10 mL of Buffer B per 60 mm/100 mm/145 mm tissue culture plate, respectively. Add 1 µg/cm^2 of human plasma fibronectin purified protein solution and gently distribute the liquid onto the plate.

1.4.3. Incubate plate at 37 °C, 5% CO_2 for 20 min.

1.4.4. Aspirate Buffer B and wash the plate with PBS prior to culturing cells.

2. Isolation of endothelial cells from VM patient tissue

2.1. Isolation of EC from solid VM tissue

NOTE: VM tissue is resected by debulking surgery[12, 13] under an IRB approved protocol.

2.1.1. Wash VM tissue (tissue sample weight typically ranges between 0.5 g and 1.5 g) in 5% PSG in PBS.

2.1.2. Transfer tissue to a 100 mm cell culture dish, mince tissue sample into small pieces using sterile surgical dissection tools, and transfer into a 50 mL conical tube.

2.1.3. Add 100 µL of collagenase A stock solution to 5 mL of Buffer A for a final concentration of 1 mg/mL, then add this to the tissue and digest the minced tissue at 37 °C for 30 min while shaking contents every 5 min.

2.1.4. Carefully grind the digested tissue at RT using a 6 mm, smooth-surface pestle grinder within the 50 mL conical tube.

2.1.5. Continue to carefully grind digested tissue using a pestle while adding 5 mL of cold PBS supplemented with 0.5% bovine serum albumin (BSA) and 2% PSG. Repeat this step four times.

2.1.6. Filter the solution through a 100 µm cell strainer into a 50 mL conical tube to remove tissue fragments.

2.1.7. Centrifuge cell suspension for 5 min at 400 x *g* at RT. Proceed to step 2.3.

2.2. Isolation of VM EC from lesional blood obtained from patient sclerotherapy

2.2.1. Obtain human VM lesional blood from sclerotherapy under an IRB approved protocol.

2.2.2. Dilute lesional blood (sample volume typically ranges between 0.5 mL and 5 mL) in PBS to a final volume of 40 mL.

2.2.3. Centrifuge cell suspension for 5 min at 200 x *g* at RT.

2.3. Initial cell plating

2.3.1. Discard supernatant and resuspend the single-cell pellet in 1 mL of EGM.

2.3.2. Add 9 mL of complete EGM onto fibronectin-coated (1 µg/cm²) 100 mm plates and seed 1 mL of cell suspension.

2.3.3. Incubate cells at 37 °C in a humidified 5% CO_2 atmosphere.

2.3.4. Every other day remove 2 mL of media and add 2 mL of sterile filtered FBS.

2.3.5. Once cell cultures reach a confluency of 40–50% change the medium to complete EGM. It typically takes between 2–3 weeks for the cells to reach this confluency.

2.3.6. Observe daily for the appearance of EC colonies. They can be recognized by their typical "cobblestone-like" morphology (**Figure 1A**). Between 3–5 EC-colonies appear in each sample.

2.3.7. Change the medium every other day for another 5–7 days until individual EC colonies start to touch one another.

2.4. Manual isolation of individual EC colonies

2.4.1. To harvest EC colonies, wash the plate with 5 mL of PBS and manually aspirate with a serological pipette.

2.4.2. Take the plate to a microscope and circle the locations of multiple EC colonies using a marking pen both on lid and bottom before returning it to laminar flow hood.

2.4.3. Detach EC colonies by pipetting 50 µL of 0.05% trypsin-EDTA solution on the marked areas.

2.4.4. Using a small cell scraper or a pipette tip, gently scrape cells from plate.

2.4.5. Tilt the plate to the nearest edge, and rinse with 1 mL of EGM to neutralize per marked area and collect cells.

2.4.6. Count the number of cells using a hemocytometer or automated cell counter.

2.4.7. Plate the collected EC colonies at a density of 1 x 10^4 cells/cm^2 onto fibronectin-coated (1 µg/cm^2) cell culture dishes containing fresh EGM. The next day, change medium to complete EGM.

2.4.8. Change the medium to complete EGM every other day for 2–3 weeks until cells reach 80% confluency.

2.4.9. Trypsinize EC with 2 mL of pre-warmed 0.05% trypsin-EDTA per 100 mm dish at 37 °C for 2 min and neutralize trypsin by adding 4 mL of EGM.

2.4.10. Collect the cell suspension into one 15 mL conical tube and centrifuge at 400 x *g* at RT for 5 min. Aspirate the supernatant and resuspend cells in 2 mL of EGM.

2.4.11. Count the number of cells using a hemocytometer or automated cell counter. Typical cell numbers obtained are 1 x 10^6 cells per 60 mm cell culture plate or 2 x 10^6 cells per 100 mm tissue culture plate.

2.4.12. Pellet the cells by centrifugation at 400 x *g* at RT for 5 min and aspirate the supernatant. Proceed to step 3.2.1.

3. Endothelial cell selection and expansion

3.1. Preparation of anti-CD31-conjugated magnetic beads

3.1.1. Vortex the vial containing the anti-CD31-conjugated magnetic beads for 30 s. The volume of beads required is 20 µL/2x10^6 cells.

3.1.2. Wash 20 µL of anti-CD31-conjugated magnetic beads with 1 mL of wash solution containing 0.1% BSA in PBS in a 1.5 mL microcentrifuge tube.

3.1.3. Place the microcentrifuge tube in a cell isolation magnet for 1 min.

3.1.4. Aspirate the supernatant and repeat the washing step.

3.2. Endothelial cell purification and plating

3.2.1. Resuspend cell pellet obtained in 2.4.11 in 500 µL of wash solution containing 0.1% BSA in PBS.

3.2.2. Add cell solution to microcentrifuge tube containing magnetic beads and resuspend thoroughly.

3.2.3. Incubate for 20 min at 4 °C with gentle tilting.

3.2.4. Add 500 µL of 0.1% BSA in PBS and mix well.

3.2.5. Place the tube on a cell isolation magnet for 1 min.

3.2.6. Gently aspirate all of the liquid, which contains the CD31-negative fraction of cells without touching the beads.

3.2.7. Wash the bead pellet containing the CD31-positive cell fraction with 1 mL of 0.1% BSA in PBS and repeat magnetic separation.

3.2.8. Repeat washing and magnetic separation step for a minimum of 3 times to purify CD31-positive cell fraction.

3.2.9. Resuspend purified endothelial cells into a 15 mL conical tube and spin down for 5 min at 400 x *g* at RT.

3.2.10. Remove supernatant and resuspend cell pellet in 1 mL of EGM.

3.2.11. Add 9 mL of complete EGM onto fibronectin-coated (1 µg/cm²) 100 mm plates and seed 1 mL of cell suspension. Note that some magnetic beads are still attached to the cells in this initial seeding step. Most beads wash away during cell expansion, but small number of beads may persist in early passages.

3.2.12. Incubate cells at 37 °C in a humidified 5% CO_2 atmosphere.

3.2.13. Change the medium every other day until cells reach 80 % confluency.

3.3. Endothelial cell expansion

3.3.1. Once cells reach 80 % confluency, detach with 2 mL of 0.05% trypsin-EDTA per 100 mm dish at 37 °C for 2 min.

3.3.2. Neutralize trypsin by adding 4 mL of EGM and collect cells into one 15 mL conical tube.

3.3.3. Centrifuge at 400 x *g* at RT for 5 min.

3.3.4. Aspirate the supernatant, resuspend cells in 2 mL of EGM, and count the number of cells with a hemocytometer or by automated cell counting.

3.3.5. Seed cells at a density of 1 x 10^4 cells/cm² into 145 mm fibronectin-coated (1 µg/cm²) tissue culture plates.

3.3.6. Continue to passage cells until the desired cell number has been met. Consider that a confluent 145 mm tissue culture plate contains about 8–9 x 10^6 cells and the number of cells

needed is 2.5 x 10^6 cells per injection. Only cells between passage 3 and 8 should be considered for the xenograft.

4. VM patient-derived xenograft protocol

NOTE: In this protocol we use 5–6 week old, male immunodeficient, athymic nude Foxn1^{nu} mice. All animal procedures must be approved by the Institutional Animal Care and Use Committee (IACUC).

4.1. Preparation of materials on the day before injection

4.1.1. Pre-chill syringes, needles, and pipet tips in -20 °C freezer overnight.

4.1.2. Slowly thaw the basement membrane extracellular matrix (BMEM) overnight on ice bucket placed at 4 °C to avoid increased viscosity of the gel.

4.2. Preparation of cell suspension for injection

4.2.1. Trypsinize EC with 5 mL of pre-warmed 0.05% trypsin-EDTA per 145 mm dish at 37 °C for 2 min.

4.2.2. Neutralize trypsin by adding 5 mL of EGM and collect cells into one 15 mL conical tube.

4.2.3. Centrifuge at 400 x *g* at RT for 5 min.

4.2.4. Aspirate the supernatant, resuspend cells in 3 mL of EGM, and count the number of cells using a hemocytometer or automated cell counter.

4.2.5. Determine the total number of cells that are needed for all planned injections.

NOTE: The recommended number of cells is 2.5 x 10^6 cells per injection. A total of 2 injections are typically performed in each mouse for technical duplicates. It is necessary to calculate a 10% excess of cell number to account for loss during transfer into the syringe.

4.2.6. Transfer the volume containing the calculated cell number into a new 50 mL conical tube and pellet cells by centrifugation at 400 x *g* at RT for 5 min.

4.2.7. Aspirate the supernatant, leaving a small volume (about 50–70 µL) to loosen the pellet.

4.3. Syringe preparation

4.3.1. Calculate an excess volume of 20 µL/injection to account for loss during transfer to the syringe. Resuspend the cell pellet with 220 µL of BMEM per injection on ice. The injected volume of cell suspension will be 200 µL per lesion.

4.3.2. Mix the cell suspension thoroughly on ice to obtain a homogenous cell suspension and avoid creating bubbles.

4.3.3. Using a 1 mL pipet and 1 mL syringe, simultaneously pipet BMEM-cell mixture into the syringe opening by suction force while pulling plunger of syringe.

4.3.4. Luer lock a 26G x 5/8 inch sterile needle to the syringe and keep prepared syringes flat on ice prior to injection.

4.4. Subcutaneous injection into mouse

4.4.1. Anesthetize the mice with 5% isoflurane/oxygen mixture at a flow rate of 1 L/min using an isoflurane vaporizer. Ensure proper sedation of animals (e.g., unresponsiveness to toe pinches). Maintain anaesthesia via continuous administration of 1.5% isoflurane/oxygen delivered via nose cone.

4.4.2. Place mice on their stomach, exposing the back region where grafting will occur and disinfect the injection region with 70% ethanol.

4.4.3. Gently roll the prepared syringe to resuspend any settled cells. Flick bubbles to the needle end of the syringe and expel a small volume of the cell suspension to ensure the removal of all bubbles.

NOTE: Two injections can be performed for each mouse – on the left and the right side of mouse back.

4.4.4. Pinch and create a 'tent-like' structure using your thumb and index finger and insert the needle subcutaneously right under the skin. Ensure that the needle is only skin deep by releasing pinched skin to prevent injection into muscle tissue.

4.4.5. Holding needle at 45° angle carefully inject 200 µL of the cell-suspension to create a small spherical mass (**Figure 1C**).

4.4.6. Record mouse weight with a scale, ear tag the mouse, and return to cage.

4.4.7. Monitor mice following sedation to ensure they return to normal activity.

4.5. Lesion growth monitoring

4.5.1. Using a caliper, measure the length and width of each plug (**Figure 1D**).

4.5.2. Document measurements every other day up until lesion collection.

5. Tissue collection and processing

5.1. Euthanize mice 9 days post-implantation in a CO_2 chamber and check vital signs to confirm death. Perform cervical dislocation on the mice as a secondary method to euthanize the mouse.

5.2. Harvest the xenograft lesion/plug from the flank of the mouse by dissection using surgical forceps and scissors.

NOTE: In order to prevent rupturing the blood-filled vessels within the plug, it is important to avoid touching the lesion plug with dissection tools and leave excessive surrounding tissue such as skin attached to the plug.

5.3. Immerse the resected lesion in PBS to wash.

5.4. Set up a camera stage with a camera. Align plugs onto a cutting board with a ruler. Take an image of all plugs to record gross vascularity of lesions (**Figure 1E**).

5.5. Fix plugs by submerging in 10% formalin overnight at RT.

5.6. Wash plugs in PBS the following day and move them into 70% ethanol.

5.7. Process lesion plugs for paraffin embedding (pathology core).

6. Lesion sectioning

6.1. Use a microtome to cut 5 µm sections from the collected murine lesions onto positively charged slides.

NOTE: For the subsequent analysis, sections in the center of the plug (about 50–70 µm into the tissue) are of importance.

6.2. Melt paraffin at 60 °C for 1 h prior to staining.

6.3. De-paraffinize and re-hydrate tissue sequentially under a chemical fume flow hood. Therefore, incubate slide in xylene for 10 min, 100% ethanol (EtOH) for 5 min, 90% EtOH for 3 min, and 80% EtOH for 3 min.

6.4. Rinse slide in deionized water for 5 min.

7. Hematoxylin and Eosin (H&E)

7.1. Incubate sections in Hematoxylin for 2 min.

7.2. Place slides in a staining jar and rinse in a sink by a steady stream of tap water until water is clear.

7.3. De-hydrate slides by incubating tissue sequentially in 70% EtOH for 1 min, 80% EtOH for 1 min, 90% EtOH for 1 min, 100% EtOH for 1 min, and fresh 100% EtOH for 1 min

7.4. Stain sections in Eosin Y for 30 s.

7.5. Rinse in fresh 100% EtOH until solution is clear.

7.6. Incubate slide in xylenes for 2 min. Let slides dry for 5–10 min under the fume hood.

7.7. Dispense a drop of permanent, non-aqueous mounting medium over xenografts sections and place coverslip on top.

7.8. Allow slides to dry overnight before imaging.

8. Immunohistochemistry

8.1. Prepare antigen retrieval buffer (Tris-EDTA) by weighing 0.6 g of Tris-base and 1 mL of 0.5 M EDTA to 500 mL of deionized water. Adjust pH to 9.0 using 1 M HCl. Add 250 µL of Tween-20.

8.2. Incubate de-paraffinized tissue slides (as obtained in step 6.2–6.3) in a beaker with antigen retrieval buffer, stirring on a heating block, for 20 min at 95 °C.

8.3. Remove the beaker from heating block, allow solution to cool to 35 °C then wash in PBS for 3 min.

8.4. Block tissue sections in 5% normal horse serum in PBS for 30 min at RT.

8.5. Prepare a biotinylated Ulex europaeus agglutinin-I (UEA-I) working solution by diluting 20 µg/mL of biotinylated UEA-I in 5% normal horse serum in PBS.

8.6. Pipet 50–100 µL of UEA-I working solution per section and incubate for 1 h at RT in a humidifying chamber.

8.7. Wash slides two times in PBS for 3 min.

8.8. Quench slide sections in 3% hydrogen peroxide for 5 min at RT.

8.9. Wash slides two times in PBS for 3 min.

8.10. Prepare 5 µg/mL of Streptavidin horseradish peroxidase-conjugated in 5% normal horse serum in PBS.

8.11. Pipet 50–100 µL on each tissue slide and incubate for 1 h at RT in a humidifying chamber.

8.12. Wash slides two times in PBS for 3 min.

8.13. Prepare 3,3'Diaminobenzidine (DAB) solution according to manufacturer's instructions and add 50–100 µL per section.

8.14. Incubate sections for 10–15 min, checking and monitoring for development of stain every 2–5 min.

8.15. Wash slides three times in PBS for 3 min.

8.16. Add a drop of Hematoxylin and incubate for 3 min.

8.17. Place slides in a staining jar and rinse in a sink by a steady stream of tap water until water is clear.

8.18. Sequentially incubate slide in 80% EtOH for 1 min, 90% EtOH for 1 min, 100% EtOH for 1 min, and xylene for 2 min.

8.19. Let slides dry for 5–10 min under the fume hood.

8.20. Dispense a drop of permanent, non-aqueous mounting medium over xenografts sections and place coverslip on top.

8.21. Allow slides to dry overnight before imaging.

9. Analysis of human-derived Vascular Channels

NOTE: Vascularity of VM lesions is quantified by measuring vascular area and vascular density. Only UEA-I positive, human-derived vascular channels are considered for quantification.

9.1. Take four to five images per lesion section with a bright field microscope at a 20x magnification (high power fields [HPF]). Take HPF images in an x-plane pattern within the lesion section to avoid overlap (**Figure 1F**). Include a scale bar on the images taken.

9.2. Open and the HPF images in Image J (**File** > **Open**). Calibrate the pixels of the scale bar as follows. Use the straight line tool and go over the scale bar. To convert the measured pixels into mm click on **Analyze** > **Set scale**.

9.3. Click on **Analyze** > **Set Measurements** and select **Area**.

9.4. Measure the total field area in a HPF using **Analyze** > **Measure**. Save this measurement for quantification in step 9.8.

9.5. Using the freehand selections tool, manually outline UEA-I$^+$ vascular channels.

NOTE: A vascular channel is defined as any area that is lined with UEA-I$^+$ - EC that may contain blood cells.

9.6. Click on **Analyze** > **Measure** to quantify the outlined UEA-I+ vascular area (mm^2/HPF).

9.7. Repeat this measurement for all five HPF taken within one plug.

9.8. Average the total vascular area of all five HPF (divide by 5). The obtained vascular area per HPF is subsequently divided by the HPF field area (in mm^2, step 9.5) and expressed as a percent (%).

9.9. For quantification of vascular density, count the number of UEA-I$^+$ vascular channels of each HPF taken. The vascular density is the average number of UEA-I$^+$ vascular channels counted per HPF area (vessels/mm^2).

REPRESENTATIVE RESULTS:

This protocol describes the process of generating a murine xenograft model of VM based on the subcutaneous injection of patient-derived EC into the back of immunodeficient nude mice. A scheme summarizing the steps from VM-EC isolation to dissection of lesion plug is presented in **Figure 2**. Endothelial cell colonies can be harvested within 4 weeks after initial cell isolation from VM tissue or lesional blood. The day after injection, the xenograft lesion plug covers a surface area of approximately 80–100 mm^2. In our hands, lesion plugs with TIE2/PIK3CA-mutant EC are visibly vascularized and perfused within 7–9 days from injection [14, 15] (**Figure 1C,D**). However, the extent of lesion growth is variable and reflects on patient and sample heterogeneity.

Lesion plugs closely recapitulate the histopathological features of human VM tissue: enlarged vascular channels lined by a thin layer of endothelial cells (**Figure 1F–H**). These vascular structures typically contain erythrocytes, confirming functional anastomoses with the host mouse vasculature (**Figure 1F–H**). Immunohistochemical staining using the human specific lectin UEA-I can confirm that cells lining vascular lesions are derived from human implanted cells rather than mouse vasculature (**Figure 1H**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative results. (**A**) Representative image of primary mixed cell culture three weeks after isolation from VM tissue before EC selection. Typical endothelial cell colony (EC) and contaminating fibroblast (FB). (**B**) Image of a purified (CD31 bead-selection) endothelial cell culture from VM patient-derived tissue. Scale bar = 200 µm. (**C**) The lesion will form a spherical structure. Vascularization is visible due blueish color through the skin of nude mice. (**D**) Dashed

lines show how lesion size is recoded by measuring the length (L) and the width (W) using a caliper. (**E**) Photo of visibly vascularized, xenograft lesion explant at day 9. Scale bar = 1 cm. (**F**) Representative image of a lesion plug section. The x-plane pattern in which five high power field images are taken for quantification are indicated by white dashed boxes. Scale bar = 1000 µm. (G–H) Representative images of VM lesion plug sections. (**G**) Hematoxylin and Eosin staining and (**H**) immunohistochemistry of human specific lectin UEA-I. Scale bar = 100 µm.

Figure 2: Schematic of the workflow to generate a patient-derived xenograft of VM. (**A**) Endothelial cells isolated from patient VM lesion solid tissue or lesional blood are plated and, when 80% confluency is reached, are selected by anti CD31-conjugated immunomagnetic beads and expanded. (**B**) For subcutaneous injection of EC, on day 0, skin on the backside of the mouse is pinched using forefinger and thumb to create a tent-like structure. Lesions are measured at day 1 and then every other day (red arrows) using a caliper through experimental day 9. Lesions are dissected and processed for histological analysis.

DISCUSSION:

Here, we describe a method to generate a patient-derived xenograft model of VM. This murine model presents an excellent system that allows researchers to gain a deeper understanding of pathological lumen enlargement and will be instrumental in developing more effective and targeted therapies for the treatment of VM. This can be easily adapted to investigate other types of vascular anomalies such as capillary lymphatic venous malformation[16]. There are several steps that are crucial for the successful generation of reproducible vascular lesions. First, the patient-derived endothelial cells must be pure (without the presence of other cell types) and growing exponentially at the time of injection. Contaminating fibroblast or other mesenchymal non-EC can be easily recognized by elongated morphology as shown in **Figure 1A**. On rare occasion, it is possible that even after purification using anti-CD31 antibody conjugated magnetic beads, a small number of non-EC remain in the culture. These cultures require further purification with endothelial specific cell surface markers. As an alternative approach, single cell clonal expansion of endothelial cells is possible. This would reinsure the homogeneity of mutant-EC as all of the cells within one culture would derive from one single cell. However, this approach is not recommended for VM-derived EC as cells tend to top their proliferation capabilities and convert into a senescent phenotype after 9–10 passages. It is critical to use cells between passages 3–8 for xenograft experiments and to not passage cells the day before the injection.

The xenograft model can be modified to investigate other vascular anomalies carrying different activating mutations. Moreover, as patient tissue samples are difficult to access for some laboratories, the xenograft model can be adapted by using EC, such as human umbilical cord blood cells (HUVEC), genetically engineered to express the mutation/s known to cause dysfunctional vascular growth[15, 17].

The number of cells recommended for the injection in the xenograft is 2.5 x 10^6 cells/200 µL of BMEM. However, if the cell number is insufficient it is possible to either reduce the number of injections per animal to one or to reduce the injection volume to a minimum of 100 µL. For the latter, it is however important to maintain the cell density ratio e.g., 1.25 x 10^6 cells/100 µL

BMEM. When working with BMEM, all the steps must be performed on ice to avoid solidification of the cell suspension before injection. During injection, it is important and that the needle is inserted at an angle of 45° directly under the skin and away from the muscle tissue, as injecting into muscle impedes lesion reproducibility and makes the lesion dissection difficult. A total of two injections can be performed on each mouse—one on the right and one on the left side of each animal. The second injection in the same mouse can serve as a technical replicate. More injections on the back are not recommended as lesions grow over time and might interfere with each other. For statistical analysis in pre-clinical studies comparing xenograft plugs of treated versus untreated (vehicle only) mice, we recommend the use of a minimum of 5 animals (10 xenograft plugs) per study group. If available, the second injection could alternatively be used as a 'internal control' using non-mutant EC. We have used primary non-mutant EC, such as HUVEC, as a control and have shown that these cells formed a negligible number of small channels[14, 15]. Furthermore, in these HUVEC control lesion plugs, we have noticed infiltration of murine-derived vascular channels into the plug after day 9. If the experimental design requires longer incubation times, these infiltrating channels can be easily excluded from analysis by staining for a human-specific marker such as human-specific CD31 antibody or Ulex europaeus agglutinin I (UEA-I) that does not cross react with mouse.

To ensure that the lesion does not become a burden to animal health and wellbeing it is important to observe lesion size, record mouse weight daily, and pay attention to any side complications such as bleeding and bruising. If the lesion volume exceeds 500 mm^3, the experiment has to be terminated.

When vascular lesions are enlarged and perfused, extreme attention must be paid during dissection to avoid rupturing the lesion. It is important to avoid touching the lesion plug with dissection tools and leave excessive surrounding tissue (such as skin) attached to the plug. This prevents collapse of the vascular structures within the xenograft plug which would interfere with accurate analysis.

Finally, to maintain consistency, it is important that the initial histological analysis begins in the center of the plug (about 50–70 µm into the tissue) rather than the border regions where anastomosing mouse vasculature might be present. It is highly recommended to stain the tissue sections with a human-specific EC marker, such as UEA-I (**Figure 1H**) or an alternative human-specific antibody which will not cross-react with mouse, in order to confirm that vascular structures are formed by human-derived EC rather than invading mouse EC.

ACKNOWLEDGMENTS:
The authors would like to thank Nora Lakes for proofreading. Research reported in this manuscript was supported by the National Heart, Lung, and Blood Institute, under Award Number R01 HL117952 (E.B.), part of the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DISCLOSURES:

The authors have no conflicts-of-interests to disclose.

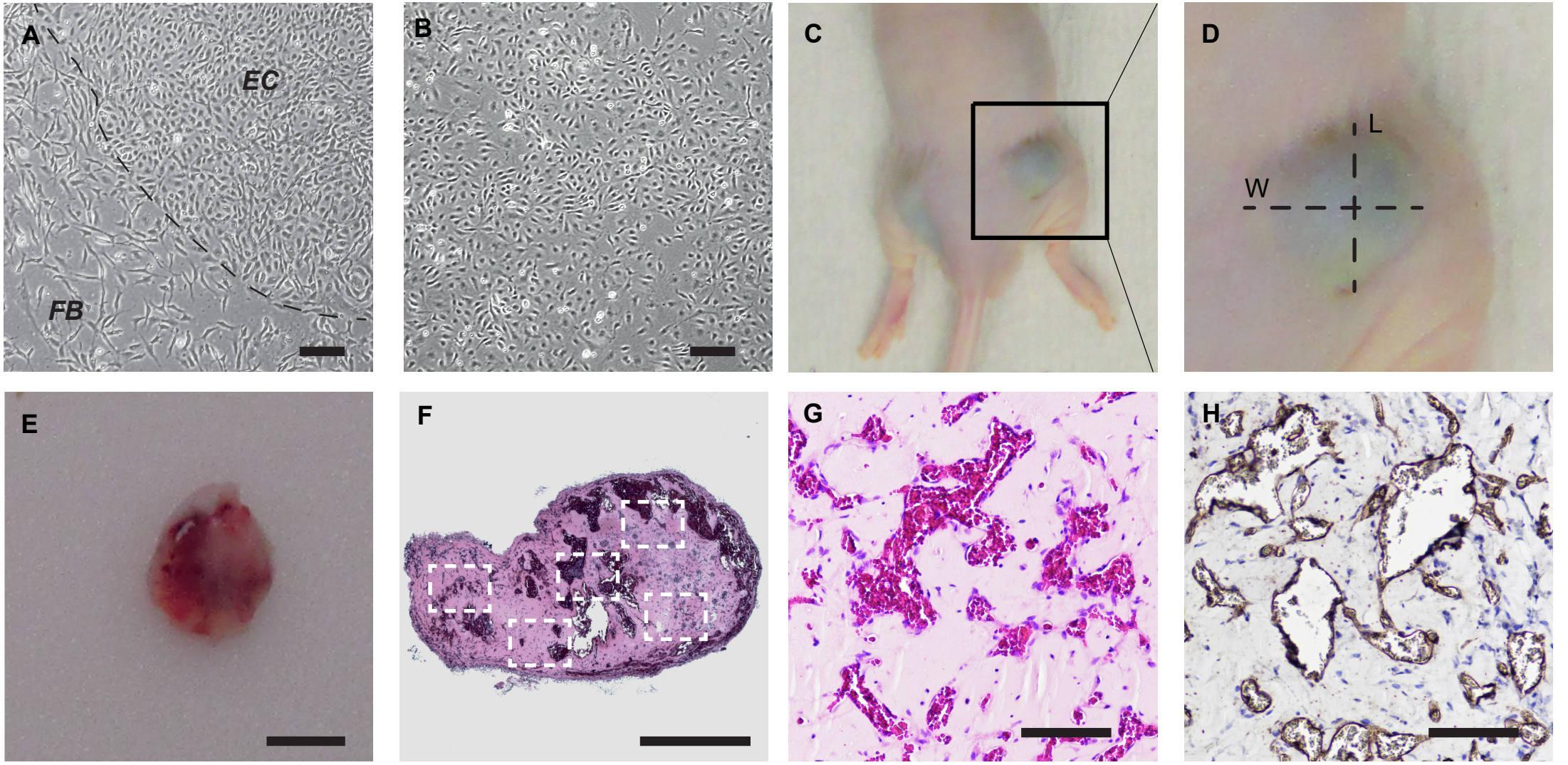
REFERENCES:

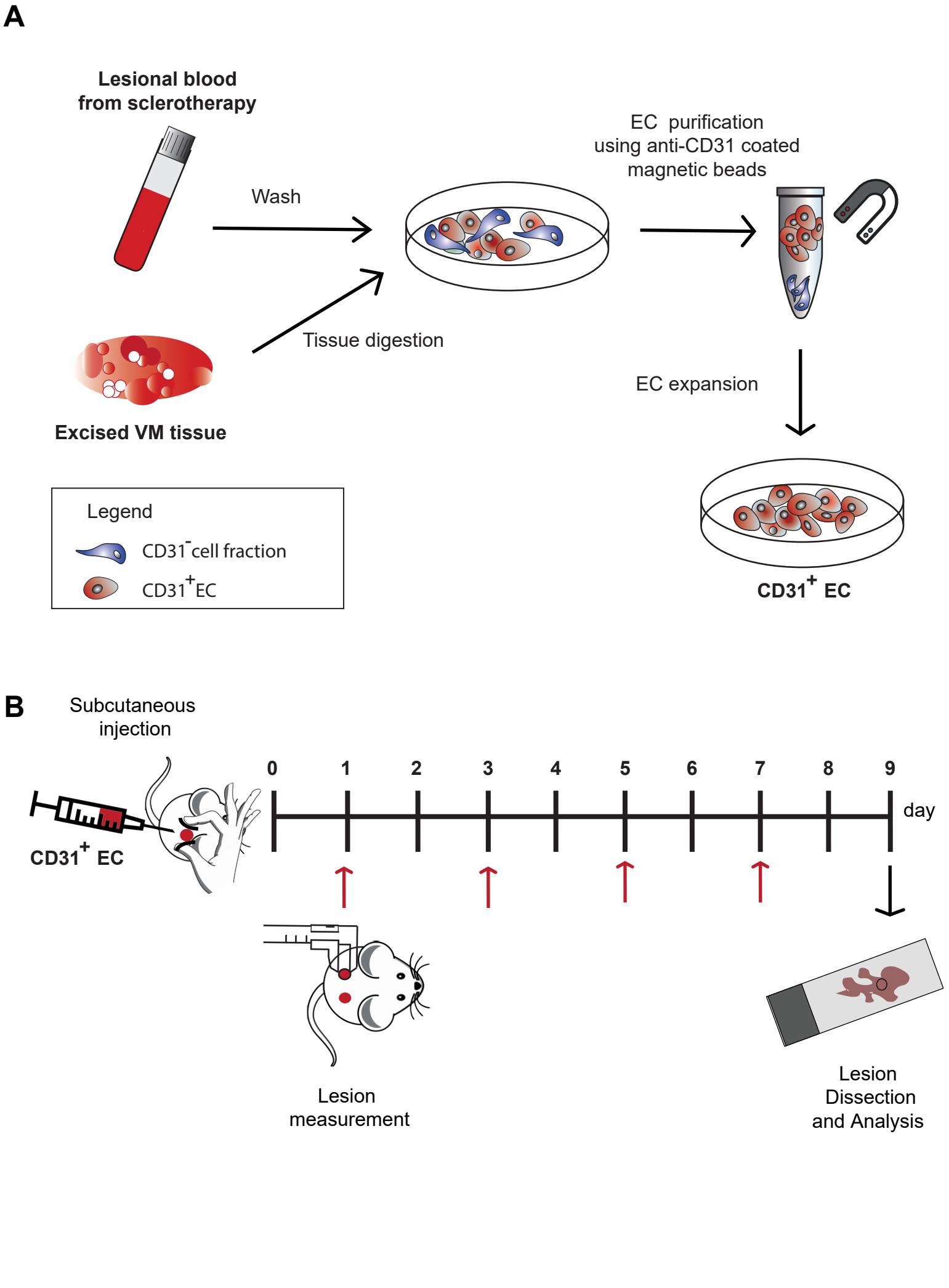
1. Dompmartin, A., Vikkula, M., Boon, L.M. Venous malformation: update on aetiopathogenesis, diagnosis and management. *Phlebology: The Journal of Venous Disease*. **25** (5), 224–235, doi: 10.1258/phleb.2009.009041 (2010).
2. Limaye, N. et al. Somatic mutations in angiopoietin receptor gene TEK cause solitary and multiple sporadic venous malformations. *Nature Genetics*. **41** (1), 118–124, doi: 10.1038/ng.272 (2009).
3. Castel, P. et al. Somatic PIK3CA mutations as a driver of sporadic venous malformations. *Science Translational Medicine*. **8** (332), 332ra42, doi: 10.1126/scitranslmed.aaf1164 (2016).
4. Limaye, N. et al. Somatic Activating PIK3CA Mutations Cause Venous Malformation. *The American Journal of Human Genetics*. **97** (6), 914–921, doi: 10.1016/j.ajhg.2015.11.011 (2015).
5. Castillo, S.D. et al. Somatic activating mutations in Pik3ca cause sporadic venous malformations in mice and humans. *Science Translational Medicine*. **8** (332), 332ra43-332ra43, doi: 10.1126/scitranslmed.aad9982 (2016).
6. Stratman, A.N. et al. Endothelial cell lumen and vascular guidance tunnel formation requires MT1-MMP-dependent proteolysis in 3-dimensional collagen matrices. *Blood*. **114** (2), 237–247, doi: 10.1182/blood-2008-12-196451 (2009).
7. Okada, Vaeteewoottacharn, Kariya Application of Highly Immunocompromised Mice for the Establishment of Patient-Derived Xenograft (PDX) Models. *Cells*. **8** (8), 889, doi: 10.3390/cells8080889 (2019).
8. Byrne, A.T. et al. Interrogating open issues in cancer precision medicine with patient-derived xenografts. *Nature Reviews Cancer*. **17** (4), 254–268, doi: 10.1038/nrc.2016.140 (2017).
9. Allen, P., Melero-Martin, J., Bischoff, J. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. *Journal of Tissue Engineering and Regenerative Medicine*. **5** (4), e74, doi: 10.1002/term.389 (2011).
10. Allen, P., Kang, K.-T., Bischoff, J. Rapid onset of perfused blood vessels after implantation of ECFCs and MPCs in collagen, PuraMatrix and fibrin provisional matrices. *Journal of Tissue Engineering and Regenerative Medicine*. **9** (5), 632–636, doi: 10.1002/term.1803 (2015).
11. Nowak-Sliwinska, P. et al. Consensus guidelines for the use and interpretation of angiogenesis assays. *Angiogenesis*. **21** (3), 425–532, doi: 10.1007/s10456-018-9613-x (2018).
12. Roh, Y.N. et al. The results of surgical treatment for patients with venous malformations. *Annals of Vascular Surgery*. **26** (5), 665–673, doi: 10.1016/j.avsg.2011.12.004 (2012).
13. Marler, J.J., Mulliken, J.B. Current management of hemangiomas and vascular malformations. *Clinics in Plastic Surgery*. **32** (1), 99–116, doi: 10.1016/j.cps.2004.10.001 (2005).
14. Goines, J. et al. A xenograft model for venous malformation. *Angiogenesis*. **21** (4), 725–735, doi: 10.1007/s10456-018-9624-7 (2018).
15. Li, X. et al. Ponatinib Combined With Rapamycin Causes Regression of Murine Venous Malformation. *Arteriosclerosis, thrombosis, and vascular biology*. **39** (3), 496–512, doi: 10.1161/ATVBAHA.118.312315 (2019).

16. Le Cras, T.D. et al. Constitutively active PIK3CA mutations are expressed by lymphatic and vascular endothelial cells in capillary lymphatic venous malformation. *Angiogenesis*. 1–18, doi: 10.1007/s10456-020-09722-0 (2020).
17. Boscolo, E. et al. Rapamycin improves TIE2-mutated venous malformation in murine model and human subjects. *Journal of Clinical Investigation*. **125** (9), 3491–3504, doi: 10.1172/JCI76004 (2015).

Figure

Click here to access/download;Figure;Figure 1.pdf





Name of Material/Equipment

Athymic nude mice, (Foxn1-nu); 5-6 weeks, males
Biotinylated Ulex europeaus Agglutinin-I (UEA-I)
Bottle top filter (500 ml; 0.2 µM)
Bovine Serum Albumin (BSA)
Calcium cloride dihydrate ($CaCl_2.2H_2O$)
Caliper
CD31-conjugated magnetic beads (Dynabeads)
Cell strainer (100 µM)
Collagenase A
Conical Tube; polypropylene (15 mL)
Conical Tube; polypropylene (50 mL)
Coplin staining jar
Coverglass (50 X 22 mm)
DAB: 3,3'Diaminobenzidine Reagent (ImmPACT DAB)
Dulbecco's Modification of Eagle's Medium (DMEM)
DynaMag-2
Ear punch
EDTA (0.5M, pH 8.0)
Endothelial Cell Growth Medium-2 (EGM2) Bulletkit (basal medium and supplements)
Eosin Y (alcohol-based)
Ethanol
Fetal Bovine Serum (FBS) , HyClone
Filter tip 1,250 µL
Filter tip 20 µL
Filter tip 200 µL
Formalin buffered solution (10%)
Hemacytometer (INCYTO; Disposable)
Hematoxylin
Human plasma fibronectin purified protein (1mg/mL)
Hydrogen Peroxide solution (30% w/w)
ImageJ Software
Isoflurane, USP
magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)

Basement Membrane Matrix (Phenol Red-Free; LDEV-free)
Microcentrifuge tube (1.5 mL)
Microscope Slide Superfrost (75mm X 25mm)
Needles, 26G x 5/8 inch Sub-Q sterile needles
Normal horse serum
Penicillin-Streptomycin-L-Glutamine (100X)
Permanent mounting medium (VectaMount)
Pestle Size C, Plain
Phosphate Buffered Saline (PBS)
Scale
Serological pipettes (10 ml)
Serological pipettes (5ml)
Sodium carbonate (Na_2CO_3)
Streptavidin, Horseradish Peroxidase, Concentrate, for IHC
Syringe (60ml)
SYRINGE FILTER (0.2 µM)
Syringes (1 mL with Luer Lock)
Tissue culture-treated plate (100 X 20 mm)
Tissue culture-treated plate (145X20 mm)
Tissue culture-treated plates (60 X 15) mm
Tris-base (Trizma base)
Trypan Blue Solution (0.4 %)
Trypsin EDTA, 1X (0.05% Trypsin/0.53mM EDTA)
Tween-20
Wheaton bottle
Xylenes

Company	Catalog Number	Comments/Description
Envigo	069(nu)/070(nu/+)	Subcutaneous injection
Vector Laboratories	B-1065	Histological anlaysis
Thermo Fisher	974106	Cell culture
BSA	A7906-50MG	Cell culture; Histological analysis
Sigma	C7902-500G	Cell culture
Electron Microscopy Sciences	50996491	Lesion plug measurment
Life Technologies	11155D	EC separation
Greiner	542000	Cell culture
Roche	10103578001	Cell culture
Greiner	07 000 241	Cell culture
Greiner	07 000 239	Cell culture
Ted Pella	21029	Histological anlaysis
Fisher Scientific	12545E	Histological anlaysis
Vector Laboratories	SK-4105	Histological anlaysis
Corning	10-027-CV	Cell culture
Life Technologies	12321D	EC separation
VWR	10806-286	Subcutaneous injection
Life Technologies	15575-020	Histological anlaysis
Lonza	CC-3162	Cell culture
Thermo Scientific	71211	Histological anlaysis
Decon Labs	2716	Histological anlaysis
GE Healthcare	SH30910.03	Cell culture
MidSci	AV1250-H	Multiple steps
VWR	10017-064	Multiple steps
VWR	10017-068	Multiple steps
Sigma	F04586	Lesion plug dissection
SKC FILMS	DHCN015	Cell culture
Vector Hematoxylin	H-3401	Histological anlaysis
Sigma	FC010-10MG	Cell culture
Sigma	H1009	Histological anlaysis
		Analysis
Akorn Animal Health	59399-106-01	Subcutaneous injection
Sigma	M1880-500G	Cell culture

Corning	356237	Subcutaneous injection
VWR	87003-294	EC separation
Fisher Scientific	1255015-CS	Histological anlaysis
Becton Dickinson (BD)	BD305115	Subcutaneous injection
Vector Laboratories	S-2000	Histological anlaysis
Corning	30-009-CI	Cell culture
Vector Laboratories	H-5000	Histological anlaysis
Thomas Scientific	3431F55	EC isolation
Fisher Scientific	BP3994	Cell culture
VWR	65500-202	Subcutaneous injection
VWR	89130-898	Cell culture
VWR	89130-896	Cell culture
Sigma	223530	Cell culture
Vector Laboratories	SA-5004	Cell culture
BD Biosciences	309653	Cel culture
Corning	431219	Cell culture
Becton Dickinson (BD)	BD-309628	Subcutaneous injection
Greiner	664160	Cell culture
Greiner	639160	Cell culture
Eppendorf	30701119	Cell culture
Sigma	T6066	Histological anlaysis
Life Technologies	15250061	Cell culture
Corning	25-052-Cl	Cell culture
Biorad	170-6531	Histological anlaysis
VWR	16159-798	Cell culture
Fisher Scientific	X3P-1GAL	Histological anlaysis

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Em?

1) Since human tissues is used, please add a human research ethics statement at the start of your protocol.
R: The statement was added at the beginning of the protocol (line 99-100)

2) 1.1.1: what is the concentration of each it.
R: The growth factors are sold as part of a commercially available kit. The concentration of the growth factors is not indicated. Even upon request, the manufacturer was not able to provide this information.

3) 2.1.1: what kind of surgery is performed for VM acquisition? Please cite references.
R: Venous malformations are commonly resected by debulking surgery. We edited the text accordingly and included references (Roh YN, 2012 Ann Vasc Surgery; Marler JJ, 2005 Clin Plast Surgery).

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
R: We revised accordingly.

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Wheaton, Matrigel,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
R: Commercial sounding language such as "Wheaton" and "Matrigel" were removed from the manuscript and replaced with generic names.

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
R: We removed trademark symbols/names.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using

figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
R: All of the presented figures have not been published before.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
This manuscript details a murine xenograft model of venous malformations (VM) using human derived endothelial cells from patients harboring two of the most prominent mutations linked to VMs (TIE2 and PIK3CA hyper activating mutations). The authors present detailed methods on the isolation, purification, maintenance, and expansion of patient derived VM ECs for the purpose of transplanting and creating human derived VMs in immunocompromised mice. VMs are relatively understudied and have very few animal models for investigation; thus, this type of experimental analysis is valuable for VM research and potentially testing therapeutic measures. Overall, the methods were clearly explained and easy to follow. I think a few minor items could be addressed to make the procedures even more straightforward and helpful.

Major Concerns:
No major concerns.

Minor Concerns:
1. Line 133: Could the authors include what was used to adjust the PH of the buffer to 9.
R: The PH of the coating buffer is adjusted with HCl 1M . This information is now included in the text. (Section 1.4.1)

2. Line 166: It would be helpful if this sentence could be coordinated with the next step/section of the process, such as adding " (proceed to 3.2.1)" or something to that effect.
R: We thank the reviewer for pointing this out. After the centrifugation step (in 2.1.8) a reference to subsequent step 2.3 was included for clarification.

3. Line 188-189: Could the authors note how long it usually takes the cultures to grow to a confluency of 40-50%.
R: It typically takes between 2 and 3 weeks for the cells to reach this confluency. The text has been edited to include this information (Section 2.3.5).

4. Section 4, Lines 194-212: It would be nice if it could be noted whether multiple EC colonies from the same dish can be isolated or whether this is not recommended.
R: It is recommended to pool multiple EC colonies. A clarification was added (Section 2.3.6) and a new image was added to Figure 2 (A) showing a typical EC colony.

5. Line 242: Is one supposed to aspirate all of the solution with our disturbing the beads.

If so, perhaps it could be written as: Gently aspirate all of the liquid, which contains the CD31 negative fraction of cells, without touching the beads.
R: That is correct and was clarified in the manuscript (Section 3.2.6 and 3.2.7).

6. Line 302-303: Add the volume that should be used to resuspend the cells.
R: The cells are resuspended in 3 mL of medium. This information was added in this revised manuscript (Section 4.2.4)

7. Line 309-310: It might be helpful to know approximately how many 50 ml conical tubes with pelleted cells can be obtained from a typical dish of ECs (145 mm dish or whatever size is used) sitel?
R: A confluent 145 mm cell culture dish contains about 8-9 x 10^6 cells. This number of cells is sufficient for at least 3 xenograft injections. This information is now included in Section 3.3.6.

8. If so, this might be a good place to mention the use of two-sided injections in the same mouse with one side serving as a "internal" control.
R: In the case of VM patient-derived cells we cannot include “healthy” control cells of the same donor. This is because so far we have not had success in isolating non-mutant ECs from VM tissue and cannot obtain healthy tissue from patients. Our group has used other primary non-mutant ECs such as HUVEC as internal control cells. These cells are forming significantly smaller and less vascular channels (Goines et al. 2018, Angiogenesis). The choice and possibility of internal controls is based on the experimental design of the study in which the model is used. If available; control cells (“healthy” or non-mutant control cells) could be included as internal control within the same animal. If planning to inject control cells, those cells will need to be expanded and prepared the same way as mutant EC.
Additionally, for pre-clinical experiments, the second injection could serve as a technical duplicate of cells of the same patient within the same animal.
A clarifying sentence was added to Section 4.2.5. and this point has been addressed in the discussion.

9. Line 366-367: Harvesting of the xenograft doesn't mention whether to include the skin or not. However, the discussion talks about including the skin during harvesting for integrity purposes. This should be include in the procedure details as well.
R: Thank you for pointing this out. This important detail was added to the procedures (Section 5.2)

10. Line 417: Could the authors include what was used to adjust the PH to 9.
R: The pH of the Tris-EDTA buffer is adjusted with 1M HCl The information is now included (Section 8.1)

11. Line 475: I am unfamiliar what the term "x-plane" means in this sentences. Could the authors clarify this description.
R: The term x-plane describes the pattern in which the five images (High power fields) are taken within one lesion. For clarification an image was added (Figure 2F) visualizing this pattern (Step 9.2).

12. Line 552-553: The use of cells for xenografts being within 2 to 5 passages is important and should be include in the procedures, not just the discussion.

R: Thank you for pointing this out. This information is missing in the protocol section. The cells should be used between passage 3 and 8. This information was corrected in the discussion and also included in Step 3.3.6.

13. Line 565-566: This mentions the number of animals and xenografts to be used in an experiment for statistical significance. I'm wondering how "internal controls" on the same mouse (one-she) fit into this or whether internal side controls on the same mouse are even used.
R: The choice and possibility of internal controls is based on the experimental design of the study in which the model is used. If available; control cells ("healthy" or "non-mutant control cells") could be included as internal control within the same animal. In pre-clinical or drug treatment studies, the second injection could serve as a technical duplicate of cells of the same patient within the same animal. The model is in this case used for the analysis of vascular channels formed in treated vs untreated (vehicle-only) mice

Reviewer #2:
Manuscript Summary:
This is an excellent article by Schrenk, Goines, and Boscolo that clearly describes the protocol to develop a xenograft mouse model of venous malformation. The authors have done a careful job at describing all the critical steps required to reproduce this experimental approach. The technique is of great interest in the field of angiogenesis and vascular anomalies and nicely complements the existence of other genetically engineered mouse models. I have made some comments to try to improve this already excellent protocol.

Major Concerns:
None

Minor Concerns:
- Could the authors include an approximate range of the size of the lesions used for the isolation? This seems to be an important detail, since the authors indicate that these cells can not be passaged indefinitely. Similarly, for the approach using blood from sclerotherapy, could the authors indicate the range of volumes that work well for this protocol? Also, what is the approximate number of cells that are isolated in this step? The authors indicate that they use 1 mL, but they don't mention the concentration of such cell suspension.
R: The size/volume of samples varies greatly and depends on the patient's status and severity of disease (how much tissue had to be excised). Furthermore, we only obtain a small portion of the resected tissue as most of it needs to be sent for pathological assessments. In general, tissue specimens we obtain range between 0.5g -1.5g. In our hands, the tissue processing always leads to a successful EC isolation no matter the initial size of tissue obtained. Lesional blood from sclerotherapy can be in the range of 0.5 mL and 5 mL. The isolation of ECs from lesional blood is not always successful but no correlation between sample volume and EC-isolation success has been noticed.
Regarding initial cell number, we do not perform cell counting. This is because the initial cell suspension is a mixture of different cell types including red blood cells present within the sample. Therefore the cell number is not suggestive of how many EC colonies will be growing. A large number of these initial cells will not attach and will be removed when the medium is changed.

- Indicate the reason by which hydrocortisone is not added to the cell media.
R: In our hands as well as reported by other groups (Kraling BM, 1998, In vitro Cell Dev Biol) the addition of hydrocortisone is not necessary for EC survival and proliferation. Furthermore, in previous publications hydrocortisone in the presence of heparin (also included in our medium) has been shown to inhibit angiogenesis (Folkman J, 1986, Cancer research). However, since hydrocortisone is part of the cell culture medium bullet kit that we use, we wanted to clarify that we are not using this component of the supplied supplements.

- How many passages can these cells tolerate before cell injection?
R: This is an important point. Cells should be used between passage 3 and passage 8. The information is now included in section 3.3.6.

-Can these tumors be expanded once engrafted in the mouse as it has been done in PDX for tumors?
R: We have not tried to expand lesions in a secondary host. However, this should be feasible based on Kang KT, 2011 Blood.

- Please specify the nature of the pestle used. Is it a Douncer homogenizer?
R: A teflon tissue homogenizer (6 mm, smooth-surface pestle grinder) that fits into a 50 mL conical tube was used in our studies. This information was included in section 2.1.5.

- The authors comment that cells can be injected in the "hind legs". Yet, the figure seems to indicate the flank of the mouse. Please clarify; it seems intuitive that the cells would be injected in the flanks and the authors mean above the hind legs. .
R: Yes, cells are injected in the flank and not hind legs – We edited the text accordingly.

Reviewer #3:
Manuscript Summary:
The manuscript provides a detailed protocol for generating xenograft murine models of venous malformation, by injecting cultured endothelial cells that were isolated directly from patients' lesions. Overall, the steps provided could be reasonably followed and lead to the anticipated results. The VM mouse model would be an asset in understanding the endothelial functional defect of venous malformations (and possibly other vascular malformations/syndromes) and to test potential drug candidates for treatment.

However, additional details could be included to clarify certain aspects of the protocol. Some specific points:

1. Generally, approximate average time periods (e.g. 5-7 days) for the in vitro culture would help, particularly with the pre-CD31-Dynabead selection cultures.
R: For the pre-CD31-Dynabead selection, it typically takes between 2-3 weeks for the EC colonies to appear and another 5-7 days for the colonies to grow to a size where those colonies start touching one another. At this point the EC colonies are ready to be harvested. For clarification average time periods have been added in section 2.3.5 and 2.3.6.

2. Point 2.4.6 (line 208-09), where the plate size to use is dependent on the number of isolated EC cultures, is ambiguous. How many EC colonies could one expect overall? How many would be needed to decide to grow on the larger-sized plate?

R: Approximately 3-5 EC colonies can be obtained from each sample. This information was added to the protocol (Step 2.3.6). A cell counting step was added (Step 2.4.6). The cell density in which the cells have to be plated is $1x10^4$ cells/cm^2 . This important information was added to the manuscript in step 2.4.7.

3. For point 3.2.1 (Line 231), how were the ECs detached and pelleted? Trypsinization, manual scraping, etc?
R; The cells are detached by trypsinization. These missing steps are now included as 2.4.9 – 2.4.11. A reference to these steps was added under 3.2.1.

4. It seems like a step is missing from the Dynabead separation of the endothelial cells to the culturing (point 3.2.7-8, lines 246-50). There is no mention of if/how the CD31+ cells are detached from the beads. Are the beads still attached when the ECs are cultured? If so, would it act as a contaminant in the culture or seen in the plug?
R: The beads are still attached to the cells when the cells are initially plated but then release during culture. A small number of beads might still be present within the first few passages of these cells. Sometimes, the beads can even be found within the xenograft plug. However they have been proven to be non-toxic and therefore do not interfere with subsequent analysis. The Information was added to under 3.2.11.

5. The approach to analyze the human-derived vascular channels (section 9) is not clear. When the images are opened in ImageJ, what is meant to "stack" the images? Also, for point 9.8 (lines 491-92), what constitutes as the values for the "total area of the lesion" and "the total area of a single image" could be better defined.
R: Thank you for pointing this out. The description of the vascular area quantification has been revised and is now described in greater detail. See step 9.2 -9.9

Major Concerns:
Firstly, there is discrepancy regarding identification of ECs in vitro. Point 2.3.6 (line 191-2) mentions that the mixed cell culture should be "observed daily for appearance of EC colonies", then refers to Figure 2A as an example of the cobblestone-like appearance typical of EC colonies. However, Figure 2A is noted as a purified (assumingly post-selection) EC culture in the figure legend. It would be more helpful to show an image of the true mixed cell culture so that one can visualize the various types of cells and ensure the correct colonies are chosen during the manual isolation step.
R: That is a good observation. We added a new figure showing a mixed culture containing endothelial cells and non-endothelial cells before purification (Figure 2A). In the discussion we now refer to this image.

Also, in the discussion, the authors mention that contaminating non-endothelial cells could still be present in isolated EC cultures, which are "easily recognized by elongated morphology" (Lines 546-47). However, it has been published that one of the pathological features of cultured ECs overexpressing TIE2 and PIK3CA is an elongated shape (Limaye, et al. AJHG 2015 and Natynki et al. HMG 2015); in Figure 2A, a few ECs appear to demonstrate this trait. Differentiation between a contaminating non-EC and a mutant EC would need to be clarified.
R: That is correct. In respect to “normal” non-mutant endothelial cells, VM-derived ECs can show an elongated morphology as described in the above mentioned references. However, there are strong differences in mutant EC morphology and the morphology of contaminating fibroblasts. To clarify this, the new image in figure 2 (Figure 2A) shows an unpurified

primary culture that allows clear identification of such contaminating fibroblasts (FB) in contrast to VM-ECs (EC).

Secondly, a disclaimer was mentioned within the "REPRESENTATIVE RESULTS" that it's not recommended that xenograft lesions do not exceed 9 days due to increased murine infiltration that complicates analyses (Line 507-8). It would be good to elaborate what complications arise and possible strategies to overcome it (as in the discussion). It also raises questions on the ability for the model to be used for preclinical screening since many therapies would likely need a longer time period to evaluate effects.
R: We would like to describe in better detail what we are referring to. While in mutant-EC xenografts this is not a problem as all of the enlarged, malformed vessels are of human origin (as shown in Goines J et al., 2018,Angiogenesis), when injecting control cells such as HUVEC we have noted infiltration of host, murine-derived vascular channels into the plug. When comparing mutant-EC xenograft with control HUVEC xenograft, it is important to know these changes happen around/after day 9. Notably, this 'problem' could be overcomed by a staining for human-specific marker such as human-specific CD31 antibody or Ulex europaeus agglutinin I (UEA-I). Finally, because in mutant-EC xenografts recruitment of murine EC is negligible and only present at periphery of the plug where anastomosis occurs, pre-clinical studies are possible and have been performed by our group for up to 6 weeks (Boscolo E., 2015 Journal of Clinical Investigation; Li X et al., 2019 Arterioscler Thromb Vasc Biol). A clarifying sentence was included in the result section.

Minor Concerns:
The authors recommend that 2.5x106 cells per injection. Should there be insufficient numbers of cells grown, what would be the minimal number of cells that could be injected and still produced lesions within matrigel plugs?
R: The number of cells of $2.5x10^6$ cells per injection is necessary for a successful xenograft. If there are fewer cells available, it is recommended to reduce the number of injections/per animal. Alternatively, the injection-volume could be reduced to 100uL. In this case it is very important to keep the cell density equal ($2.5x10^6$ cells /200uL BMEM). A paragraph was added to the revised discussion.

Reviewer #4:
Manuscript Summary:
The authors present a well-written and detailed protocol on how to generate a murine xenograft model of venous malformation, based on the subcutaneous injection of patient-derived endothelial cells containing hyper-activating TIE2 and/or PIK3CA gene mutations. Overall, the manuscript meets the Jove Journal standard and scope. However, by answering key questions along the text or providing some extra information, the protocol presented here could be improved

Major Concerns:

*** Which control cells (non-mutant) are used for the xenograft model? What is the difference between the histology of the lesion formed from EC isolated from a human VM or control cells (non-mutant)? Without this comparison it is not easy to appreciate whether EC isolated from VMs reproduce the disease or it is simply a vessel growth assay.**

R: In the case of patient-derived cells we cannot include “healthy” control cells of the same donor. This is because so far we have not had success in isolating non-mutant ECs from VM tissue and cannot obtain healthy tissue from patients. We have used other primary non-mutant ECs such as HUVEC as a control and have shown that these cells are forming only a negligible number of small channels (Goines et al. 2018, Angiogenesis). A paragraph addressing this point was added to the discussion.

*** How do authors know that all EC carry the driver mutation? Is this culture heterogenous?**

R: This is a very good question: Isolated cells are routinely sequenced by Sanger-Sequencing as we have published recently (Goines et al. 2018, Angiogenesis). The cultures are heterogeneous in the sense that they arrive from a pool of several colonies and not from single cell clones. Our attempts to purify mutant ECs from non-mutant EC by single cell-derived clonal expansion were not successful. For this reason, we hypothesized that non-mutant EC from the same patient have lower clonogenic and thus proliferative potential compared to mutant EC. Taken this together, we speculate that after few passages our cultures are mostly composed by mutant EC .

*** It would be good to include a picture of a non pure isolation (mixed of ECs and fibroblasts) for the reader to understand what none pure looks like.**

R: This is a good point. We added a figure showing a culture of mixed cells before EC purification (Figure 2A).

Minor Concerns:

*** There are 3 groups which simultaneously discovered that venous malformations are cause by mutations in PIK3CA, this should be more clearly reflected in text (Limaye et al, and Castel et al, Castillo et al).**

R: Thank you for pointing this out. We included these references (3-5) and included a sentence to the introduction to specify that our protocol is also relative to TIE2 and PIK3CA mutations as our xenograft model can be performed with PIK3CA-mutated EC, as previously shown in Goines J et al., 2018, Angiogenesis.