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.