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TITLE:

In vitro Three-Dimensional Sprouting Assay of Angiogenesis using Mouse Embryonic Stem Cells for Vascular Disease Modeling and Drug Testing

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KEYWORDS:

mouse embryonic stem cells, sprouting angiogenesis, endothelial cell specification, vessel maturation, drug testing, disease modeling

SUMMARY:

This assay utilizes mouse embryonic stem cells differentiated into embryoid bodies cultured in 3D-collagen gel to analyze the biological processes that control sprouting angiogenesis *in vitro*. The technique can be applied for testing drugs, modeling diseases, and for studying specific genes in the context of deletions that are embryonically lethal.

ABSTRACT:

Recent advances in induced pluripotent stem cells (iPSC) and gene editing technologies enable the development of novel human cell-based disease models for phenotypic drug discovery (PDD) programs. Although these novel devices could predict the safety and efficacy of investigational drugs in humans more accurately, their development to the clinic still strongly rely on mammalian data, notably the use of mouse disease models. In parallel to human organoid or organ-on-chip disease models, the development of relevant *in vitro* mouse models is therefore an unmet need for evaluating direct drug efficacy and safety comparisons between species and *in vivo* and *in vitro* conditions. Here, a vascular sprouting assay that utilizes mouse embryonic stem cells differentiated into embryoid bodies (EBs) is described. Vascularized EBs cultured onto 3D-collagen gel develop new blood vessels that expand, a process called sprouting angiogenesis. This model recapitulates key features of *in vivo* sprouting angiogenesis—formation of blood vessels from a pre-existing vascular network—including

endothelial tip cell selection, endothelial cell migration and proliferation, cell guidance, tube formation, and mural cell recruitment. It is amenable to screening for drugs and genes modulating angiogenesis and shows similarities with recently described three-dimensional (3D) vascular assays based on human iPSC technologies.

INTRODUCTION:

In the past three decades, target-based drug discovery (TDD) has been widely employed in drug discovery by the pharmaceutical industry. TDD incorporates a defined molecular target playing an important role in a disease and relies on the development of relatively simple cell culture systems and readouts for drug screening[1]. Most typical disease models used in TDD programs include traditional cell culture methods such as cancer cells or immortalized cell lines grown within artificial environments and non-physiological substrates. Although many of these models have provided viable tools for identifying successful drug candidates, the use of such systems can be questionable owing to their poor disease relevance[2].

For most diseases, the underlying mechanisms are indeed complex and various cell types, independent signaling pathways, and multiple sets of genes are often found to contribute to a specific disease phenotype. This is also true for inherited diseases where the primary cause is a mutation in one single gene. With the recent advent of human induced pluripotent stem cell (iPSC) technologies and gene editing tools, it is now possible to generate 3D organoids and organ-on-chip disease models that could better recapitulate the *in vivo* human complexity[3,4]. The development of such technologies is associated with a resurgence in interest in phenotypic drug discovery (PDD) programs[1]. PDD can be compared to empirical screening, as they do not rely on knowledge of the identity of a specific drug target or a hypothesis about its role in disease. The PDD approach is now increasingly recognized to strongly contribute to the discovery of first-in-class drugs[5]. Because the development of human organoid and organ-on-chip technologies is still in its infancy, it is expected that IPSC models (complemented with innovative imaging and machine-learning tools[6,7]) will provide, in the near future, multiple novel complex cell-based disease models for drug screening and associated PDD programs to overcome the poor productivity of the TDD approach[8,9].

While human organoid and organ-on-chip models can provide important insights into disease complexity and to the identification of novel drugs, bringing drugs into new clinical practice also strongly relies on data from animal models to assess their efficacy and safety. Among them, genetically modified mice are certainly the most preferred mammalian models. They have many advantages as they have a relatively short generation time for mammals, have many similar phenotypes to human diseases, and can be easily genetically manipulated. They are therefore extensively used in drug discovery programs[10]. However, bridging the gap between mice and humans remains an important challenge[11]. The development of *in vitro* mouse models equivalent to human organoid and organ-on-chip models could at least partially fill this gap as it will allow direct drug efficacy and safety comparisons between *in vivo* mouse and *in vitro* human data.

Here, a vascular sprouting assay in mouse embryoid bodies (EBs) is described. Blood vessels are

composed of endothelial cells (inner lining of vessel walls), mural cells (vascular smooth muscle cells), and pericytes[12]. This protocol is based on the differentiation of mouse embryonic stem cells (mESCs) into vascularized EBs using hanging droplets that recapitulate *de novo* endothelial cell and mural cell differentiation[13,14]. Mouse ESCs can be easily established in culture from isolated day 3.5 mouse blastocysts having different genetic backgrounds[15]. They also provide possibilities for clonal analysis, lineage tracing, and can be easily genetically manipulated to generate disease models[13,16].

As blood vessels nourish all organs, it is not surprising that many diseases if not all, are associated with changes in the microvasculature. In pathological conditions, endothelial cells can adopt an activated state or can become dysfunctional resulting in mural cell death or migration away from blood vessels. These can result in excessive angiogenesis or in-vessel rarefaction, can induce abnormal blood flow and defective blood vessel barrier leading to immune cell extravasation, and inflammation[12,17–19]. Research for the development of drugs modulating blood vessels is, therefore, high, and multiple molecular players and concepts have already been identified for therapeutic targeting. In this context, the protocol described is particularly suitable for building disease models and for drug testing as it recapitulates key features of *in vivo* sprouting angiogenesis, including endothelial tip and stalk cell selection, endothelial cell migration and proliferation, endothelial cell guidance, tube formation, and mural cell recruitment. It also shows similarities with recently described 3D vascular assays based on human iPSC technologies[20].

PROTOCOL:

1. Media preparation and culture of mESC

1.1. Prepare conditioned medium +/- (CM+/-) using the supplement 1x Glasgow MEM (G-MEM BHK-21) medium with 10% (vol/vol) heat-inactivated Fetal Bovine Serum (FBS), 0.05 mM β-mercaptoethanol, 1x non-essential amino acids (NEAA 1x), 2 mM L-glutamine, and 1 mM sodium pyruvate.

1.2. Prepare conditioned medium +/+ (CM+/+) using the supplement CM+/- medium with Leukemia Inhibitory Factor (LIF) (1,500 U/mL) and 0.1 mM β-mercaptoethanol.

1.3. Prepare conditioned medium +/+ in the presence of two inhibitors (CM+/+ 2i) using the supplement CM+/+ medium with 1 µM PD0325901 and 3 µM CHIR99021.

1.4. Prepare 0.1% gelatin solution by mixing 25 mL of pre-warmed 2% gelatin solution in 500 mL of phosphate-buffered saline without calcium and magnesium (DPBS).

1.5. Prepare 10x Trypsin Versene Phosphate (TVP 10x) buffer by mixing Trypsin (2.5%) with TVP 1x (9.5% 10x DPBS, 1 mM EDTA, 0.01% chicken serum, 0.01% Trypsin (2.5%) in H_2O) (1:10 ratio, vol/vol).

NOTE: Filter all the solutions through a 0.22 µm pore filter. Store the culture medium at -20 °C for long term and keep other reagents at 4 °C for up to 3 weeks (see **Table of Materials**).

1.6. Coat two 12-well cell culture plates with 0.1% gelatin solution (500 µL per well) and incubate them for 30 min in a CO_2 incubator (37 °C, 5% CO_2, humid atmosphere).

1.7. Wash the gelatin-coated plates with PBS and add 500 µL of CM-/- medium.

1.8. Thaw two vials of 1 x 10^6 cryopreserved irradiated mouse embryonic fibroblasts (MEFs) at 37 °C and transfer the cell suspension to a conical tube with 5 mL of CM+/- medium.

1.9. Centrifuge the cells at 200 x *g* for 5 min at room temperature (RT). Aspirate the medium and gently resuspend the cell pellet in 12 mL of CM+/- medium at a concentration of 1.67 x 10^6 cells per mL.

1.10. Seed 500 µL of MEF suspension in a 12-well cell culture plate (2.4 x 10^5 cells per cm^2) and incubate the plate overnight (o/n) in a CO_2 incubator.

1.11. Thaw one vial of cryopreserved mESCs (1 x 10^6) in CM+/+ 2i medium.

1.12. Seed the mESC suspension on a pre-washed 12 well plate with MEFs in 1 mL of CM+/+ 2i medium and transfer the plate to a CO_2 incubator. Refresh the medium daily.

1.13. At 70% confluency, wash the mESC colonies with DPBS. Add 150 µL of warm 10x TVP buffer and incubate at RT for 30 s to initiate enzymatic dissociation.

1.14. Carefully remove the TVP buffer, resuspend the cells in 1 mL of CM+/+ 2i medium and dissociate the colonies into single cells by gentle pipetting.

1.15. Passage the cells by transferring them to a new 12-well cell culture plate with MEFs (splitting ratio: 1:3–1:5). Mix gently to distribute the cells and incubate in a CO_2 incubator.

1.16. Refresh the culture medium (2–3 mL) and observe the cell growth/morphology daily. Repeat serial passaging at 70% confluency every 2 days. Switch to CM+/+ medium for two passages before initiating cell differentiation.

2. EB formation in hanging drop

2.1. Prepare fresh mesoderm differentiation medium by supplementing the CM+/- medium with basic Fibroblast Growth Factor (bFGF) (50 ng·mL^{-1}) and with Bone Morphogenetic Protein 4 (BMP-4) (5 ng·mL^{-1}) and keep it at 4 °C until use.

2.2. Coat one well of the 6-well cell culture plate with 500 µL of 0.1% gelatin solution and place it in a CO_2 incubator for 30 min.

2.3. Wash the gelatin-coated plates with PBS and add 500 µL of CM+/- medium.

2.4. To obtain a pure population of mESCs, trypsinize the cell culture plate with 10x TVP buffer for 30 s at RT, resuspend the cells in 1 mL mesoderm differentiation medium and then transfer them to the gelatin-coated 6-well plate for 30 min allowing the MEFs to attach while the mESCs stay in suspension.

2.5. Collect the cell suspension in a 50 mL conical tube and count the cells using a Neubauer hemocytometer and Trypan blue dye for a live/dead cell exclusion.

2.6. Centrifuge the cells at 200 x *g* for 5 min at RT. Remove the supernatant and resuspend the cell pellet in mesoderm differentiation medium to reach 4.55 x 10^4 cells per mL.

2.7. Fill the bottom of 94 mm low attachment polystyrene dishes with 15 mL of sterile water.

NOTE: Four dishes containing hanging drops (1.6 x 10^5 cells in 3.52 mL of medium) will be required for testing one particular condition using the 3D sprouting angiogenesis assay.

2.8. Transfer the cell suspension into a sterile plastic reservoir and load four positions of a multichannel pipette with 22 µL of cell suspension per channel (1 x 10^3 cells per 22 µL drop).

2.9. Lift and invert the lid of the 94 mm dish and place it on the clean surface of the flow cabinet with the inner side facing upwards.

2.10. Deposit 40 drops of the cell suspension on the inner surface of each lid. Carefully invert the lid without disturbance and place it back on the dish, so that the drops face the water.

2.11. Incubate the dishes in a CO_2 incubator. Consider this as differentiation day 0. Maintain the plates for 4 days to form EBs.

3. Competition assay for the tip cell position

3.1. Culture one fluorescent and one non-fluorescent mESC line as previously described[13]. As an example, 7AC5/EYFP mESCs labeled in yellow and R1 mESCs are used.

3.2. Prepare mosaic EBs by mixing equal amounts of the two mESC lines (1:1 ratio) and incubate the hanging drop dishes in a CO_2 incubator as described in step 2.

4. Floating EBs culture for vascular differentiation

4.1. Before collecting EBs from the hanging drops, prepare the following.

4.1.1. Prepare 5% agar solution in H_2O and sterilize it by autoclaving (20 min at 120 °C).

4.1.2. Use the warm 5% agar solution to prepare G-MEM BHK-21 medium containing 1% agar and quickly pour 3 mL in one of the 60 mm polystyrene dishes. Allow the agar to solidify for 1 h at RT. Store the dishes at 4 °C until use.

4.2. Prepare fresh 2x vascular differentiation medium by supplementing the CM+/- medium with bFGF (100 ng·mL^{-1}) and VEGF-A (50 ng·mL^{-1}). Store the medium at 4 °C until use.

4.3. Collect the hanging drops in a 15 mL conical tube using a P1000 pipette and remove the supernatant after a few minutes of EB sedimentation.

4.4. Resuspend the EBs in 3 mL of 2x vascular differentiation medium, transfer the EB suspension to one agar-coated dish and distribute the EBs homogenously to avoid aggregation.

4.5. Incubate the dishes in CO_2 incubator and refresh the medium every 2 days until day 9 using 1x vascular differentiation medium in the presence of bFGF (50 ng·mL^{-1}) and VEGF-A (25 ng·mL^{-1}).

4.6. Alternatively, add Platelet Derived Growth Factor-BB (PDGF-BB) (10 ng·mL^{-1} on day 4 and 5 ng·mL^{-1} on day 6 and day 8) to the vascular differentiation medium to promote mural cell differentiation.

5. Flow cytometry analysis

5.1. Collect the 9-day-old EBs in a 15 mL conical tube using a P1000 pipette and wash them once with warm PBS.

5.2. Add 1mL of G-MEM BHK-21 medium containing 0.2 mg·mL^{-1} of collagenase A and incubate the cells in a CO_2 incubator for 5 min.

5.3. Dissociate the EBs by gently pipetting up and down with a P1000 pipette.

5.4. Stop the collagenase activity by adding 1 mL of G-MEM BHK-21 medium with 10% FBS.

5.5. Centrifuge the cells at 200 x *g* for 5 min at RT.

5.6. Resuspend the cells in 500 µL of PBS with 2% FBS.

5.7. Count the cells using a Neubauer hemocytometer.

5.8. Resuspend 400,000 cells in 100 µL of PBS with 2% FBS per staining condition.

5.9. Incubate the cells for 45 min at 4 °C with the following antibodies: APC conjugated rat anti-mouse PECAM-1 antibody (clone MEC13.3) and FITC conjugated rat anti-mouse CD45

(clone 30-F11) or isotype control antibodies.

5.10. Wash the cells twice with 1 mL of PBS containing 2% FBS.

5.11. Resuspend the cells to reach a final concentration of 5 x 10^6 cells per mL.

5.12. Filter the cells using a round bottom polystyrene test tube, with a cell strainer snap cap.

5.13. Analyze 20,000 PECAM-1 (+) events by flow cytometry.

6. 3D sprouting angiogenesis assay and immunofluorescence staining

6.1. At day 9, prepare a sprouting medium by adding 10% FBS (vol/vol), bFGF (50 ng·mL^{-1}), VEGF-A (25 ng·mL^{-1}), human recombinant Erythropoietin (hEPO) (20 ng·mL^{-1}), human interleukin-6 (IL-6) (10 ng·mL^{-1}), 0.05 mM β-mercaptoethanol, NEAA (1x), L-glutamine (1x), sodium pyruvate (1x), type I rat tail collagen (1.25 mg·mL^{-1}), and NaOH (3.1 mM) to GMEM BHK-1 medium.

6.2. To avoid collagen gelation, maintain the sprouting medium on ice until use.

6.3. To evaluate the effect of pro/anti-angiogenic molecules, add the selected drug or vehicle to the sprouting medium at the selected concentration.

6.4. Collect 9-day-old EBs from one 60 mm agar dish in a 15 mL conical tube (equivalent to one condition) and remove the supernatant after a few minutes of sedimentation.

6.5. Cover the bottom of a 35 mm culture dish with 1 mL of the sprouting medium and incubate at 37 °C for 5 min to induce gelation.

6.6. Resuspend the EBs in 2 mL of cold sprouting medium.

6.7. Transfer the suspension to the 35 mm culture dish coated with the first layer of sprouting medium.

6.8. Distribute the EBs all over the plate and ensure they are at an equal distance from each other. Incubate the dish in a CO_2 incubator. The first sprout formation happens within 24–48 h.

6.9. At day 12, the collagen gel containing EBs is carefully transferred to a glass slide (75 x 26 mm) using a spatula.

6.10. Remove the excess liquid using a pipette (P1000) and dehydrate the gel by placing a gauze sheet of nylon linen and absorbent filter cards (gel blotting paper) on top of the gel. Apply pressure by placing a weight (250 g) for 2min. Carefully remove the nylon/filter papers and allow the slides to air dry for 30 min at RT.

6.11. Wash the slides three times with PBS for 5 min at RT.

6.12. Fix the EBs using zinc solution (see **Table of Materials)** o/n at 4 °C. Alternatively, fix the mosaic fluorescent EBs with Paraformaldehyde (PFA) (4%) o/n at 4 °C in the dark.

6.13. Remove the fixative. Wash the slides five times with PBS for 5 min at RT.

6.14. Permeabilize the EBs in PBS containing 0.1% Triton-X100 for 15 min at RT.

6.15. Remove the permeabilization solution. Wash the slides five times with PBS for 5 min.

6.16. Incubate the EBs in the blocking buffer (PBS with 2% Bovine Serum Albumin, BSA) for 1 h at RT.

6.17. To stain the endothelial sprouts, use a rat anti-mouse anti-PECAM-1 primary antibody (1:100 dilution) in blocking buffer o/n at 4 °C.

6.18. Wash the slides five times with PBS for 5 min.

6.19. Incubate the slides with goat anti-rat Alexa 555 secondary antibody in blocking buffer (1:250 dilution) and when required, with FITC-conjugated anti-α-SMA antibody in blocking buffer (1:250 dilution) to stain mural cells for 2 h at RT in the dark.

6.20. Wash the slides three times with PBS for 5 min and one time with H_20 before mounting them.

7. Confocal imaging, morphometric, and quantitative analysis of EB endothelial sprouts

7.1. Acquire high-resolution images of the immunostained EBs by focal plane merging (z-stacking) using a confocal microscope. Use 10x magnification objective to image whole EBs.

7.2. Analyze the images acquired using ImageJ to evaluate the morphology and quantify the characteristics of PECAM-1 positive endothelial sprouts according to established quantification methods[13,14,21].

7.2.1. Calculate the mean number of endothelial sprouts per EBs by manually counting the total sprout number per individual EBs.

7.2.2. Measure the individual sprout lengths using the ImageJ drawing tool. Define the base of the endothelial sprout starting at the EB core area and manually draw a line until the sprout tip end.

7.2.3. Calculate the mean number of tip cells per sprout by manually counting the number of tip cells per individual sprout and then calculate the mean per EB.

7.2.4. Calculate the filopodia orientation by manually determining the axis of the parent sprout and measure the acute angle between them using the ImageJ software angle tool. Calculate the number of sprouts with an orientation >50° and divide it by the total number of sprouts of the EB of interest.

NOTE: The angles always ranged from 0° to 90°.

7.3. Acquire high-resolution images of the immunostained EBs using a confocal microscope. Use 40x magnification objective to realize high-resolution images of single endothelial sprouts.

7.4. Quantify the vessel coverage of PECAM-1 positive EC sprouts surrounded by α-SMA positive MCs using the ImageJ software.

7.4.1. Split the merged images into separate red and green channels.

7.4.2. Convert the images into its binary form.

7.4.3. Measure the total cellular area of the sprout occupied separately by PECAM-1 (endothelial cells labeled in red) and by α-SMA (mural cells labeled in green) positive cells.

7.4.4. Generate the merged image using the image calculator function and the AND operator. Measure the total cellular area of the image. To calculate the coverage, divide the area of the colocalized image by the area of the PECAM-1 binary image.

7.5. To analyze the cell competition for endothelial tip/stalk cell position of sprouts developed by mosaic EBs, manually score the number of tip cells and mark their genotypic origin based on the fluorescence signal. Calculate the mean values per EB.

REPRESENTATIVE RESULTS:

The protocol overview of the blood vessel sprouting assay is shown in **Figure 1**. Nine-day-old EBs derived from three independent 129/0la mESC lines (Z/Red, R1, and E14) were enzymatically dissociated into single cells using collagenase A. Cells were stained for PECAM-1 and analyzed by Fluorescence-activated cell sorting (FACS) as described. All the cell lines exhibited robust endothelial differentiation, and no differences in their ability to differentiate into endothelial cells were observed. All the cell lines produced about 10.5% ± 1.3% of endothelial cells (**Figure 2A**). The relative expression levels of PAN-endotelial cell markers in the PECAM1 (+) cell populations were also quantified. The mRNA expression levels of all the analyzed markers (*Flk1*, *Flt1*, *Flt4*, *Eng*, *Tie2*, and *Cdh5*) were comparable between the cell lines and the experiments, confirming the robustness of the differentiation protocol (**Figure 2B**). PECAM1 (+) cell populations only expressed very low mRNA levels of arterial (*Notch1* and *Efnb2*) or venous (*Couptf2* and *Ephb4*) markers supporting the relatively immature state of the

endothelial cells that were generated by the protocol (**Figure 2C**).

The ability of the vascular sprouting EB model to screen for drugs modulating angiogenesis was then demonstrated (**Figure 3**). DC101 and DAPT (N-[N-(3,5-difluorophenacetyl)-Lalanyl]-S-phenylglycine t-butylester) were tested. These compounds are widely used in mice to respectively block angiogenesis by inhibiting VEGFR2 activity or to promote endothelial tip cell differentiation and the formation of a dense vascular plexus by targeting Notch signaling (**Figure 3A**). Both VEGF and Notch signaling pathways are key regulators of sprouting angiogenesis *in vivo*. The effects of various concentrations of DC101 and DAPT in EBs plated in collagen I were evaluated. High doses of DC101 ranging from 6–30 $mg \cdot L^{-1}$ inhibited both the number and the length of the vessel sprouts while DAPT had opposite effects at the dose of 1 $\mu mol \cdot L^{-1}$ (**Figure 3B–C**). High magnification pictures of the vessel sprouts stained for PECAM1 of EBs cultured in the presence of DAPT is also provided (**Figure 3D**). DAPT even at low doses ranging from 0.5–1 $\mu mol \cdot L^{-1}$ strongly increased the number of endothelial tip cells with vessel sprouts that had a misguidance phenotype (**Figure 3D–F**). High dose of DAPT also led to vessel coalescence and the formation of large and flat endothelial cell areas without organization (**Figure 3A**). The results confirmed the ability of the model to test drugs that either promote or inhibit angiogenesis.

To confirm that this model is suitable for mimicking vascular diseases, the confocal images of EBs derived from *Acvrl1*$^{+/-}$ mESCs are provided. *Acvrl1* gene encodes for ALK1 (Activin Receptor-like Kinase 1), a receptor specifically expressed in endothelial cells that if mutated is responsible for the development of a vascular rare disease with angiodysplasia named Hereditary Hemorrhagic Telangiectasia (HHT). A high magnification picture of the *Acvrl1*$^{+/-}$ endothelial sprouts revealed that they had more endothelial tip cells and more branches per sprout that were at random angles relative to the parent vessels. These confirmed *in vitro* a misguidance phenotype as observed in HHT mice (**Figure 4**).

By forming chimeric EBs that contain differentiated mESC fluorescently-labeled and mESC of a particular genotype, an alternative protocol to study the process of endothelial tip selection is included (**Figure 5A–C**). A confocal image of PECAM1 labeled vessel sprouts identified the genotypic origin of the leading endothelial tip cells (**Figure 5B**). Mixtures of a wild-type YFP (yellow fluorescent protein) mESC line at 1:1 ratio with one unlabeled mESC wild-type line consistently led to the equal contribution of each population to the leading endothelial tip cells (**Figure 5C**).

This protocol is also suitable for quantifying mural cell coverage of the vessel sprout. EBs that undergo angiogenesis were fixed and stained for PECAM1 (endothelial cells, red) and for α-Smooth Muscle Actin (α-SMA) (mural cells, green) (**Figure 5D**). A high magnification image revealed how one individual vessel sprout was surrounded by mural cells (**Figure 5E**, left). Binary transformation was performed after color channel separation (**Figure F–G**) to quantify the ratio of PECAM (+) vessel covered by α-SMA (+) mural cells using the ImageJ software (**Figure 5H**).

FIGURE AND TABLE LEGENDS:

Figure 1: Timeline of the protocol procedures.

Figure 2: Characterization of ECs derived from mESC vascular differentiation inside EBs. (**A**) Flow cytometric analysis of CD31 expression from 9-day-old EBs and quantification of the percent of Pecam-1 (+) cells. (**B–D**) mRNA expression levels of *Pecam-1*, *Flk1*, *Flt1*, *Flt4*, *Eng*, *Tie2*, *Cdh5*, *Notch1*, *EfnB2*, *Couptf2*, and *EphB4* in sorted endothelial cells from 9-day-old EBs. Error bars represent the mean ± standard error of the mean (SEM).

Figure 3: 3D sprouting angiogenesis assay for drug testing. (**A**) Confocal images of three representative EBs stained for Pecam-1 (white, endothelial cells) treated with vehicle alone, DAPT (0.5 $\mu mol \cdot L^{-1}$, 1.0 $\mu mol \cdot L^{-1}$, 5.0 $\mu mol \cdot L^{-1}$) or DC101 (3 $mg \cdot L^{-1}$, 6 $mg \cdot L^{-1}$, 30 $mg \cdot L^{-1}$). (**B**) Quantification of the number of sprouts per EB. (**C**) Quantification of the sprout length. (**D**) On top-left panel, high magnification of endothelial sprout showing the network complexity and the branch point counting on two different layers from the same EB, on the bottom-left panel a schematic illustration representing the measurement method of endothelial sprout orientation. On the top-right panel, high magnification of endothelial sprouts from the vehicle alone and on the bottom-right panel, high magnification of endothelial sprouts from DAPT (0.5 $\mu mol \cdot L^{-1}$) condition. (**E**) Quantification of the number of tip cells per sprout. (**F**) Quantification of the percent of filopodia within angle >50°. All bars represent mean ± SEM and p values from unpaired, one-way ANOVA test. ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 4: Defective vessel sprouting in Hereditary Hemorrhagic Telangiectasia EBs. On the top panel, confocal images of representative 12-day-old EBs from *Acvrl1*$^{+/+}$ and *Acvrl1*$^{+/-}$ genotypes stained for Pecam-1 (white, endothelial cells). On the bottom panel, high magnification of *Acvrl1*$^{+/-}$ endothelial sprouts (white box from top panel) showing numerous tip cells (red arrows), endothelial branch points (blue dots) and sprouting misguidance phenotype (green arrows).

Figure 5: Studying tip/stalk cell position and vessel maturation using the 3D sprouting assay. (**A**) Confocal image of a representative 12-day-old chimeric EB made from R1 wild-type cell line mixed 1:1 with 7ACS/EYFP wild-type cell line stained for Pecam-1 (red, endothelial cells). Red arrows indicate tip cells from the R1 cell line and green arrows indicate tip cells from 7ACS/EYFP cell line. (**B**) High magnification of an endothelial sprout showing the distribution of R1 and 7ACS/EYFP endothelial cell along the sprout. (**C**) Quantification of the relative genotyping tip cells from representative wild-type EBs. (**D**) Confocal image of a representative 12-day-old EB stained for Pecam-1 (red, endothelial cells) and α-SMA (green, mural cells). (**E**) High magnification of an endothelial sprout (white rectangular dotted box from D) showing mural/endothelial cell interaction. (**F–G**) Images of the colored endothelial sprout and their associated binary transformed images. (**H**) Binary image of co-localized Pecam-1 and α-SMA staining. Ratio of endothelial cell sprouts covered by mural cells. All bars represent mean ± SEM.

DISCUSSION:

This protocol describes an unbiased, robust, and reproducible 3D EB-based vascular sprouting assay that is amenable to screening for drugs and genes modulating angiogenesis. This method offers advantages over many widely used two dimensional (2D) assays using endothelial cell cultures such as Human Umbilical Vein Endothelial Cells (HUVECs) to monitor migration (lateral scratch assay or the Boyden chamber assay)[22,23] or proliferation (counting cell number, detection of DNA synthesis, detection of proliferation markers, or metabolic assays)[24] in that it uniquely allows the study of both endothelial and mural cell differentiation and their organization into a vascular network mimicking key steps of sprouting angiogenesis. These steps include the endothelial tip cell selection, proliferation of the stalk cells, spatial orientation and migration of the vessel sprout, and the recruitment of mural cells to the nascent blood vessel[25]. It also offers advantages to many 3D angiogenesis models. The fibrin bead[26,27] or collagen gel assay[28–30] mimicking the tubulogenesis commonly use HUVECs or Endothelial Colony Forming Cells derived Endothelial Cells (ECFC-EC) as they have a high proliferative rate but are not suitable for mouse primary endothelial cells that are difficult to maintain in culture. The *ex vivo* retina explant[31] or vascularized micro-organ assay[32] can recapitulate well all the blood vessel formation steps but they have complex experimental procedures and are not suitable for high throughput drug or genetic screening. This is also true for the *ex vivo* aortic ring assay[33,34] and for many *in vivo* assays such as tumor implanted in mice or loss of function studies in mice that have often high cost and difficulties in obtaining large amount of data[35]. This protocol also nicely complements similar *in vitro* angiogenesis assays using human iPSCs allowing comparisons between mouse and human data. Although it is important to note that human iPSC-derived endothelial cells show less ability to sprout than the mouse cells[36,37].

The method developed here has also some limitations. It cannot evaluate the effects of fluid flow on blood vessel maturation, vessel permeability and does not produce nascent blood vessel in a specific tissue environment in comparison to recent microfabricated devices that are under development. Indeed, organ-on-chip technologies that combine microfluidics with tissue engineering can provide cultured endothelial cells with a microenvironment similar to that *in vivo*[38,39]. Microfluidic systems contain the correct extracellular matrix composition and are designed to produce mechanical signals such as shear stress. Some are designed to incorporate mural cells and other supportive cells of a given tissue or can generate chemical gradients. They contain networks of micron-scale fluid filled channels that are similar in size and in structure to the blood capillaries. The organ-on-chip technologies also enable the quantification of specific vascular functions, including the permeability and the trans endothelial electrical resistance. Although organ-on-chip technologies offer promise, they are as far beyond the research expertise of most biology laboratories, still need proper standardization, and require specialized fabrication techniques. Commercialization of organ-on-chip technology manufacture is only beginning, and these systems are considered cost-and-time prohibitive for pharmaceutical companies at present[40,41].

There are several critical steps that should be taken into account. Use high-quality cells which robustly expresses well-accepted markers (Nanog, Oct4, Sox2, and SSEA-1) of the pluripotent state. It is essential to carefully monitor their growth, mES cell shape, and the size and

morphology of mES colonies. As karyotypic stability is stochastic in cultured cells, reassessing it after extensive passaging is essential. It is recommended to use products already tested for mESC culture and test MEF feeders, Fetal calf serum, and all chemical compounds for several passages to detect whether mESCs maintain their cellular properties or whether they differentiate or acquire an epiblastic phenotype. The medium should be refreshed daily and mESC colonies should not be allowed to become too large and dense. Finally, mESCs need to be cultured at least for two passages without 2i before differentiation to ensure the best yield of endothelial cells.

The endothelial differentiation includes two important steps: the formation of EBs using the hanging drop method and their culture under floating conditions in the presence of vascular differentiation medium[13,14]. Movement of the hanging drop dishes should be minimized to achieve uniform cell aggregation. The number of mESCs used to form an aggregate in most cases, ranges between 800–1,000 cells, but it may need to be optimized if mESCs have a different genetic background than 129/ola to ensure an optimal differentiation into vascularized EBs. When cultured under floating conditions, EBs need to be carefully distributed and avoid movement that favors EB clumping.

EBs are finally cultured in collagen I gel to form vessel sprouts. Angiogenic medium should be freshly prepared and once mixed with collagen I must be maintained on ice to avoid spontaneous gelation. In case of drug testing, drugs are added in the cold mixture at the right concentration during this step. Adjusting the pH with NaOH before resuspending the EBs is crucial, otherwise collagen acidity will cause cell toxicity. Finally, EBs should be spread at equidistance from each other to ensure reproducible results.

In conclusion, this method introduces a 3D vascular sprouting assay based on mESC that has the required robustness and scalability to be used for genetic screening as recently described by Elling U. et al*.* that generated a large haplobank of hemi/homozygous mutant mESC[16] and for phenotypic drug discovery program.

ACKNOWLEDGMENTS:

This work was supported by grants from the Nederlandse organisatie voor gezondheidsonderzoek en zorginnovatie (ZonMW 446002501), Health Holland (LSHM19057-H040), Leading Fellows Programme Marie Skłodowska-Curie COFUND, and by the Association Maladie de Rendu-Osler (AMRO).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

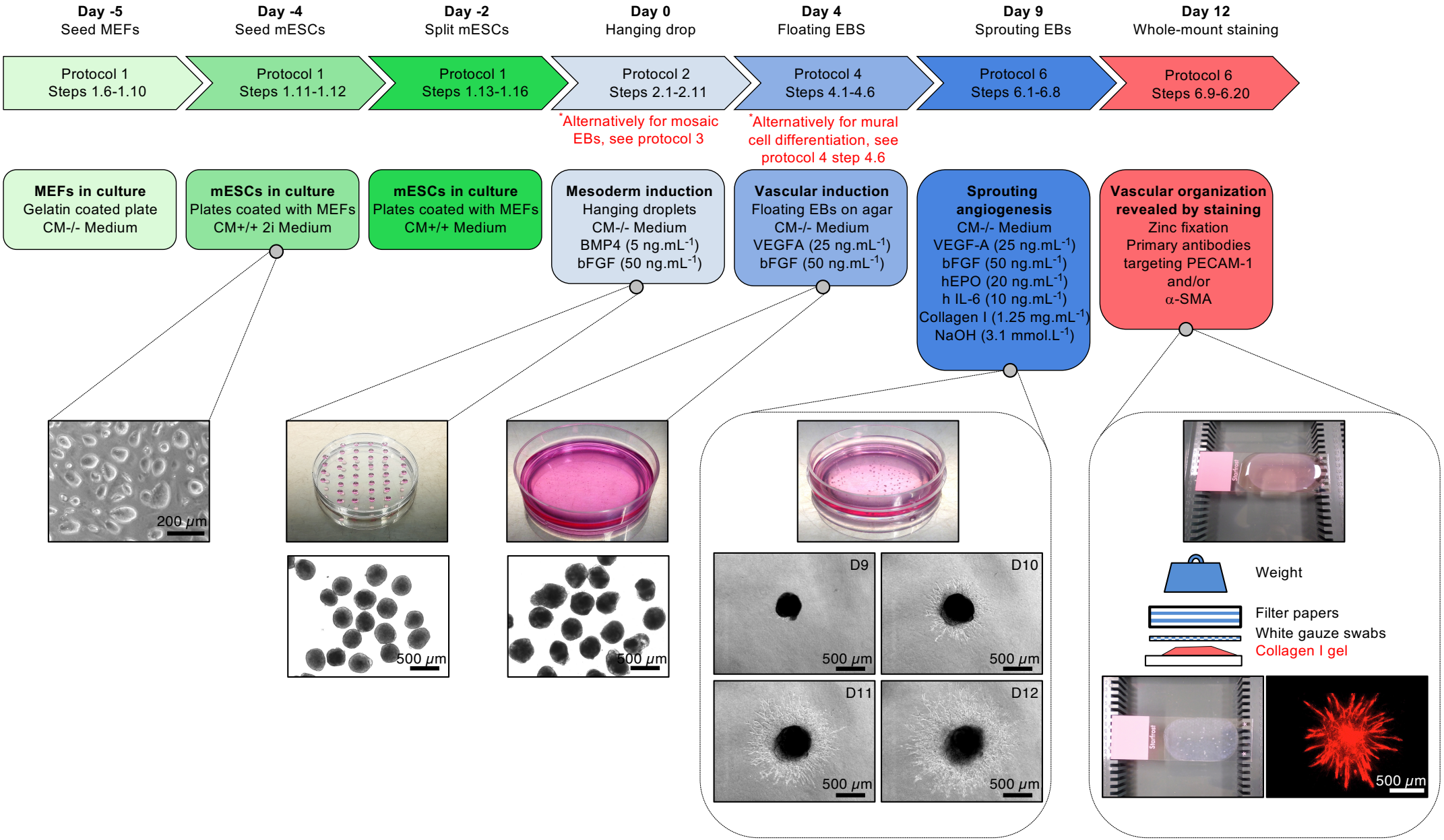


Figure 2

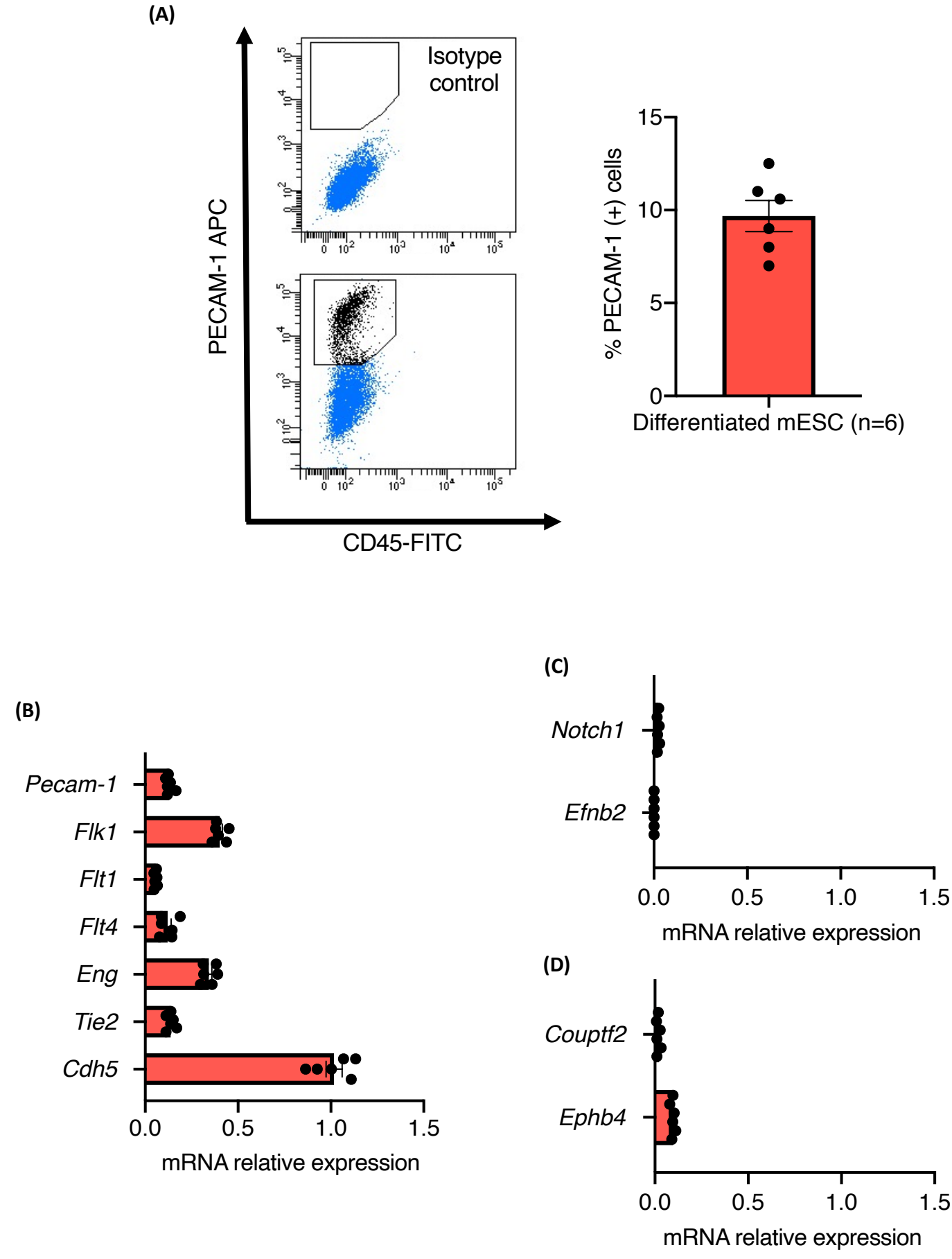


Figure 3

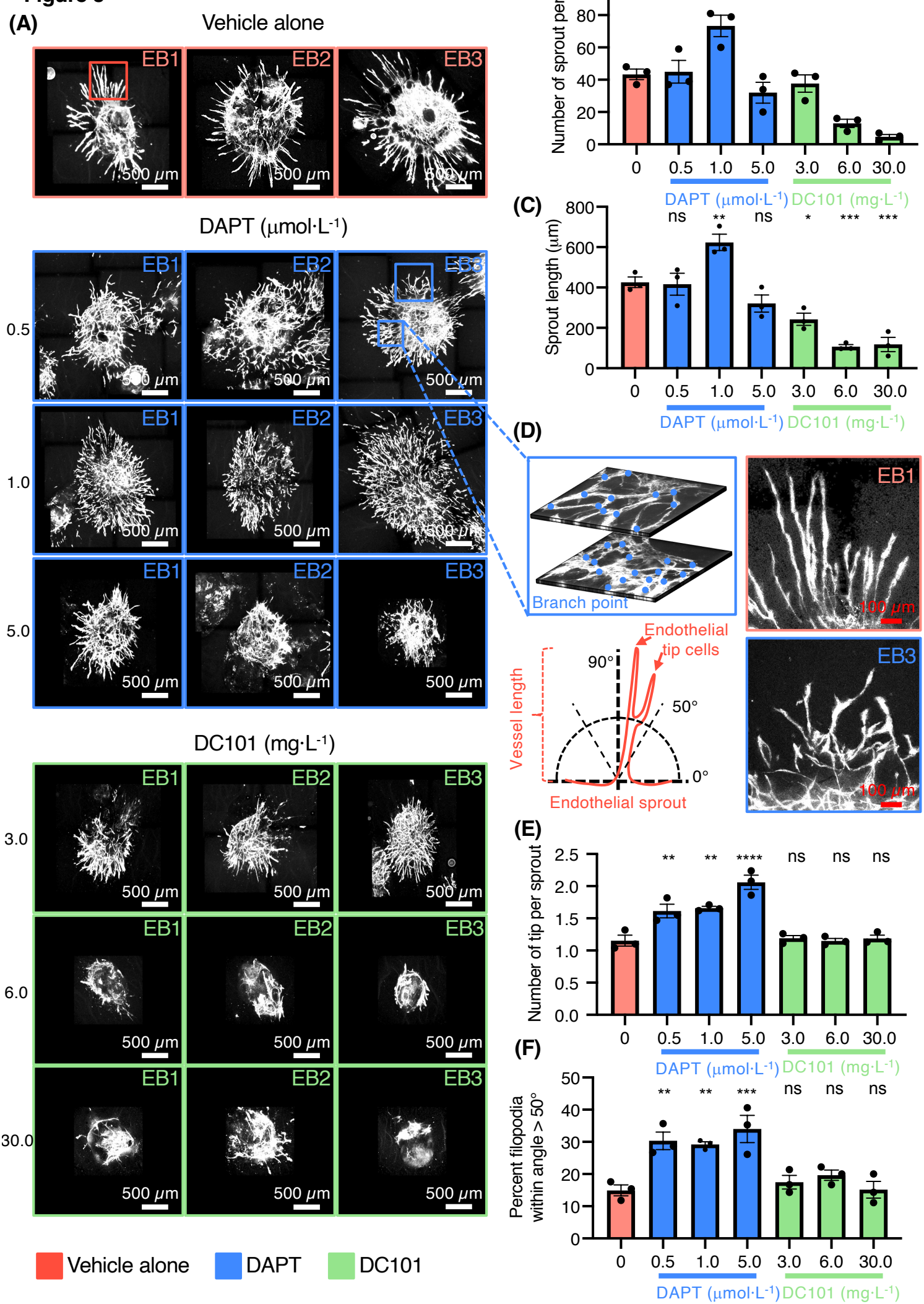
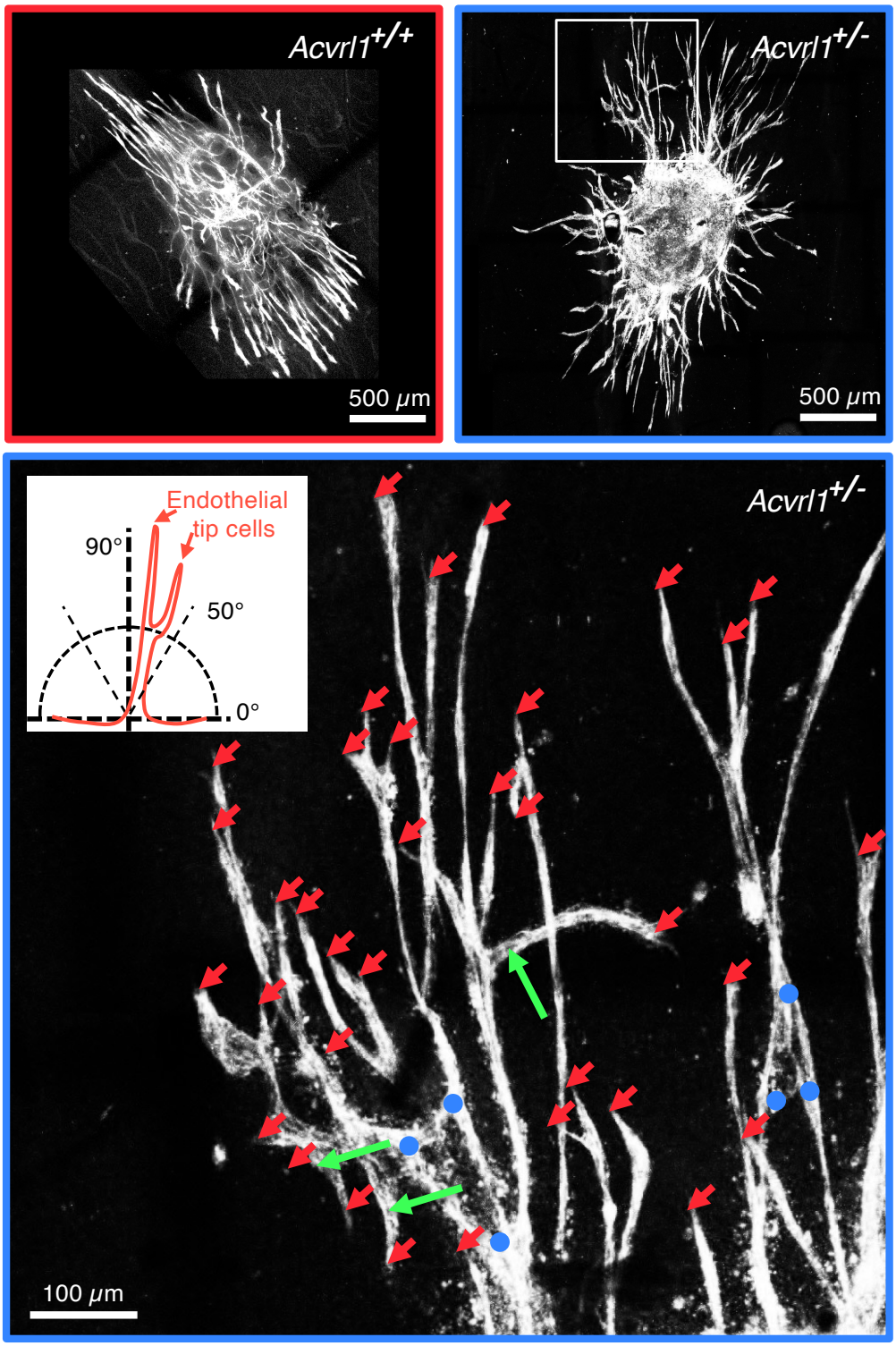


Figure 4



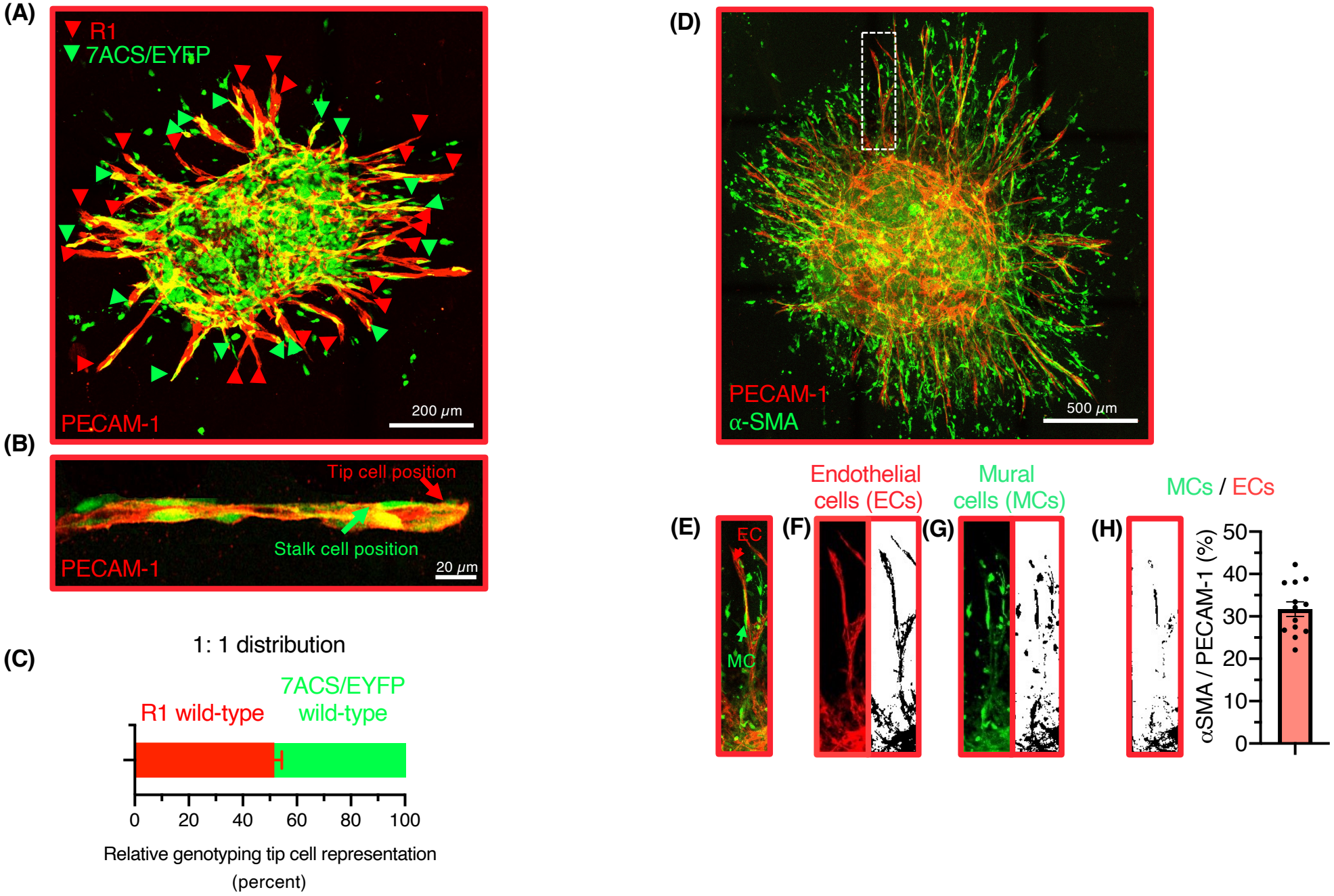


Table of Materials

Click here to access/download;Table of Materials;Table of Materials-62554_R1.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2-mercaptoethanol	Milipore, Merck	805740	Biohazard: adequate safety instructions should be taken when handling
Agar Noble	Difco, BD Pharmigen	214220	
Alexa Fluo 555 goat anti rat IgG	Life technologies	A21434	
APC conjugated rat anti-mouse PECAM-1 antibody (clone MEC13.3)	BD Biosciences	551262	
APC Rat IgG2a κ Isotype Control (Clone R35-95)	BD Biosciences	553932	
Axiovert 25 inverted phase contrast tissue culture microscope	ZEISS		
Basic Fibroblast Growth Factor-2 (bFGF)	Peprotech	450-33	
Benchtop Centrifuge, Allegra X-15R	Beckman Coulter	392932	
Biosafety cabinet BioVanguard (Green Line)	Telstar	133H401001	
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A9418	
Cell counting chamber, Buerker, 0.100mm	Marienfeld	640211	
Cell culture dishes 60 x 15mm	Corning	353802	
Cell culture dishes, 35 x 10 mm	Corning	353801	
Cell culture plates 12-well	Corning	3512	
CFX96 Touch Real-Time PCR Detection System	Biorad	1855196	
Chicken serum	Sigma-Aldrich	C5405	
CHIR-99021 (CT99021) HCl	Selleckchem	S2924	
Collagen I, High Concentration, Rat Tail, 100mg	Corning	354249	
Collagenase A	Roche	10103586001	
Confocal Laser Scanning Microscope, TCS SP5	Leica		
Cover glasses, 24 x 50 mm	Vwr	631-0146	
DAPT γ-secretase inhibitor	Sigma Aldrich	D5942	
DC101 anti mouse VEGFR-2 Clone	BioXcell	BP0060	
DC101 isotype rat IgG1	BioXcell	BP0290	
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2438-5X	Biohazard: adequate safety instructions should be taken when handling
DPBS (10x), no calcium, no magnesium	Gibco, Thermofisher scientific	14200067	
EDTA 40 mM	Gibco, Thermofisher scientific	15575-038	
Embryonic stem-cell Fetal Bovine Serum	Gibco, Thermofisher scientific	16141-079	Should be lot-tested for maximum ES cell viability and growth. Heat inactivate at 60°C and store at −20 °C for up to 1 year
Eppendorf Microcentrifuge 5415R	Eppendorf AG	Z605212	
Erythropoietin, human (hEPO), 250 U (2.5 µg) (1 mL)	Roche	11120166001	
ESGRO Recombinant Mouse LIF Protein (10^7 units 1 mL)	Milipore, Merck	ESG1107	
Falcon tubes 15 mL	Greiner Bio-One	188271	
Falcon tubes 50 mL	Greiner Bio-One	227270	
Filter tip ,clear ,sterile F.Gilson, P-200	Greiner Bio-One	739288	
Filter tip ,clear ,sterile F.Gilson, P10	Greiner Bio-One	771288	
Filter tip ,clear ,sterile F.Gilson, P1000	Greiner Bio-One	740288	
FITC conjugated anti-α Smooth Muscle Actin (SMA) (clone 1A4)	Sigma Aldrich	F3777	
FITC conjugated rat anti-mouse CD45 (clone 30-F11)	Biolegend	103107	
FITC Rat IgG2b, κ Isotype Ctrl Antibody (clone RTK4530)	Biolegend	400605	
Fluorscent mounting media	DAKO	S3023	
Gascompress	Cutisoft	45846	
Gauze Cutisoft 10 x 10 cm	Bsn Medical	45844_00	
Gel blotting paper, Grade GB003	Whatman	WHA10547922	
Gelatin solution, type B	Sigma-Aldrich	G1393-100 ml	
Glasgow's MEM (GMEM)	Gibco, Thermofisher scientific	21710082	
IHC Zinc Fixative	BD Pharmigen	550523	
IncuSafe CO2 Incubator	PHCBi	MCO-170AICUV-PE	
Interleukin-6, human (hIL-6)	Roche	11138600001	
L-Glutamine 200 mM	Gibco, Thermofisher scientific	25030-024	
MEM Non-Essential Amino Acids Solution (100x)	Gibco, Thermofisher scientific	11140035	
Microscope slide box	Kartell Labware	278	
Microscope slide, Starfrost	Knittel glass	VS113711FKB.0	
Mm_Cdh5_1_SG QuantiTect Primer Assay	Qiagen	QT00110467	
Mm_Eng_1_SG QuantiTect Primer Assay	Qiagen	QT00148981	
Mm_Epha4_1_SG QuantiTect Primer Assay	Qiagen	QT00093576	
Mm_Ephb2_1_SG QuantiTect Primer Assay	Qiagen	QT00154014	
Mm_Flt1_1_SG QuantiTect Primer Assay	Qiagen	QT00096292	
Mm_Flt4_1_SG QuantiTect Primer Assay	Qiagen	QT00099064	
Mm_Gapdh_3_SG QuantiTect Primer Assay	Qiagen	QT01658692	
Mm_Kdr_1_SG QuantiTect Primer Assay	Qiagen	QT00097020	
Mm_Notch1_1_SG QuantiTect Primer	Qiagen	QT00156982	
Mm_Nr2f2_1_SG QuantiTect Primer Assay	Qiagen	QT00153104	
Mm_Pecam1_1_SG QuantiTect Primer	Qiagen	QT01052044	
Mm_Tek_1_SG QuantiTect Primer Assay	Qiagen	QT00114576	
Mouse (ICR) Inactivated Embryonic Fibroblasts (2 M)	Gibco, Thermofisher scientific	A24903	Store vials in liquid nitrogen (195.79 °C) indefinitely
Mouse embryonic stem cell line 7AC5/EYFP (ATCC SCRC-1033)	ATCC	SCRC-1033	Generated by Dr A Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave, Toronto, Ontario, M5G 1X5, Canada. [Hadjantonakis, A. K., et al. *Mechanisms of Development* . **76** (1–2), 79–90 (1998)].
Mouse embryonic stem cell lines Acvrl1 +/- and Acvrl1 +/+			Generated at Leiden University Medical Centre [Thalgott, J.H. et al. *Circulation* . **138** (23), 2698–2712 (2018)].
Mouse embryonic stem cells line E14			Provided by M Letarte laboratory and generated according to Cho, S. K., et al. *Blood* . **98** (13), 3635–3642 (2001).
Mouse embryonic stem cells line R1 (ATCC SCRC-1011)	ATCC	SCRC-1011	Generated by Dr A Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave, Toronto, Ontario, M5G 1X5, Canada. [Nagy, A., et al. *Procedings of the National Academy of Sciences of the United States of America* . **90** (18), 8424–8428 (1993)].
Mouse embryonic stem cells line Z/Red (strain 129/Ola)			Generated by Dr A Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave, Toronto, Ontario, M5G 1X5, Canada [Vintersten, K., et al. *Genesis* . **40** (4), 241–246 (2004)].
NanoDrop 1000 UV/VIS Spectrophotometer	Thermo Fischer Scientific	ND-1000	
PD0325901	Selleckchem	S1036	
PDGF-BB, Recombinant Human	Peprotech	100-14B	
Pecam-1 antibody, Rat Anti-Mouse	BD Biosciences	550274	
Penicillin-streptomycin (10,000 U/mL)	Gibco, Thermofisher scientific	15140122	
Petri dish, PS, 94/16 mm, standard ,with vents, sterile	Greiner Bio-One	633181	
Pipetboy acu 2	Integra-Biosciences	155 019	
Pipetman G Multichannel P8 x 200G	Gilson	F144072	
Pipetman G Starter Kit, 4 Pipette Kit, P2G, P20G, P200G, P1000G	Gilson	F167360	
Recombinant Human BMP-4 Protein	R&D Systems	314-BP	
RNeasy Plus mini Kit	QIAGEN	74134	
Serological pipettes, 10 mL	Greiner Bio-One	607 180	
Serological pipettes, 25 mL	Greiner Bio-One	760 180	
Serological pipettes, 5 mL	Greiner Bio-One	606 180	
Sodium hydroxide (NaOH)	Merck	106498	
Sodium pyruvate 100 mM	Gibco, Thermofisher scientific	11360039	
Test tubes 5ml round-bottom with cell-strainer cap	Corning	352235	
Thermal cycler, T100	Biorad	1861096	
Triton X-100 (BioXtra)	Sigma Aldrich	T9284	
Trypan Blue Solution, 0.4%	Gibco, Thermofisher scientific	15250061	
Trypsin (2.5%)	Gibco, Thermofisher scientific	15090046	
Vacuum Filter/Storage Bottle System, 500 mL	Corning	430758	
$VEGFA^{165}$, recombinant murine	Peprotech	450-32	
Water, Sterile	Fresenius-Kabi	B230531	
Waterbath, Lab-Line Digital	Thermo Fischer Scientific	18052A	

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your reference
datum April 15, 2021
subject letter
pages 9

to Dr. Amit Krishnan
Review editor
Journal of Visualized Experiments

Dear Dr. Amit Krishnan,

Thank you for the input into our manuscript "*In vitro* three-dimensional sprouting assay of angiogenesis using mouse embryonic stem cells for vascular disease modeling and drug testing". We are submitting a revised manuscript in which we have addressed all points raised by yourself and the referees and include a point by point response.
We hope that you and the referres will agree and we look forward to your response.
Best regards
Franck Lebrin

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We apologize for the spelling and grammar errors. They have been corrected.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

This has been corrected as suggested.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Please refrain from using bullets or dashes.

This has been corrected as suggested.

4. Line 115-125: The Protocol should contain only action items that direct the reader to do something. For example: “Prepare conditioned medium using supplement 1X Glasgow MEM (G-MEM BHK-21) medium 116 with 10% (vol/vol) heat-inactivated Fetal Bovine Serum (FBS), 0.05 mM β-mercaptoethanol, 117 1X non-essential amino acids (NEAA 10x), 2 mM L-glutamine and 1 mM sodium pyruvate”.

This has been corrected as suggested.

5. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Corrected.

6. Please title case and italicize journal titles and book titles in the Reference section. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

We apologize. This has been corrected.

7. Figure 3: Please include scalebars in all the images of the panel. Include the details of the magnification in the Representative Results section to make it more informative.

Thank. Figure 3 has been modified as requested.

Reviewer #1:

This paper by Galaris and colleagues aimed to describe the putative use of mouse ESC-derived embryoid bodies for vascular specific phenotypic assays like sprouting or characterisation of the endothelial specification. The authors suggest that these assays can be useful assets for drug discovery and development. They also argue for the benefits of vascularised (mouse) EBs as a 3D model. Although I do not share the authors' enthusiasm for rodent lines in human drug development; nevertheless, the protocols are well written with convincing figures. Specific question:

1. The authors claim that this protocol is "an unbiased, robust and reproducible three-dimensional EB-based assay". Therefore, if any steps of the protocol may be scalable or require manual steps? Was there any difference in mESC lines that the authors used here to manufacture a large number of homogenous EBs?

No. Our lab has worked with multiple 129/Ola mESC lines over the past decade. All wild-type lines studied have been differentiated as described in this protocol to form endothelial sprouts in collagen I gel. As far, they showed similar ability to form EBs (size and shape evaluated by light microscopy), produced the same yield of endothelial cells and have similar ability to sprout when plated in collagen I. Moreover, we have also characterized the CD31+ population isolated from day-9-old EBs generated from different 129/Ola mESC lines by RNA sequencing and found that these cells had similar mRNA expression profiles (not included in the present protocol).

2. Was there a potential difference between lines? By this protocol, was the size and structure of EBs reproducible?

Yes. We have not observed obvious difference in term of size and EB shape between lines. For clarity, photos are now included in figure 2.

3. Is there a reliable way to count cells in 3D constructs? Chamber does not seem to appropriate.

No. We don’t count EBs in 3D but dissociate them for FACS analysis. We apologize for the lack of clarity. A protocol is now included in page 5, lines 246-265.

4. Line 438. It is not entirely clear how this platform is suitable for genetic screening studies. The obvious difference in species may cast doubt on its value.

Sorry for the lack of clarity. This refers to the paper published by Elling U. *et al.,* (Nature, 2017; 550(7674): 114-118) that has generated the larger haplobank of hemi/homozygous mutant mES cells by reversible mutagenesis and did show how this approach was useful to screen for genes implicated in sprouting angiogenesis.

This reference is now included and the following sentence has been added:

“to be used for genetic screening as recently described by Elling U. *et al.* that have generated a large haplobank of hemi/homozygous mutant mESC (Elling et aL., 2017, Nature) and for phenotypic drug discovery program”.

Minor:

1. The manuscript needs to be checked for multiple typos, spelling errors, English language and formatting.

We apologize and have corrected them.

2. Line 275: "(red channel)" and "(green channel)" both need rephrasing.

This has been corrected. The following sentence is now included:
“Measure the total cellular area of the sprout occupied separately by PECAM-1 (endothelial cells labeled in red) and by **α**-SMA (mural cells labeled in green) positive cells”.

3. Line 413. "Use high quality mESCs". This statement is rather vague and may require further instructions.

The referee is correct that this is vague. We have therefore added the following sentence:

“Use high quality mESCs. They should robustly express well accepted markers of the pluripotent state such as Nanog, Oct4, Sox2 and SSEA-1. It is essential to monitor carefully their growth, mES cell shape and the size and morphology of mES colonies. Because, karyotypic stability is stochastic in cultured cells, it should also be reassessed after extensive passaging”.

4. Regarding reference 35 (consensus guidelines on angiogenesis), I would suggest referring to this better and describing novelties/similarities versus the already available other platforms.

We thank the referee for his comment. However, we believe that the recent review summarizing the various angiogenesis models including advantages and limitations for each method by Nowak-Sliwinska, P. *et al.* nicely summarize issues regarding each

model of angiogenesis including mice and microfluidics combined with vascularized OoC. Off course, it was not our intention to ignore the recent data on vascularized OoC in fact these systems are one of the most interesting discoveries on angiogenesis models. We have therefore included additional references related to these specific technologies.

Reviewer #2:

In this manuscript, Galaris et al. explain the embryoid body model derived from mouse embryonic stem cells (mESCs) to recapitulate key features of in vivo sprouting angiogenesis, which has been reported previously, both in mouse and human models. The main relevance of this work is the detailed method in which the authors show its relevance in exploiting to screen for drugs and genes modulating angiogenesis. The manuscript is well written and focused. I have only minor concerns with this work.

Line 77. Whist? Whilst?
The referee is correct, it should read whilst and not whist.

Line 79. Please, reason why human organoids and organ-on-chip model strongly rely on data from animal models to assess their efficacy and safety.

Sorry for the lack of clarity, we refer to bringing drugs into new clinical practice that rely to animal models to have access to PK and PD data. We have now modified the text to clarify this point. The following sentence has been added:

“Whilst human organoid and organ-on-chip models can provide important insights into disease complexity and to the identification of novel drugs, bringing drugs into new clinical practice also strongly rely on data from animal models to assess their efficacy and safety”.

Line 113. It is inconvenient to have the title with the number 1 and the subtitles same, 1, 2, 3…
Would at least be better to write Protocol 1. Medium and reagents for mESC cell culture (is "cell" not excessive in this case, since the C in mESC is abbreviation for cell?). Same goes for Protocol 2 etc…

The numbering and the text have been modified as requested.

Line 141. "1X106" - X missing

Corrected

Line 164. " stain in suspension" - stay? in suspension

Yes, this has been corrected

Line 184. "as an example"

Corrected

Line 192 and 193. "use a and b instead of 1 and 2 again"

Yes, this has been corrected

Line 197. "The medium can be stored it at 4…" - delete it.

This has been deleted

Line 203. Here and throughout the manuscript basic fibroblast growth factor is abbreviated b-FGF or even bFGF2 (in flow chart and table of materials). To my knowledge it is either bFGF or FGF2.

We apologize for these inconsistencies and have now corrected them.

Line 203. "in the presence of … "

Corrected

Line 213 . "NAOH" - NaOH

Corrected

Line 232. Protocol 8-2: This is an interesting approach to dehydrate the gel - researchers may be more interested in this procedure as the dehydration is often problematic. Please, address this method in more detail or have a figure legend in Fig1. In addition, the table of materials used does not include the items used.

This is a good point. We have included in the table of materials all information and we will use the movie to clearly describe the procedure to dehydrate the gel.

Line 236. "Wash the slides 3 times".

Corrected

Line 237. "Fixate" - fix.

Corrected

Line 261. "EB soma" - ?

We apologize for the lack of clarity. The following sentence has been added: “Define the base of the endothelial sprout starting at the EB core area and manually draw a line until the sprout tip end”.

Line 276. "colocalize" - merged?

Corrected

Line 287. "Collagenase A" - Collagenase A has not been mentioned before in the protocol - only TVP.

Line 288. "analyzed by FACS as described" - has FACS been described before?

The referee is correct. We have now included a short paragraph describing the FACS analysis and the cell dissociation method including the use of collagenase A. All antibodies and chemical compounds are now included in the supplementary table

Line 237. "mRNA expression levels of all markers that have been analyzed (….) were comparable" - What does this mean? They are not comparable but just present.

We apologize for the lack of clarify. We have now made clear that we do compare the expression levels of pan-endothelial cell markers between 3 independent mESC lines. We have now included the following sentence: “The mRNA expression levels of all analyzed markers (*Flk1*, *Flt1, Flt4, Eng, Tie2* and *Cdh5*) were comparable between lines and experiments confirming the robustness of the differentiation protocol (**Figure 2B)”**.

Line 237. "Activin like Kinase Receptor 1"- Activin Receptor-like kinase 1

This has been corrected

Line 323. "genotypic origing"- genotypic origin

This has been corrected

Line 329. "Binamy"- Binary

Corrected

Line 349. Please, use top right and bottom right to depict vehicle alone and DAPT, respectively.

The referee is correct. The text has been changed as follow: “On the top right panel, high magnification of endothelial sprouts from vehicle alone and on the bottom right panel, high magnification of endothelial sprouts from DAPT (0.5µmol.L^{-1}) condition.

Line 329. "12 days old"- 12 day old (more of this, please check in find)

We apologize and have now corrected them

Line 356. "Acvrl1…. models"- mice

Corrected

Line 367. "representation from representative wild-type EBs"- Delete representation

Corrected

Line 367. "representative12 days old EB"- representative 12 day old EB

Corrected

Line 380. "HUVECS"- HUVECs

Corrected

Line 384. "sprounting"- sprouting

Corrected

Line 387. "It also offer advantages to many three-dimentional angiogenesis"- "It also offers advantages to many three-dimentional angiogenesis models

Corrected

Line 392. "much more difficult to implement"- Please, explain why?

This refers to the complexity of the procedures when compared to most *in vitro* models. We have now modified the text as follow for clarity: “The *ex vivo* retina explant[31] or vascularized micro-organ assay[32] can recapitulate well all blood vessel formation steps but they have complex experimental procedures and are not suitable for high throughput drug or genetic screening”.

Line 398. "and do not produce"- and does not produce

Corrected

Line 417. "not let to become"- not allowed to become

Corrected

Line 419. "the best production of endothelial cells"- what is best production here? Quality of ECs or the best yield of ECs - or both?

The referee is correct that is vague. We have corrected and made clear that it was the yield.

Line 422. "in the presence"

Corrected

In general in discussions, it would be an advantage to mention similar approaches done

This point is well taken and the text has been modified to include recent data on human iPSCs as follow: “This protocol also nicely complement similar *in vitro* angiogenesis assays using human iPSCs allowing comparisons between mouse and human data. Although it is important to note that human iPSC-derived endothelial cells show less ability to sprout than the mouse cells (Belair G et al., acta Biomater.; Bezenah J. et al., 2018)”.

Line 430. "EBs are finally cultured onto collagen I gel"- is it onto or in?

Corrected.

Figure 2B - Pecam-1 vs Cdh5 - not consistent (Pecam-1, CD31), please, be consistent

Figure 2B refers to mouse gene and is therefore labeled *cdh5* and *Pecam1*

Figure 2C - might be nice to depict arterial vs venous genes on the Figure.

It has been modified as suggested and data are now provided as figure 2C and 2D.