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Generation of Human Blood Vessel Organoids from Pluripotent Stem Cells

Nicolas Werschler[1,2], **Josef Penninger**[1,2,3,4]

[1] School of Biomedical Engineering, University of British Columbia [2] Life Sciences Institute, University of British Columbia [3] Department of Medical Genetics, University of British Columbia [4] Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA)

Corresponding Author

Nicolas Werschler
nico.werschler@ubc.ca

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Abstract

An organoid is defined as an engineered multicellular *in vitro* tissue that mimics its corresponding *in vivo* organ such that it can be used to study defined aspects of that organ in a tissue culture dish. The breadth and application of human pluripotent stem cell (hPSC)-derived organoid research have advanced significantly to include the brain, retina, tear duct, heart, lung, intestine, pancreas, kidney, and blood vessels, among several other tissues. The development of methods for the generation of human microvessels, specifically, has opened the way for modeling human blood vessel development and disease *in vitro* and for the testing and analysis of new drugs or tissue tropism in virus infections, including SARS-CoV-2. Complex and lengthy protocols lacking visual guidance hamper the reproducibility of many stem cell-derived organoids. Additionally, the inherent stochasticity of organoid formation processes and self-organization necessitates the generation of optical protocols to advance the understanding of cell fate acquisition and programming. Here, a visually guided protocol is presented for the generation of 3D human blood vessel organoids (BVOs) engineered from hPSCs. Presenting a continuous basement membrane, vascular endothelial cells, and organized articulation with mural cells, BVOs exhibit the functional, morphological, and molecular features of human microvasculature. BVO formation is initiated through aggregate formation, followed by mesoderm and vascular induction. Vascular maturation and network formation are initiated and supported by embedding aggregates in a 3D collagen and solubilized basement membrane matrix. Human vessel networks form within 2-3 weeks and can be further grown in scalable culture systems. Importantly, BVOs transplanted into immunocompromised mice anastomose with the endogenous mouse circulation and specify into functional arteries, veins, and arterioles. The present visually guided

protocol will advance human organoid research, particularly in relation to blood vessels in normal development, tissue vascularization, and disease.

Introduction

Vascular dysfunction and blood vessel diseases present with marked complications in organ functions. Cardiovascular disease (CVD) is the leading cause of death worldwide[1] and is also the primary contributing factor to increasing healthcare costs in the United States. CVD case numbers are increasing annually, and rising numbers of these cases are occurring in younger age groups (20-45 years)[2]. Multiple *in vivo* models have been developed to explore the development and maturation of blood vessels, vascular disease, and endothelial dysfunction[3,4]. Currently, methods combining single and multiple lineage-defined cells either derived from stem cells or isolated from adult tissues *in vivo* can create vascular networks that replicate aspects of human vascular function and anatomy[5,6]. Blood vessels have emerged as one of the first functional systems during development from the mesoderm, and they organize either through a process of assembly called "vasculogenesis" or by expansion and branching from pre-existing vessels, which is termed "angiogenesis"[7].

Leveraging the power of developmental biology and self-directed assembly, Wimmer et al. reported the first self-organizing 3D human blood vessel organoids from hPSCs that exhibit the functional, morphological, and molecular characteristics of human microvasculature[8]. Similar to human vasculature[9], these hBVOs are generated and present with an endothelium, a continuous basement membrane, and surrounding mural cells[8,10]. The hBVOs can be transplanted *in vivo* and anastomosed with the endogenous circulation. They can also undergo maturation *in vitro* and serve as models for cardiovascular diseases (i.e., diabetes)[8] or tissue tropism in virus infections such as SARS-CoV-2[11]. While we previously published a written protocol[10], there exists no available video protocol for this otherwise complex technique.

Through a concise stepwise progression, hBVO formation is accomplished through aggregate formation, mesoderm induction using a WNT agonist, Chiron (CHIR99021), and bone morphogenic protein - 4 (BMP4)[12,13], vascular induction *via* vascular endothelial growth factor A (VEGFA) and Forskolin (Fors)[12], and embedding in a custom sprouting matrix[8,10]. Vascular maturation and network formation follow the embedding of the aggregates in the sprouting matrix. These human vessel networks form within 2-3 weeks and can be removed from the sprouting matrix and further grown in scalable culture systems for up to 6 months. Here, optically guided procedures are provided for the formation and application of human stem cell-derived vasculature.

Protocol

All experiments performed herein used the commercially available H9 human iPSC line. Common commercially and non-commercially available human pluripotent stem cell lines (i.e., H9, NC8) have also been tested and proven effective for the generation of human blood vessel organoids using this protocol. For details, please refer to our previously published reports[8,10].

1. Media and reagent formulation for the generation of human blood vessel organoids

1. Prepare the aggregation medium.
    1. Mix 40 mL of knock-out DMEM/F12 (low osmolality medium without L-glutamine or HEPES buffer optimized for human ESC and iPSC growth), 10 mL of knock-out serum replacer (KOSR), 0.5 mL of 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl, 0.5 mL of non-essential amino acids (NEAA), 35 µL of beta-mercaptoethanol (BME, 100 µL of 2-mercaptoethanol in 10 mL of sterile PBS), and Y-27632 (cell-permeable and selective inhibitor of Rho-associated, coiled-coil containing protein kinase [ROCK][14] [10 mM] at 1:200) (see **Table of Materials**).
2. Prepare N2B27 (N2 and B27 supplemented basic medium).
    1. Mix 25 mL of DMEM/F12, 25 mL of neurobasal media, 1 mL of B27 supplement, 0.5 mL of N2 supplement, 250 µL of 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl, and 35 µL of beta-mercaptoethanol (see **Table of Materials**).
3. Prepare the mesoderm induction medium.
    1. Supplement N2B27 medium with ChiR (12 µM, GSK3a/b inhibitor stimulating mesoderm induction) and BMP-4 (30 ng/mL, MSX2 activator inducing mesoderm lineage).
    NOTE: Stock (ChiR): 10 mM (use 1:833, or 1.2 µL/mL N2B27). Stock (BMP-4): 100 µg/mL (use 1:3333, or 0.3 µL/mL N2B27) (see **Table of Materials**).
4. Prepare vascular induction medium.
    1. Supplement N2B27 medium with VEGFA (100 ng/mL) and forskolin (2 µM) (see **Table of Materials**).
5. Prepare the extracellular matrix (ECM).
    1. Use 1 mL of ECM for one well of a 12-well plate (for embedding 30-50 aggregates). Of the 1 mL of ECM solution used for each well, use 0.5 mL to create a bottom layer, "layer 1", which will serve as an ECM foundation, and 0.5 mL plus aggregates for the top layer, "layer 2". Using this approach provides, through the ECM suspension, sufficient space and support for the human blood vessel networks to sprout in all directions.
    NOTE: Overall, 1 mL of ECM contains 500 µL of purified bovine collagen type I, 250 µL of solubilized basement membrane matrix secreted by Engelbreth-Holm-Swarm mouse sarcoma cells (see **Table of Materials**), and 250 µL of basic matrix solution (step 1.5.2). Prepare it fresh and keep on ice until ready to use.
    2. To prepare the basic matrix solution for four 12-well plates (48 wells), mix 5.627 mL of 0.1 N NaOH, 2.498 mL of 10x DMEM, 473 µL of HEPES, 368 µL of 7.5% sodium bicarbonate, 233 µL of 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl, and 3.451 mL of Ham's F-12 (see **Table of Materials**).
    NOTE: The leftover basic matrix solution can be placed in a capped 50 mL high-clarity polypropylene conical tube and stored at 4 ˚C for use for up to 2 months.
6. Prepare the sprouting medium.
    1. Specifically formulate 50 mL of flexible serum-free medium (SFM) to support the development of human hematopoietic cells, a 1.3 mL aliquot of

the flexible serum-free medium nutrient supplement, 15% fetal bovine serum (FBS), 250 µL of penicillin-streptomycin, 500 µL of 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl, VEGFA (100 ng/mL), and FGF2 (100 ng/mL) (see **Table of Materials**).

7. Prepare the blocking buffer.

    1. Mix 0.5 g of bovine serum albumin, 1.5 mL of fetal bovine serum, 250 µL of Tween 20, 250 µL of Triton X-100, 500 µL of sodium deoxycholate (1% wt/vol stock), and 47.5 mL of 1x phosphate-buffered saline (see **Table of Materials**). Pipette up and down until all the components are well incorporated and the solution is clear.

2. Maintenance and culture of human pluripotent and induced pluripotent stem cell

1. Perform cell culture using LDEV-free reduced growth factor basement membrane matrix (1:50 dilution)-coated 6-well tissue culture plates with stem cell culture medium (see **Table of Materials**).
2. Once the cells reach ~70% confluency, passage them using 1 mL of mammalian cell dissociating enzyme (see **Table of Materials**) for 3-4 min at 37 ˚C.

    NOTE: Splitting the cells at a ratio of 1:6 enhances the cell viability and achieves confluency within 2-4 days for most human stem cell lines. To increase the cell viability, ROCK inhibitor Y-27632-supplemented (10 mM, 1:1,000) culture medium is used for 24 h post passaging.
3. Change the culture medium daily until a confluency of 70% is reached.

    NOTE: For the present study, at 70% confluency, one well of a 6-well cell culture plate contains around 1 million H9 hPSCs.

3. Day 0 - Generation of pluripotent aggregates from a single-cell suspension

NOTE: A confluency of 70% in two wells of a 6-well culture plate will yield approximately 175 hBVOs.

1. Using either a pipette or vacuum system, aspirate the culture medium, replace it with 1 mL of cell dissociation reagent (see **Table of Materials**), and incubate for 5 min at 37 ˚C.
2. While the cells are under the cell dissociation reagent, prepare the necessary volume of aggregation medium (step 1.1) in a 15 mL conical tube as needed for the desired number of wells in a 6-well ultra-low attachment culture plate (see **Table of Materials**).
3. Aspirate the 1 mL of cell dissociation reagent, and suspend the cells in 1 mL of aggregation medium. Create a single-cell suspension with gentle up-down pipetting prior to counting the samples.

    CAUTION: hPSCs are quite sensitive to mechanical stress and cannot tolerate aggressive pipetting.
4. Count the cells using an automated cell counting device[10] or a standardized system under a microscope. Calculate the cell number needed for the experiment.

    NOTE: Depending on the cell line, 200,000 cells/well to 300,000 cells/well is considered ideal for aggregate formation (i.e., 4 wells = 800,000 to 1,200,000 cells in total).
5. Add the appropriate volume of the cell suspension to the aggregation medium in the 15 mL high-clarity polypropylene conical tube. Gently pipette the diluted cell suspension up and down to ensure a homogenous cell distribution.

6. Pipette 3 mL of the diluted cell suspension into each desired well of the 6-well ultra-low attachment culture plate.

7. Place the plate in the incubator (37 ˚C, 5% CO_2, >90% humidity), and avoid opening and closing the incubator doors as much as possible.
   NOTE: This initial step is best completed in the evenings or during low incubator traffic. Even slight vibrations can affect the size and shape of the aggregates, which can impair the results.

4. Day 1 - Mesoderm induction of aggregates

1. Ensure that 24 h post seeding, small aggregates comprising 2-10 cells are visible under the microscope. Collect the aggregates, and allow them to settle prior to changing the medium to the mesoderm induction medium (step 1.3).

2. Set up one 15 mL high-clarity polypropylene conical tube per well of the 6-well ultra-low attachment culture plate.

3. Use a circular motion (i.e., like an orbital shaker) to gather the aggregates in the center of each well, use a 1 mL pipette to gently collect the aggregates and the surrounding medium from each well, place them into their corresponding 15 mL high-clarity polypropylene conical tubes, and allow the aggregates to sediment at room temperature.

4. Once collected, set a timer for 1 h, which is the time required for most cell lines/aggregates to sediment at this stage.
   NOTE: If the time is less than 1 h, one might lose any aggregates that may not have settled to the bottom of the 15 mL conical tube.

5. Once the hour has passed, with caution, aspirate the supernatant with a pipette or a high-sensitivity aspirating pump. Ensure the aggregates remain undisturbed at the bottom of the 15 mL conical tube.

6. Resuspend the aggregates of each 15 mL conical tube in 2 mL of mesoderm induction medium, and place them back in their respective wells of the 6-well ultra-low attachment culture plate.

7. Place the plate back in the incubator (37 ˚C), and leave it until day 4.
   NOTE: If the aggregates attach and grow onto or into one another, using a 1 mL pipette, gently pipette each well of aggregates up and down once per day to keep the aggregates similar in size.

5. Day 4 - Vascular induction and priming of the aggregates

1. Collect the aggregates, and allow them to settle prior to changing the medium to the vascular induction medium (step 1.4).

2. Set up one 15 mL conical tube per well of the 6-well ultra-low attachment culture plate.

3. Use a circular motion (i.e., like an orbital shaker) to gather the aggregates in the center of each well, use a 1 mL pipette to gently collect the aggregates and the surrounding medium from each well, and place them into their corresponding 15 mL conical tubes.

4. Once collected, set a timer for 30 min to allow the aggregates to sediment.
   NOTE: A time of 30 min is required for most cell lines at this stage. If the time is less than 30 min, there is a risk of losing any aggregates that may not have settled to the bottom of the 15 mL conical tube.

5. After 30 min, with caution, aspirate the supernatant with a pipette or a high-sensitivity aspirating pump. Ensure the aggregates remain undisturbed at the bottom of the 15 mL conical tube.

6. Resuspend the aggregates of each 15 mL conical tube in 2 mL of vascular induction medium, and place them back in their respective wells of the 6-well ultra-low attachment culture plate.

7. Place the plate back in the incubator (37 ˚C), and leave it until day 6.

   NOTE: Using a 1 mL pipette, gently pipette each well of aggregates up and down once per day to keep the aggregates similar in size and prevent them from growing into or attaching to one another.

6. Day 6 - Aggregate embedding and vessel sprout induction

1. While working on ice, prepare the desired final volume of the ECM solution (step 1.5).

   NOTE: The following is the protocol for one well of a 12-well plate (30-50 aggregates), which can be adjusted as necessary.

   1. For one well of a 12-well plate, apply 0.5 mL of ECM as the bottom layer, and use 0.5 mL of ECM plus aggregates on the top layer. This creates the ECM "sandwich" necessary for effective 3D aggregate sprouting.

2. Pipette 500 µL of ECM into one well of a 12-well plate. Ensure that no bubbles are formed and the well bottom and side meniscus are completely coated. This comprises layer 1 of the ECM sandwich.

3. Place the plate back at 37 ˚C for 2 h.

   NOTE: A time of 2 h is necessary for effective polymerization. Any shorter time risks compromising the ECM integrity.

4. Toward the end of layer 1 polymerization, use a circular motion to gather the aggregates in the center of each well, use a 1 mL pipette to gently collect the aggregates and surrounding medium from each well, and place them into their corresponding 15 mL conical tubes.

5. Allow the aggregates to settle for 10-15 min, and aspirate the supernatant.

6. Place the aggregates in the 15 mL conical tube on ice for 5 min. While cooling, take the 12-well plate with the now polymerized layer 1 out of the incubator.

   NOTE: Cooling the aggregates helps prevent early polymerization of the layer 2 ECM.

7. Working swiftly and carefully to prevent bubble formation, resuspend the aggregates in 500 µL of ECM, and pipette the ECM-aggregate suspension atop the already polymerized layer 1. Being cautious not to touch layer 1, use a 200 µL pipette tip to gently distribute layer 2 and the aggregates around the well.

8. Place the plate back at 37 ˚C for 2 h.

   NOTE: An incubation time of 2 h is necessary for effective ECM polymerization. Any shorter time risks compromising the ECM integrity and may prevent strong adhesion between layer 1 and layer 2.

9. Add 1 mL of pre-warmed (37 °C) sprouting medium (step 1.6) to induce blood vessel differentiation. Sprouting vessels must appear 1 day to 3 days post embedding. Change the medium after 3 days and then every other day.

NOTE: The sprouting medium must be pre-warmed; otherwise, layer detachment may occur, and the ECM integrity may be impacted.

7. Day 11 - Isolation and maturation of the BVOs

1. Working under sterile conditions, use the rounded end of a sterile spatula to loosen the ECM sprouting matrix containing the vascular networks. At this stage, the gel must resemble a free-floating disk.
    1. Using sterile forceps and the rounded end of a sterile spatula, carefully transfer the gel disk (including the vascular networks) to the lid of a 10 cm culture dish.
2. Place the lid plus gel under a stereomicroscope adjusted to the desired magnification and focus, and use sterile needles to cut out single blood vessel networks, trying to limit the amount of non-vascularized ECM obtained in the process.
   NOTE: The cells naturally degrade the surrounding ECM over time; however, reducing the amount cut out with each organoid increases the image quality and the freestanding vessel network integrity.
3. Once all the organoids have been isolated from the gel, gently transfer them back into one well of an ultra-low attachment 6-well plate with 3 mL of sprouting medium . At this stage, the organoids can be left overnight, or one may immediately continue to step 7.4.
4. Use a 1 mL pipette to transfer single organoids from the 6-well plate to the wells of an ultra-low attachment 96-well plate. Once transferred, add 200 µL of pre-warmed (37 °C) sprouting medium to each well of the 96-well plate.
   NOTE: One well of vascular organoids from a 12-well plate must fill 30-40 wells of a 96-well plate.
5. At 4-6 days post isolation in the 96-well plates, ensure that the organoids possess a round and healthy morphology. At this stage, the organoids are ready to be fixed and prepared for staining.

8. Day 15 - Fixation, blocking, and staining of the BVOs

1. Using a cut 1 mL tip, transfer the organoids into a 1.5 mL microcentrifuge tube.
2. Being careful to avoid the aspiration of any BVOs, use a 200 µL or 1,000 µL pipette to remove the remaining sprouting medium in the microcentrifuge tube, and then add 1 mL of 4% PFA in PBS for 1 h.
   NOTE: Fixation on an orbital shaker (125 rpm) at room temperature is recommended. A maximum of 60 BVOs/ microcentrifuge tube is acceptable. Any more BVOs will impair the fixation and wash efficacy.
   CAUTION: PFA is a harmful chemical. Use it with caution in a fume hood, and follow the manufacturer's directions for use and appropriate disposal methods.
3. Wash the newly fixed BVOs 3x for 15 min each time with 0.25% PBS-Tween.
4. If the organoids are larger than 1 mm in diameter, permeabilize the BVOs with 1% Triton X-100 in PBS for 30-60 min at room temperature.
5. Aspirate all the 0.25% PBS-Tween, and add 1 mL of blocking buffer (step 1.7).
   NOTE: Although antibody-dependant, 2 h at room temperature on an orbital shaker (125 rpm) is sufficient to reduce non-specific antibody binding during the staining process. The organoids can be left in the blocking buffer at 4 ˚C for up to 2 weeks.

6. Remove the blocking buffer, and add the primary antibody (1:100) diluted in 1 mL of the blocking buffer. Keep overnight at 4 ˚C on an orbital shaker (12 rpm), ensuring the organoid(s) remain submerged in the primary antibody solution.

   NOTE: Overnight primary antibody incubation is suitable for the antibodies used for this study. The necessary primary and secondary antibodies and their respective dilutions are provided in the **Table of Materials**.

7. Following overnight primary antibody incubation, remove the primary antibody solution and wash 3x for 15 min each time with 0.25% PBS-Tween.

8. Add the secondary antibody (1:250) plus DAPI (1:1,000) diluted in 1 mL of blocking buffer, and incubate for 2 h at room temperature or at 4 ˚C overnight.

   NOTE: If the samples are prepared correctly (as described above), incubation for 2 h at room temperature or at 4 ˚C overnight is suitable for the antibodies used in the present study.

9. Following incubation, remove the secondary antibody solution, and wash 3x for 15 min each time with 0.25% PBS-Tween.

   NOTE: Following the staining, the organoids can be kept in PBS for up to 2 weeks.

9. Mounting of the blood vessel organoids (BVOs)

1. Glue mounting spacers onto the desired imaging coverslips (see **Table of Materials**).

2. Using a cut 1 mm pipette tip, use a 1 mL pipette to transfer single organoids into the spacer wells, and aspirate the remaining PBS.

3. Fill the well with 150-200 µL of clearing solution (see **Table of Materials**) heated to 75 ˚C, fully submerging the organoid.

   NOTE: The samples should become clear within 1 h of exposure to the warmed clearing solution.

   1. Leave the organoids to become clear in a covered box in the dark at room temperature for 6 h to overnight if the 1 h clearing time is insufficient. If leaving the sample overnight, ensure it has enough clearing solution to avoid drying out.

4. After clearing, carefully aspirate the clearing solution with a 100-200 µL pipette tip. Gently maneuver the organoid to the center of the spacer well, and add drops of mounting gel (see **Table of Materials**) (warmed to 75 ˚C in a controlled heating block) until the well is full.

5. Gently place the second coverslip on top of the mounting spacer to seal the samples in the space between the bottom and top coverslips. Let the mounting gel cure in the dark at 4 ˚C overnight.

   NOTE: Following the curing, topcoat nail polish can be used to further seal the mounted samples.

Representative Results

The steps described in this protocol were developed specifically to yield a controlled and precise method for the generation of human blood vessel organoids from hPSCs. The generation of aggregates of 30-100 µm in diameter from hPSC cultures marks the starting point of the protocol (**Figure 1**, **Figure 2B**). The aggregates are led through stepwise inductions toward mesoderm (day 1-day 4) and vascular lineages (day 4-day 6) prior to embedding (day 6) (**Figure 2A-D**), which is necessary for vessel network formation. Near-radially symmetric vessel

sprouting must be visible by d7 or d8 (**Figure 2E**) and continue through d10 (**Figure 2F**,**G**). The explantation of the hBVOs from the ECM to the 96-well ultra-low attachment plates reduces the fragility of the sprouting networks and allows for continued maintenance in suspension culture (**Figure 2H**) conditions for up to 6 months. By d15, the hBVOs exhibit an extensive and connected endothelial ($CD31^+$) network surrounded by pericytes (PDGFR-ß$^+$) and smooth muscle actin (SMA^+) (**Figure 3A-D**). A continuous collagen IV ($ColIV^+$) basement membrane envelopes the vessel networks (**Figure 3E**). Endothelial cells ($CD31^+$, VE-$Cadherin^+$) and pericytes (PDGFR-ß$^+$) comprise approximately 30%-35% and 60%-65% of the organoid cell populations, respectively[8]. The active sprouting of the vessels occurs under the direction of an endogenously organized tip cell ($CD31^+$) population that presents with typical tip cell morphology, such as excessive filipodia[8,10]. The presentation of PDGFR-ß$^+$ and SMA^+ mural cells encapsulating the endothelial vessel networks can be seen by d15 of the organoid maturation process (**Figure 3A**,**C**). Following removal from the ECM and maturation in suspension culture (i.e., d15), the hBVOs are tenable for respective analyses or transplantation under the mouse kidney capsule.

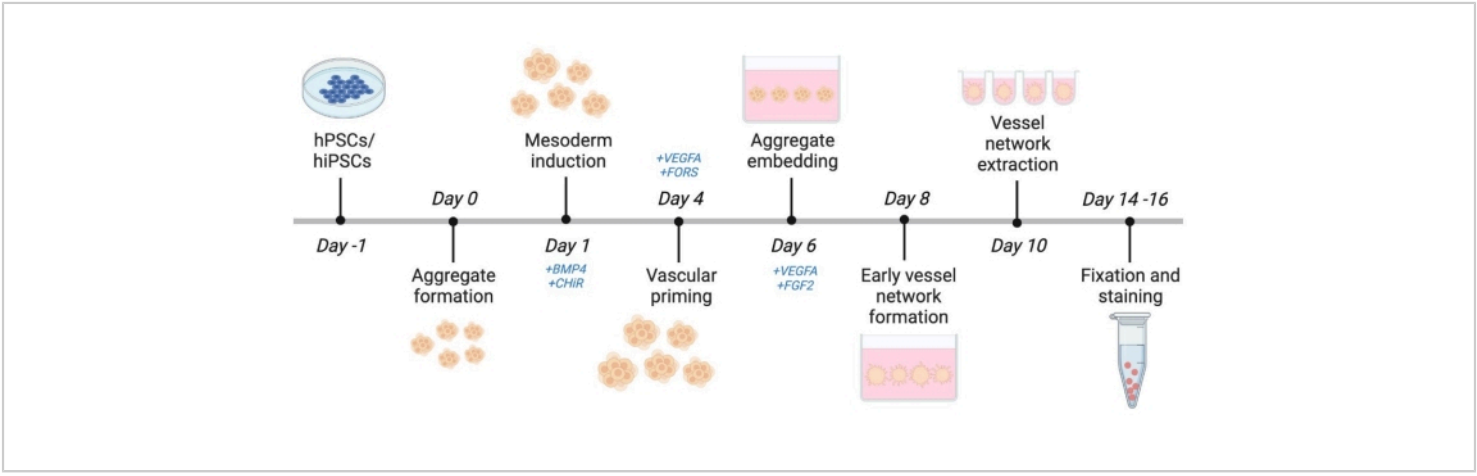

Figure 1: Schematic of the hBVO protocol highlighting the timing and stepwise progression. From seemingly homogenous hPSC or hiPSC cultures, aggregate formation occurs in the presence of the Rho-kinase inhibitor Y27632. BMP4 and CHiR-supplemented medium is used to instruct the aggregates toward the mesodermal fate. Mesoderm induction medium is replaced by VEGFA and forskolin-supplemented medium to stimulate the aggregates toward a vascular lineage. Mechanical and chemical queues achieved through the embedding of the aggregates in a collagen I and solubilized basement membrane matrix and exposure to VEGFA and FGF2-containing medium results in near-radially symmetric vascular sprouting from the aggregate body. The vessel networks generated can be removed from the collagen I and solubilized basement membrane and placed in suspension culture for further maturation, analysis, or transplantation *in vivo*.

Please click here to view a larger version of this figure.

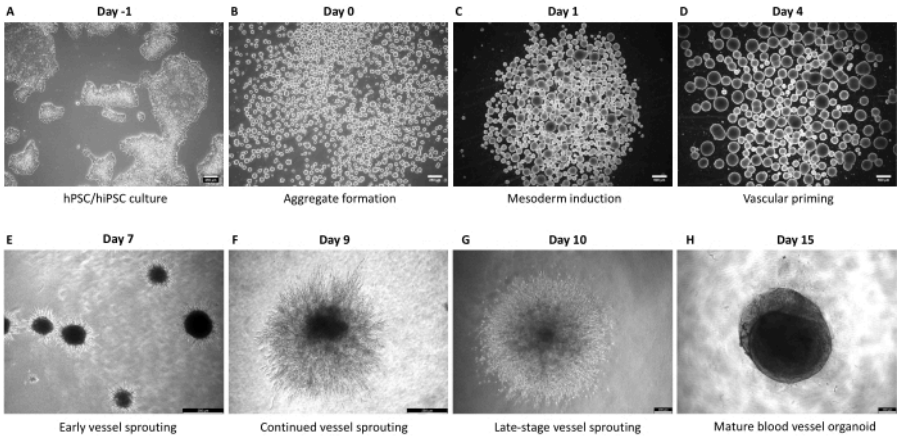

Figure 2: Stepwise progression of the hBVO generation captured under brightfield. (**A**) Typical morphology of hPSC (H9) colonies at day −1. (**B**) Generation of hPSC aggregates (day 0) on 6-well ultra-low attachment plates in the presence of Y-27632. (**C**) The mesodermal induction of aggregates using BMP4 and ChiR-supplemented medium (day 1). Subtle changes in the aggregate size and shape can also be observed. (**D**) Day 4 aggregates primed toward a vascular lineage using VEGFA and forskolin-supplemented medium. (**E**) Early vessel sprouting on day 7, one day after embedding the aggregates in the sprouting matrix and exposure to VEGFA and FGF2-supplemented sprouting medium (day 7). (**F**) Healthy organoid morphology and continued vessel sprouting on day 9. (**G**) Late-stage vessel sprouting at day 10. Ideally, the dense cell structures at the organoid center have disappeared by this time. (**H**) Typical morphology of mature (day 15) human blood vessel organoids. The removal of the surrounding matrix by cutting and maturation in ultra-low attachment 96-well culture plates shapes the organoids spherically, with the vessel networks housed internally. Scale bars: (**A**,**B**,**E**,**F**) 250 µm; (**C**,**D**) 500 µm; (**G**,**H**) 200 µm. Please click here to view a larger version of this figure.

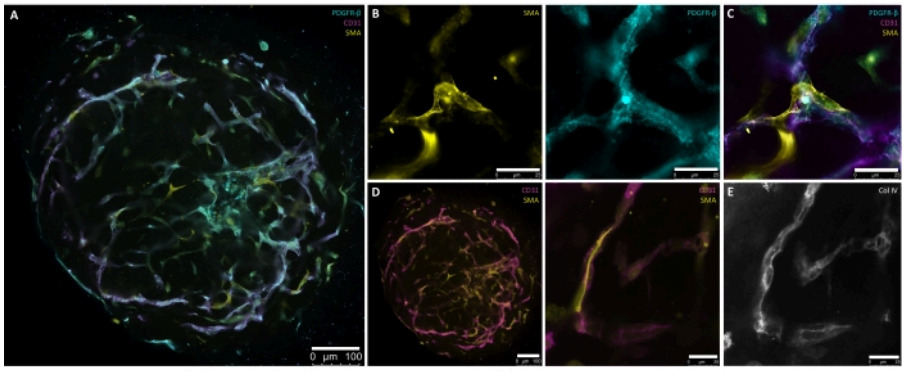

Figure 3: Whole-mount staining of mature (day 15) human blood vessel organoids. (**A**) Presentation of mature (day 15) human blood vessel organoids with self-organized endothelial ($CD31^+$, magenta) networks and surrounding pericyte (PDGFR-β^+, cyan) and alpha-smooth muscle actin (SMA^+, yellow) coverage. (**B**) Higher magnification of **A** detailing the SMA^+ (yellow) and PDGFR-β^+ (cyan) expression of the vessel networks. (**C**) Detailed presentation of endothelial ($CD31^+$, magenta), pericyte (PDGFR-β^+, cyan), and alpha-smooth muscle actin (SMA^+, yellow) interaction. (**D**) Whole-mount staining of the vessel endothelial ($CD31^+$, magenta)-smooth muscle (SMA^+, yellow) interaction in a cross-section (left) and a higher magnification (right) of mature blood vessel organoids. (**E**) Self-directed formation of a vascular basement membrane (Col IV^+, grayscale) through the close association of the mural cells and endothelial tubes. Scale bars: (**A**,**D**[left]) 100 µm; (**B**,**D**[right],**E**) 25 µm. Please click here to view a larger version of this figure.

Discussion

Recent breakthroughs in stem cell-derived organoid cultures have provided the framework for more advanced and physiologically relevant models of human vasculature. The human blood vessel organoid (hBVO) model presented here, developed in our laboratory[8,10], provides a powerful means of exploring not only further aspects of human vasculogenesis but also new avenues of disease modeling and regenerative therapy[8,10,11]. Multiple *in vivo* models have been employed to explore the development and maturation of blood vessels, vascular disease, and endothelial dysfunction[3,4]. Varying approaches combine single and multiple lineage-defined cells either derived from stem cells or isolated from adult tissues *in vivo* to create replicative human vascular networks[5,6].

The protocol presented here leverages the principle of developmental biology and self-organization to yield the first ever multi-cell lineage human blood vessel networks that can be generated, in essence, from a single hPSC[8,10].

Each stem cell line has a unique genetic makeup and differs from others in terms of its sourcing, function, and responsiveness[15]. Therefore, the protocol for human blood vessel organoids (hBVOs) was developed and optimized to ensure a robust and reproducible protocol compatibility with multiple (>12) different hPSC lines[8,10]. The method described here generates hPSC-derived blood vessel organoids over 2 weeks. However, changes to the media composition and or the techniques in hBVO generation may lead to ineffective vessel network and organoid generation.

The varying proliferation rates of individual stem cell lines also markedly impact the reproducibility of stem cell experimentation[15] and, thus, organoid cultures. For example, in generating the BVOs, more proliferative cells or higher numbers of large day 1 aggregates are inherently subject to different metabolic environments and gas and nutrient diffusion parameters. This, in turn, changes the growth factor exposure times and efficiencies, the degree of differentiation and vascular priming, and, most importantly, the ability to form vessel networks upon embedding the aggregates in the collagen 1 and solubilized basement membrane matrix.

The passive diffusion of oxygen and the administration of essential nutrients from an external environment is not ideal for the long-term cell growth of 3D organoids and tissue morphogenesis *in vitro*[16]. Although dependant on several factors (i.e., the tissue metabolic rate, the nutrient and gas bioavailability, a static or dynamic environment), a general 150 µm O_2 and nutrient diffusion limit has been established for tissues cultured *in vitro*, considering that, physiologically, human tissues present cords of living cells within 150 µm of perfused blood vessels[17]. Although effective gas and nutrient diffusion distances of 70-200 µm have also been proposed[18,19,20,21], the construct density, temperature, pH, and media composition significantly impact the diffusion efficacy. Due to the surface area optimization and integrin-beta receptor communication following day 6 embedding, aggregates of 250-300 µm in diameter perform better than those >500-600 µm in diameter, resulting in a complete vessel sprouting process and a minimally condensed organoid core. Thus, the aggregate size is crucial and can be affected by both the number of cells used during the initial seeding and the time allotted for aggregate formation. Microwell plates that allow for control over the aggregate size and number[22] are a viable alternative to the otherwise stochastic aggregate formation technique resulting from the use of ultra-low attachment 6-well plates in this protocol. Consistency in timing and managing the aggregate sizes during the first 6 days of the hBVO protocol is one of, if not the most, crucial indicators of successfully developing *bona fide* blood vessel organoids.

Medium changes on day 1 (mesoderm induction) and day 4 (vascular induction) must be completed in combination with the sedimentation of the aggregates. Although centrifugation is a tempting alternative, the additional forces applied to the weakly assembled aggerates can cause unwanted clumping, assembly, and shearing, which negatively impact differentiation, maturation, and sprouting efficacy in the later stages of the protocol. Working on ice during the day 6 embedding process is critical for preserving proper ECM polymerization and layer formation. During the aggregate embedding and sprouting induction, exposing the ECM to temperatures above 4˚C and/or an ECM pH other than 7.4 will affect not only the polymerization rates and ECM layer integrity but also the sprouting efficiency of the embedded aggregates. The elastic nature of the ECM sprouting matrix allows for easy detachment and transport from the 12-well culture dish to the sterile cutting surface. Individual organoids removed from the matrix and transferred to ultra-low attachment 96-well plates will consume the remaining surrounding ECM and retain self-organized mural cell-coated endothelial microvessel networks with a continuous basement membrane.

While not covered in this proposal, alterations to the media compositions can replicate diseases that, in turn, provoke pathological responses in hBVOs[8,11]. The boundaries of disease modeling using the hBVO system are far from well-known, and this is certainly an area that needs exploration.

The application of our blood vessel organoid technology in the vascularization of previously avascular organoid constructs[23] is also of significant impact and interest.

Disclosures

There is no relationship between the authors' financial interests and the research being presented. The blood vessel organoid technology has been licensed to Stemcell Technologies. J.M.P. is a founder and shareholder of Angios Biotech that develops blood vessel organoids as vascular transplant therapy. A patent application related to this work has been filed under Pat. No. US20200199541A1, listing Reiner A. Wimmer, Josef M. Penninger, and Dontscho Kerjaschki as inventors.

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