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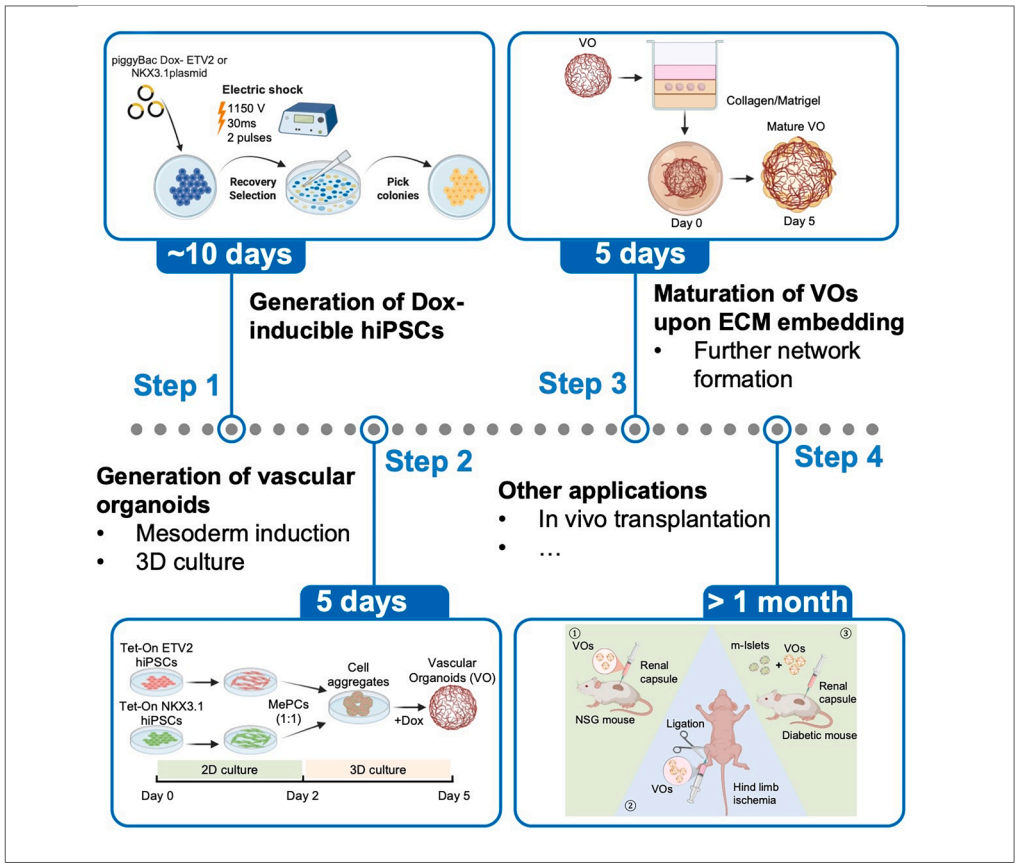
Protocol for generating human vascular organoids via orthogonal activation of ETV2 and NKX3.1



Vascular organoids (VOs) are 3D multicellular constructs that model vascular development and function. Here, we present a protocol to generate VOs from human induced pluripotent stem cells via orthogonal activation of transcription factors. We describe steps for inducing endothelial and mural lineages independently using doxycycline-inducible expression of ETV2 and NKX3.1. This protocol enables efficient formation of functional VOs within 5 days, streamlining conventional techniques and supporting applications in vascular biology, tissue engineering, and regenerative medicine.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for generating vascular organoids from human iPSCs

Steps to induce vascular lineages via doxycycline-inducible ETV2 and NKX3.1

Instructions to embed organoids in extracellular collagen-Matrigel matrices

Guidance on dissociating vascular organoids into single cells for downstream analyses

Gong & Melero-Martin, STAR Protocols 7, 104294
March 20, 2026
https://doi.org/10.1016/j.xpro.2025.104294

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Protocol for generating human vascular organoids via orthogonal activation of ETV2 and NKX3.1

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https://doi.org/10.1016/j.xpro.2025.104294

SUMMARY

Vascular organoids (VOs) are 3D multicellular constructs that model vascular development and function. Here, we present a protocol to generate VOs from human induced pluripotent stem cells via orthogonal activation of transcription factors. We describe steps for inducing endothelial and mural lineages independently using doxycycline-inducible expression of ETV2 and NKX3.1. This protocol enables efficient formation of functional VOs within 5 days, streamlining conventional techniques and supporting applications in vascular biology, tissue engineering, and regenerative medicine.

For complete details on the use and execution of this protocol, please refer to Gong et al.[1]

BEFORE YOU BEGIN

General laboratory preparation

1. Set up a standard cell culture incubator at 37°C with 5% CO_2.
2. Place an orbital shaker inside the incubator for use during organoid culture.
3. Perform all procedures under aseptic conditions, using sterile gloves, plates, tubes, tips, and standard aseptic techniques.
4. Refer to the key resources table for a complete list of materials and reagents.

Coating plates with Matrigel

Timing: ~12–16 h (overnight thaw), plus 15 min coating

5. Thaw a frozen bottle of Matrigel on ice at 4°C for 12–16 h. Once thawed, gently mix to ensure homogeneity.
6. Aliquot 300 μL of Matrigel into pre-chilled 50 mL centrifuge tubes using pre-chilled pipette tips. Store aliquots at −20°C until use.
7. To coat plates, thaw one 300 μL aliquot on ice and dilute with 50 mL of cold KnockOut DMEM. Mix thoroughly.
8. Immediately add 1 mL of the diluted Matrigel solution per well of a 6-well plate. Gently swirl to evenly coat.
9. Incubate at 20°C–25°C for 15 min before use.

Note: If not used immediately, seal plates with Parafilm and store at 4°C for up to 14 days. Bring to 20°C–25°C for 30 min prior to cell seeding.

Thawing cryopreserved human iPSCs

Timing: 1 h

10. Warm mTeSR Plus medium supplemented with ROCK inhibitor to 37°C. Ensure Matrigel-coated plates are ready before starting to minimize thawing time.

Note: Do not add ROCK inhibitor unless cells were just dissociated/single-cell seeded—in that case, include 10 μM ROCK inhibitor for the first 24 h only.

11. Quickly thaw the cryovial in a 37°C water bath by gently swirling until the frozen cell pellet is no longer visible.
12. Remove the cryovial, sterilize the exterior with 70% ethanol, and transfer it to a biosafety cabinet.
13. Transfer the contents of the cryovial into a 15 mL conical tube using a 1,000 μL pipette.
14. Slowly add 2 mL of pre-warmed mTeSR Plus medium with ROCK inhibitor dropwise while gently mixing.
15. Centrifuge at 300 × g for 5 min at 20°C–25°C.
16. Aspirate the supernatant carefully without disturbing the pellet. Resuspend cells in 2 mL of fresh mTeSR Plus medium with ROCK inhibitor.
17. Plate cells onto a Matrigel-coated 6-well plate (2 mL per well).
18. Incubate at 37°C with 5% CO_2 and 95% humidity.
19. Replace medium daily.

Passing human iPSCs

Timing: 1 h

20. Warm mTeSR Plus medium supplemented with ROCK inhibitor to 37°C. Allow Matrigel-coated plates to equilibrate at 37°C for 15 min before use.
21. Aspirate the spent medium from each well and wash cells with 1 mL of PBS per well.
22. Remove PBS, then add 1 mL of TrypLE Select Enzyme (1×) per well. Swirl gently to distribute evenly.
23. Aspirate the TrypLE solution and incubate the plate at 37°C for 3 min.
24. Add 1 mL of mTeSR Plus medium with ROCK inhibitor per well.
25. Gently dissociate cells by pipetting up and down with a 1,000 μL pipette.
26. Plate the desired volume of the single-cell suspension into Matrigel-coated plates, then add additional mTeSR Plus medium with ROCK inhibitor to a final volume of 2 mL per well.

Note: The seeding density should be adjusted depending on the downstream application.

27. Incubate at 37°C with 5% CO_2 and 95% humidity.
28. Perform daily medium changes.

Note: ROCK inhibitor should only be added on the day of passaging.

Cryopreservation of human iPSCs

Timing: 1 h

29. Aspirate the spent medium and wash each well with 1 mL of PBS.
30. Remove PBS, then add 1 mL of TrypLE Select Enzyme (1×) per well. Swirl gently to distribute.
31. Aspirate the TrypLE and incubate at 37°C for 3 min.
32. Add 2 mL of cold mFreSR medium per well and gently detach the cells by pipetting.
33. Transfer 1 mL of the cell suspension into each cryovial using a 1,000 μL pipette.
34. Place cryovials into an isopropanol-based freezing container and store at −80°C for 12–16 h.
35. Transfer cryovials to a liquid nitrogen storage tank the next day for long-term preservation.

Innovation

This protocol introduces a rapid and modular approach for generating human vascular organoids (VOs) from iPSCs via orthogonal activation of transcription factors (TFs). Unlike previous methods that rely on spontaneous or sequential differentiation, we use doxycycline-inducible ETV2 and NKX3.1 transgenes to independently drive endothelial and mural cell fates. This decouples lineage specification from microenvironmental signals and temporal constraints, enabling consistent and reproducible generation of vascular tissues.

Key innovations include: (1) a dual-piggyBac engineering strategy that permits tunable and synchronized TF activation in 3D aggregates; (2) a five-day organoid formation protocol that bypasses the need for extended culture or external patterning cues; and (3) formation of vascular networks in vitro with both endothelial and mural compartments represented, supporting downstream applications in vascular modeling and transplantation.

This protocol integrates iPSC genome engineering, mesoderm induction, and 3D self-organization into a streamlined workflow that accelerates organoid generation and improves reproducibility. It is adaptable to different iPSC lines and easily scaled, making it useful for applications in vascular biology, tissue engineering, and regenerative medicine.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-human CD31	Santa Cruz	RRID:AB_629040 Cat# sc-53411, 1:100
Rabbit anti-smooth muscle myosin heavy chain 11	Abcam	RRID:AB_2890982 Cat# Ab133567, 1:200
Donkey anti-mouse IgG AlexaFluor 568	Thermo Fisher Scientific	RRID:AB_11180865 Cat# A-10037, 1:500
Goat anti-rabbit IgG, AlexaFluor 488	Thermo Fisher Scientific	RRID:AB_2576217 Cat# A-11034, 1:500
4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	Thermo Fisher Scientific	RRID:AB_2629482 Cat# D1306, 1:5000
PE anti-human CD144 (VE-CAD)	Thermo Fisher Scientific	RRID:AB_763438 Cat#12-1449-82, 1:100
APC-anti-CD140b	BioLegend	RRID:AB_2162787 Cat# 323608, 1:100
Biological samples		
BJ273-iPSCs (BJ-iPSCs)	Schlaeger et al.[2]	N/A
Chemicals, peptides, and recombinant proteins		
PiggyBac super transposase	SBI	Cat# PB210PA-1
Y27632	Selleckchem	Cat# S1049
CHIR99021	Sigma-Aldrich	Cat# SML1046-25MG
Puromycin	InvivoGen	Cat# ant-pr-1
100X glutamax	Thermo Fisher Scientific	Cat# 35050061

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Doxycycline hyclate	Sigma-Aldrich	Cat# D9891-10G
Gelatin from porcine skin	Sigma-Aldrich	Cat# G2500-500G
L-Ascorbic acid phosphate	MilliporeSigma	Cat# A8960-5G
Penicillin-Streptomycin (P/S)	Thermo Fisher Scientific	Cat# 15140122
EGF Human	ProSpec	Cat# CYT-798
FGF 2 Human (147 a.a.)	ProSpec	Cat# CYT-557
VEGF Human	ProSpec	Cat# CYT-241
Matrigel	Corning	Cat# 354277
GenClone Fetal Bovine Serum	Genesee	Cat# 25-514
DMEM, high glucose, pyruvate	Thermo Fisher Scientific	Cat# 11995073
mTeSR Plus	STEMCELL Technologies	Cat# 100-0276
FreSR-S	STEMCELL Technologies	Cat# 5859
Advanced DMEM/F-12	Thermo Fisher Scientific	Cat# 12634028
StemPro-34 SFM (1X)	Thermo Fisher Scientific	Cat# 10639011
KnockOut DMEM	Thermo Fisher Scientific	Cat# 10829018
PuroCol Type I Collagen Solution, 3 mg/ml (Bovine)	Advanced BioMatrix	Cat# 5005
PBS, pH 7.4	Thermo Fisher Scientific	Cat# 10010049
TrypLE Select Enzyme (1X), no phenol red	Thermo Fisher Scientific	Cat# 12563029
HEPES (1 M)	Thermo Fisher Scientific	Cat# 15630080
Ham's F-12 Nutrient Mix	Thermo Fisher Scientific	Cat# 11765054
Sodium Bicarbonate 7.5% solution	Thermo Fisher Scientific	Cat# 25080094
Dynabeads CD31 Endothelial Cell	Invitrogen	Cat# 11155D
Urea	Thermo Fisher Scientific	Cat# 140750010
N,N,N′,N′-Tetrakis(2-Hydroxypropyl) ethylenediamine(Quadrol)	MilliporeSigma	Cat# 122262-1L
Triton X-100	Thermo Fisher Scientific	Cat# A16046-AE
Sucrose	MilliporeSigma	Cat# S0389-500G
BSA Bovine Serum Albumin	MilliporeSigma	Cat# A7906-100G
sodium azide	MilliporeSigma	Cat# S2002-5G
Critical commercial assays		
Neon Electroporation Kit	Invitrogen	Cat# MPK1009
Oligonucleotides		
h-GAPDH RT-qPCR forward primer: 5′- CATGTTCGTCATGGGTGTGAACCA-3′	This paper	N/A
h-GAPDH RT-qPCR reverse primer: 5′-TGGCATGGACTGTGGTCATGAGT-3′	This paper	N/A
h-ETV2 RT-qPCR forward primer: 5′-CCGACGGCGATACCTACTG-3′	This paper	N/A
h-ETV2 RT-qPCR reverse primer: 5′-CGGTGGTTAGTTTTGGGGCAT-3′	This paper	N/A
h-NKX3.1 RT-qPCR forward primer: 5′-TCACGGAGACCCAAGTGAAG-3′	This paper	N/A
h-NKX3.1 RT-qPCR reverse primer: 5′-TATACACGGAGACCAGGGAG-3′	This paper	N/A
h-OCT4 RT-qPCR forward primer: 5′-CTTGAATCCCGAATGGAAAGGG-3′	This paper	N/A
h-OCT4 RT-qPCR reverse primer: 5′-GTGTATATCCCAGGGTGATCCTC-3′	This paper	N/A
h-NANOG RT-qPCR forward primer: 5′-TTTGTGGGCCTGAAGAAAACT-3′	This paper	N/A
h-NANOG RT-qPCR reverse primer: 5′-AGGGCTGTCCTGAATAAGCAG-3′	This paper	N/A
h-SOX2 RT-qPCR forward primer: 5′-GCCGAGTGGAAACTTTTGTCG-3′	This paper	N/A
h-SOX2 RT-qPCR reverse primer: 5′-GGCAGCGTGTACTTATCCTTCT-3′	This paper	N/A
Recombinant DNA		
Dox-inducible ETV2 PiggyBac	Luo, et al.[3]	N/A
Dox-inducible NKX3.1 PiggyBac	Lee, et al.[4]	N/A
Software and algorithms		
ImageJ	US National Institutes of Health	https://imagej.nih.gov/ij/
Other		
Orbi-Shaker CO_2	Benchmark Scientific	Cat# BT4001
6-Well Cell Culture Plates Flat Bottom Wells	Genesee Scientific	Cat# 25-105
6-Well Non-Treated Plates Flat Bottom Wells	Genesee Scientific	Cat# 25-100
12-Well Cell Culture Plates Flat Bottom Wells	Genesee Scientific	Cat# 25-106
15 mL Conical Centrifuge Tubes	Genesee Scientific	Cat # 28-103

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
50 mL Conical Centrifuge Tubes	Genesee Scientific	Cat # 28-108
25 mL Wobble-not Serological Pipet	Vistalab	Cat# 4090-0025
10 mL Serological Pipets Sterile	Genesee Scientific	Cat# 12-104C
5 mL Serological Pipets Sterile	Genesee Scientific	Cat# 12-102C
1.7 mL Microtubes	Genesee Scientific	Cat# 24-282S
1000 µL Reach Olympus Premium Barrier Tips	Genesee Scientific	Cat# 24-430C
200 µL Barrier Pipette Tips	Thermo Fisher Scientific	Cat# 2069-05-HR-HID
20 µL Barrier Pipette Tips	Thermo Fisher Scientific	Cat# 2149P-05-HR
10 µL Barrier Pipette Tips	Thermo Fisher Scientific	Cat# 2139-05-HR
FastWells Incubation Chambers	Electron Microscopy Sciences	Cat# 70325-50

MATERIALS AND EQUIPMENT

Medium used		
mTeSR plus	Final concentration	Volume
mTeSR 1 Basal Medium	N/A	400 mL
mTeSR 1 5X Supplement	1X	100 mL

Note: Once prepared, store at 4°C for 4 weeks.

mTeSR plus with ROCK inhibitor	Final concentration	Volume
mTeSR 1 Basal Medium	N/A	400 mL
mTeSR 1 5X Supplement	1X	100 mL
100 mM Y27632	5 µM	25 µL

Note: Once prepared, store at 4°C for 4 weeks.

Basal medium	Final concentration	Volume
Advanced DMEM/F-12	N/A	500 mL
GlutaMAX Supplement 100X	1X	5 mL
L-Ascorbic acid phosphate 50 mg/mL	60 µg/mL	600 µL
Penicillin-Streptomycin 100X	0.4X	2mL

Note: Once prepared, store at 4°C for 4 weeks.

S1 medium	Final concentration	Volume
Basal medium	N/A	10 mL
CHIR99021	6 µM	3 µL

Note: Prepare freshly before using.

S2 medium	Final concentration	Volume
Basal medium	N/A	500 mL
EGF Human	20 µL	10 ng/mL
FGF 2 Human (147 a.a.)	250 µL	50 ng/mL
VEGF human	250 µL	50 ng/mL

Note: Once prepared, store at 4°C for 4 weeks.

S2 medium + doxycycline	Final concentration	Volume
S2 medium	N/A	10 mL
Doxycycline 0.5 mg/mL	0.5 μg/mL	10 μL

Note: Prepare freshly before using.

D10 medium	Final concentration	Volume
DMEM, high glucose, pyruvate	N/A	445 mL
FBS	10%	50 mL
Penicillin-Streptomycin 100X	1X	5 mL

Note: Once prepared, store at 4°C for 4 weeks.

StemPro-34 SFM complete medium	Final concentration	Volume
StemPro-34 SFM medium	N/A	50 mL
StemPro-34 nutrient supplement	N/A	1.3 mL
Glutamax	10%	0.5 mL
Penicillin-Streptomycin 100X	1X	0.5 mL

Note: Once prepared, store at 4°C for 2 weeks.

Setup of Neon Electroporator		
Pulse voltage	Pulse width	Pulse number
1150V	30 ms	2

Setup of Orbi-Shaker CO_2

Place the Orbi-Shaker in a CO_2 incubator and adjust the speed to 100 rpm.

STEP-BY-STEP METHOD DETAILS

Human iPSC electroporation

Timing: ~1.5 h

1. Preparation of cells.
   a. Warm mTeSR Plus medium with ROCK inhibitor to 37°C and prepare Matrigel-coated 6-well plates at least 15 min prior to starting.
   b. Rinse cells with PBS, then add 1 mL of TrypLE per well. Aspirate and incubate at 37°C for 3 min.
   c. Add mTeSR Plus with ROCK inhibitor to resuspend cells gently.
   d. Count an aliquot of the cell suspension to determine the total cell number.
   e. Transfer cells to a 1.5 mL microcentrifuge tube and centrifuge at 300 × g for 5 min at 20°C–25°C.
   f. Wash cells once with PBS, centrifuge again at 300 × g for 5 min.
   g. Aspirate PBS and resuspend the pellet in Resuspension Buffer R at a final concentration of 1 × 10^7 cells/mL.

Table 1. Plasmid Transfection Conditions for hiPSCs

Component	Amount
Plasmid DNA	1–2 μg
Cell number	1 × 10^6
Resuspension Buffer R	100 μL
Plating medium (per well)	2 mL mTeSR + ROCKi

Note: Transfect 1 million cells per electroporation reaction.

⚠ CRITICAL: Do not store cells in Buffer R for more than 15–30 min at 20°C–25°C. Prolonged exposure reduces cell viability and transfection efficiency.

h. Add 2 mL of mTeSR Plus with ROCK inhibitor into each well of a pre-warmed 6-well plate and place in the 37°C incubator until use.

2. Electroporation setup.
   a. Use the following parameters for each 6-well transfection reaction (Table 1):
   b. Use the electroporation settings and cuvette format recommended by the manufacturer (e.g., Neon, Lonza). Resuspension Buffer R is compatible with normal voltage protocols.
3. Operating the Neon Transfection System.
   a. Fill the Neon Tube with 3 mL of Electrolytic Buffer E2 (for 100 μL Neon Tip).

Note: Ensure the side electrode is completely immersed in the buffer.

   b. Insert the Neon Tube into the Neon Pipette Station until it clicks into place.
   c. Transfer the desired amount of plasmid DNA into a sterile 1.5 mL microcentrifuge tube.
   d. Add the prepared cell suspension to the DNA tube and gently mix.
   e. Insert a Neon Tip into the Neon Pipette by pressing the push-button to the second stop to open the clamp.
   f. With continued downward pressure, guide the pipette head into the Neon Tip until it clamps securely.
   g. Gently release the push-button while maintaining pressure to ensure a complete seal.
   h. Press the push-button to the first stop, immerse the Neon Tip into the DNA-cell mix, and slowly release to aspirate the mixture.

⚠ CRITICAL: Avoid air bubbles during pipetting, as they can cause arcing and reduce transfection efficiency.

   i. Insert the loaded Neon Pipette vertically into the Neon Tube in the Pipette Station until a click is heard.

Note: Ensure the metal pipette head aligns with the station groove.

   j. Set the electroporation parameters to 1150 V pulse voltage, 30 ms pulse width, and 2 pulses using the touchscreen interface, then press Start to initiate electroporation.
   k. Once the pulse is delivered, "Complete" will appear on the screen.
   l. Remove the Neon Pipette and transfer the transfected cells into a prewarmed Matrigel-coated 6-well plate by pressing the push-button to the first stop.

Note: Discard the used Neon Tip into a biohazard container by pressing the button to the second stop.

   m. Gently rock the plate to evenly distribute the cells. Incubate at 37°C in a humidified 5% CO_2 incubator.

Manual isolation of puromycin-selected iPSC colonies and clonal expansion

Timing: ~2 weeks

4. Initiate puromycin selection.
   a. Begin selection 24–48 h post-transfection when cells reach ~60–80% confluence.
   b. Aspirate the medium, gently rinse once with PBS, then add selection medium (5 μg/mL puromycin) and return to the incubator.
   c. Replace selection medium every 24 h. Monitor morphology: non-resistant cells will round up and detach; surviving colonies remain compact with well-defined borders. Continue until the non-resistant background is minimal and the resistant foci are clearly visible (typically 48–72 h).
5. Prepare colonies for manual picking.
   a. When colonies reach ~0.05–0.3 mm and appear healthy, switch to basal medium without puromycin or ROCK inhibitor for 12–24 h to allow recovery before picking.
   b. In a Matrigel-coated 96-well plate, add 150–200 μL/well of recovery medium (mTeSR Plus with ROCK inhibitor) and pre-warm in the incubator for at least 30 min.
6. Manually isolate colonies.
   a. Using a sterile P200 tip, lightly score a small rectangle around each colony.
   b. Add 20–30 μL TrypLE solution into the scored area.
   c. Incubate for 3 min at 37°C until the colony loosens.
   d. Gently triturate 3–5 times to obtain small clumps.
   e. Transfer the clumps into a pre-warmed 96-well plate with recovery medium.
7. Expand clonal lines.
   a. Incubate undisturbed for 12–16 h.
   b. Replace medium with mTeSR Plus without ROCK inhibitor on the next day and continue daily feeding.
   c. Feed daily and expand wells that reach 60%–80% confluence to 24-well, then to 6-well plates as needed.
   d. Split each clone into –Dox and +Dox (500 ng/mL) conditions and maintain Dox for 24 h.
8. Assess clonal induction and pluripotency.
   a. After 24 h induction, total RNA is harvested, reverse-transcribed to cDNA, and analyzed by qPCR to quantify transgene expression (ETV2 or NKX3.1).
      i. Normalized transcript levels to housekeeping genes (e.g., GAPDH).
      ii. Report expression as fold-change relative to untreated controls (Figures 1A–1C).
   b. Perform immunofluorescence staining on parallel cultures with appropriate primary antibodies to assess protein expression and subcellular localization.
   c. Collect RNA from non-induced cultures to verify baseline pluripotency.
      i. Quantify canonical pluripotency markers (OCT4/POU5F1, SOX2, NANOG) by qPCR and compare expression to parental hiPSCs (Figure 1D).
   d. Immunostaining the non-induced cultures for OCT4, SOX2, and NANOG, to confirm maintenance of pluripotency.
      i. Verify strong nuclear expression, compact colony morphology, and minimal spontaneous differentiation.

Generation of VOs from human iPSCs

Timing: 6 days

Note: This protocol uses two human iPSC lines: Dox-inducible ETV2-BJ-iPSCs and Dox-inducible NKX3.1-BJ-iPSCs, referred to hereafter as Dox-ETV2-iPSCs and Dox-NKX3.1-iPSCs, respectively.

9. Prepare hiPSCs for differentiation (Day −1).

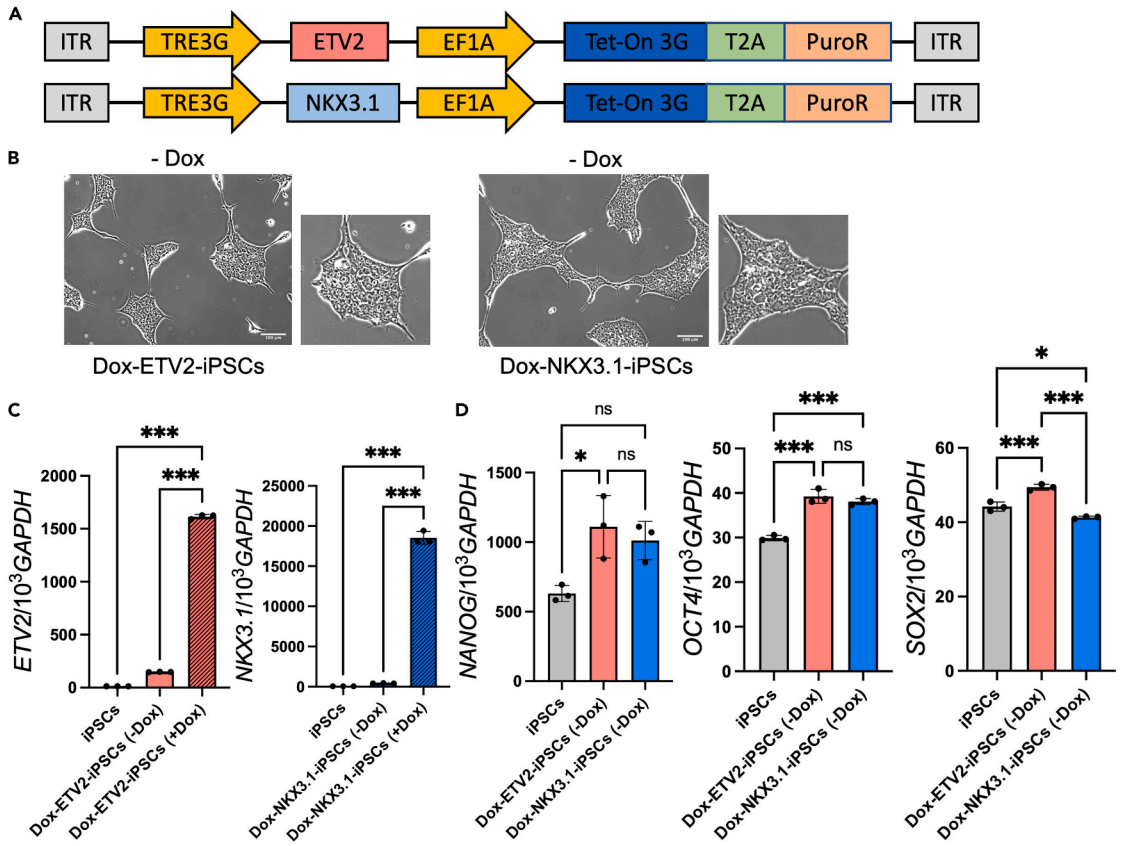

Figure 1. Generation and validation of doxycycline-inducible hiPSCs

(A) Schematic of piggyBac-based constructs for generating Dox-inducible ETV2- and NKX3.1-hiPSCs.

(B) Representative phase-contrast images of Dox-ETV2-hiPSCs and Dox-NKX3.1-hiPSCs cultured without doxycycline (–Dox). Scale bars, 100 μm.

(C) qPCR analysis confirming Dox-inducible expression of *ETV2* and *NKX3.1* in engineered hiPSCs compared with parental controls. Data are normalized to GAPDH.

(D) Pluripotency marker expression (*NANOG*, *OCT4*, *SOX2*) by qPCR showing comparable expression in Dox-inducible lines and parental hiPSCs, indicating maintained pluripotency. *p < 0.05, ***p < 0.001, ns = not significant. All data are mean ± s.e.m. (C and D). n denotes biological replicates (C and D). Statistical analysis was performed using one-way ANOVA with Bonferroni's post-test (C and D).

a. One day before differentiation, wash the hiPSCs (1 well of a 6-well plate) once with 1 mL PBS.
b. Add 1 mL TrypLE per well and incubate for a few seconds.
c. Aspirate most of the TrypLE and incubate at 37°C for 3 min.
d. Add 1 mL of DMEM medium to neutralize the TrypLE and detach the cells by gentle scraping.
e. Transfer the cells to a Matrigel-coated plate containing 2 mL of mTeSR Plus medium supplemented with 5 μM Y27632. For Dox-ETV2-iPSCs and Dox-NKX3.1-iPSCs, include 5 μg/mL puromycin (Figure 2).

10. Induce mesoderm differentiation (S1 phase).
    a. On S1–Day 0, ensure that the hiPSCs are ~30% confluent. Replace mTeSR Plus with S1 medium, consisting of basal medium supplemented with 6 μM CHIR99021 (Figure 3A).
    b. On S1–Day 1, replace with 2 mL of fresh S1 medium (6 μM CHIR99021).

⚠ CRITICAL: Handle gently, as cells are loosely attached at this stage. If seeding density is low, attachment may be even weaker, and medium changes must be performed with extra care to avoid dislodging the cells.

    c. On S1–Day 2 (also S2–Day 0), at this point, S1 differentiation is complete, and the resulting cells are referred as h-iMePCs (human-induced Mesoderm Progenitor Cells).

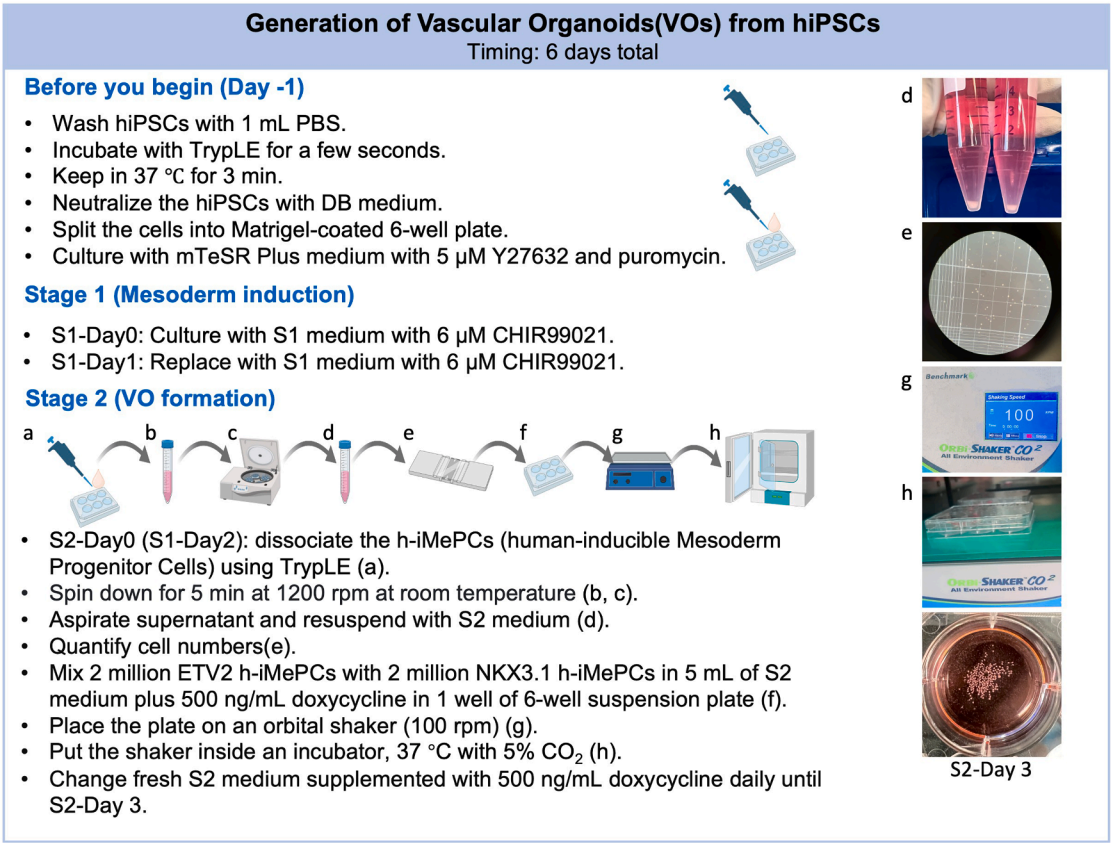

Figure 2. Schematic overview of VO differentiation protocol

Graphical workflow outlining the stepwise procedure to generate vascular organoids (VOs) from human iPSCs. "before you begin" includes plating and pre-conditioning steps. Stage 1 (Mesoderm induction) involves treatment with CHIR99021 for two days to induce human-inducible mesoderm progenitor cells (h-iMePCs). Stage 2 (VO formation) entails aggregation of mixed ETV2 and NKX3.1 h-iMePCs under doxycycline induction and orbital shaking to generate VOs. Panels were partially created with BioRender.com.

11. Prepare and seed h-iMePCs for VO induction (S2 phase).
    a. Wash once with PBS and add 1 mL TrypLE per well.
    b. Incubate at 37°C for 3 min to dissociate the cells.
    c. Neutralize with D10 medium, collect, and centrifuge at 300 × g for 5 min at 20°C–25°C.
    d. Count the cells and resuspend 2 million Dox-ETV2 h-iMePCs and 2 million Dox-NKX3.1 h-iMePCs in 5 mL of S2 medium supplemented with 500 ng/mL doxycycline.
    e. Seed into one well of a 6-well suspension plate and place on an orbital shaker (100 rpm) inside a 37°C incubator.

Note: Expect ~1–2 million h-iMePCs per well of a 6-well plate.

12. Maintain VO induction (S2-Day 1 to S2-Day 3).
    a. Replace 2–3 mL of S2 medium (+Dox) daily on S2-Day 1 and S2-Day 2. Maintain the culture on the orbital shaker throughout.

⚠ CRITICAL: When changing medium, aspirate slowly and carefully to avoid removing the organoids.

    b. On S2–Day 3, confirm completion of differentiation (Figure 3B).

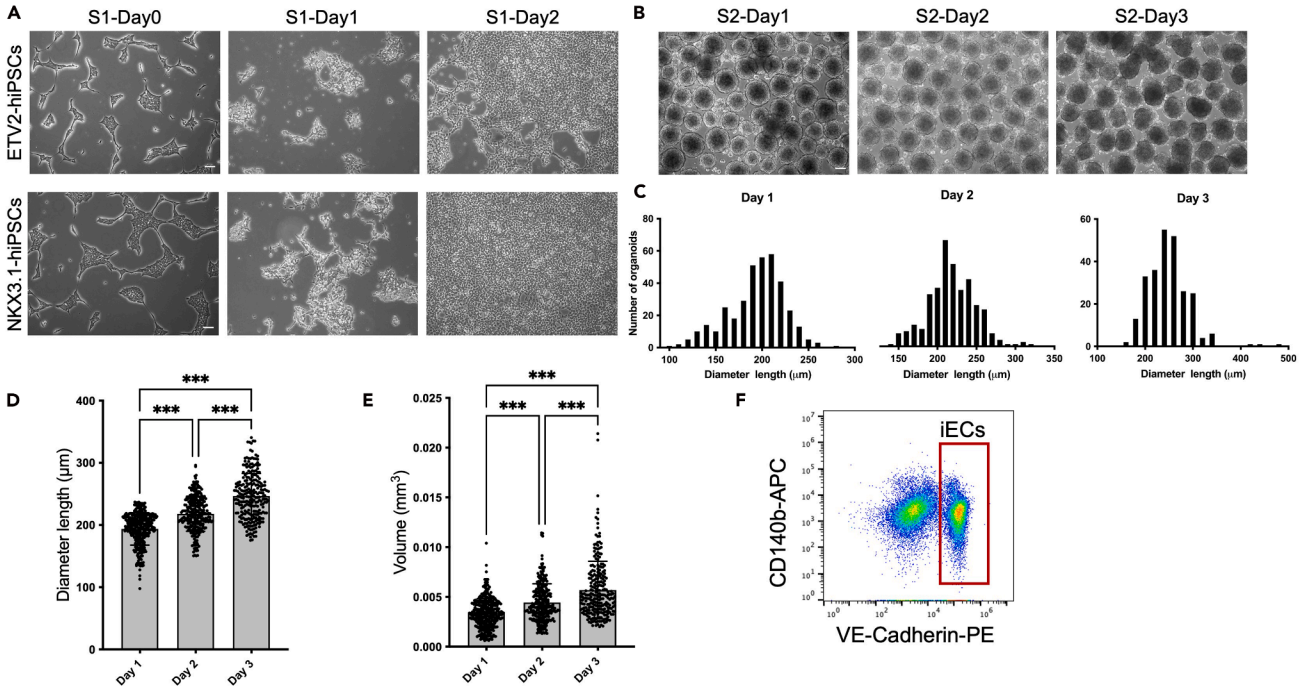

Figure 3. Stepwise differentiation of hiPSCs into VOs

(A) Morphological changes of ETV2- and NKX3.1-hiPSCs during Stage 1 mesoderm induction (S1-Day 0 to S1-Day 2). Scale bars, 100 um.

(B) Representative bright-field images of VO formation during Stage 2 (S2-Day 1–3). Scale bars, 100 m.

(C) Size distribution of VOs at Days 1–3, showing progressive increase in average diameter (~250 μm by Day 3).

(D and E) Quantification of VO diameter (D) and volume (E) across 3 days of culture. ***p < 0.001. All data are mean ± s.e.m. (D and E). n are individual VOs (n > 200). Statistics are one-way ANOVA with Bonferroni's post-test analysis.

(F) Flow cytometry analysis of dissociated VOs at S2-Day 3, showing VE-Cadherin and CD140b expression.

Dissociation of VOs into single cells

Timing: ~30 min

13. Collect and wash the VOs.
    a. Gently transfer the VO suspension to a 15 mL centrifuge tube using a wide-bore P1000 pipette.
    b. Centrifuge at 300 × g for 5 min at 20°C–25°C.
    c. Carefully aspirate the supernatant and resuspend the pellet in 5 mL PBS. Repeat step 13b.
    d. Remove the supernatant completely, taking care not to disturb the pellet.
14. Dissociate the VOs.
    a. Resuspend the organoid pellet in 2 mL of TrypLE per pellet (from one well of a 6-well plate).
    b. Place the tube on an orbital shaker inside the incubator.
        i. Every ~3 min, gently swirl the tube to accelerate dissociation, and observe VO morphology under a microscope.
        ii. Continue alternating incubation and observation until ~10 min, when aggregates begin to fragment.
    c. Gently triturate the suspension 5–10 times using a wide-bore P1000 pipette tip to promote single-cell release. Immediately check the dissociation status under a microscope.

⚠ CRITICAL: Complete dissociation within 10 min. Do not expose cells to TrypLE for extended periods.

15. Neutralize digestion.
    a. Immediately neutralize the digestion with pre-warmed D10 medium once the VOs are fragmented, gently invert to mix, and proceed to centrifugation or washing.

Isolation of iECs and iMCs from dissociated VOs

Timing: 10 min (for steps 16a–16d)

Timing: 10 min (for step 16e)

Timing: 30 min (for step 16f)

16. Prepare cells for isolation.
    a. After dissociation and neutralization, filter the cell suspension through a 70 μm strainer into a fresh 15 mL tube.
    b. Rinse the strainer with 2 mL of cold sorting buffer (PBS without Ca^{2+}/Mg^{2+} + 0.5% BSA + 2 mM EDTA).
    c. Centrifuge at 300 × g for 5 min at 20°C–25°C. Aspirate the buffer completely.
    d. Resuspend the pellet in cold sorting buffer and keep the cells on ice.
    Decision point: Choose either **Branch A** (qPCR from whole VO cells) or **Branch B** (CD31 magnetic isolation).
    Branch A – Harvest of whole VO cells for qPCR.
    e. Collect whole VO cells for RNA extraction.
        i. Wash once with ice-cold PBS.
        ii. Lyse immediately in RNA lysis buffer.
        iii. Snap-freeze on dry ice and store at −80°C, or proceed directly to RNA extraction.
    Branch B – $CD31^+$/$CD31^-$ magnetic isolation.
    f. Enrich CD31+ or CD31-populations.
        i. Aliquot the desired number of cells from step 16d.
        ii. Centrifuge at 300 × g for 5 min. Remove supernatant, wash the pellet in 10 mL isolation buffer, centrifuge again, and resuspend in 1 mL isolation buffer. Transfer to a sterile 1.5 mL tube.
        iii. Add 5 μL of anti-CD31 Dynal beads and incubate for 5 min at 4°C with gentle intermittent mixing to allow binding of $CD31^+$ cells.
        iv. Place the tube in a magnetic cell concentrator for 1 min. $CD31^+$ cells will attach to the magnet; collect the supernatant containing the $CD31^-$ fraction.
        v. Wash the $CD31^-$ fraction with PBS buffer, then either pellet and lyse for RNA extraction or resuspend in SmGM-2 medium for culture.
        vi. Remove the tube from the magnet, add 0.5 mL isolation buffer, mix gently, and return to the magnet for 1 min. Discard the supernatant ($CD31^-$ wash). Repeat this wash step 3 times total.
        vii. After the final wash, either pellet and lyse $CD31^+$ cells for RNA extraction or resuspend in EGM2 medium for reseeding onto 1% gelatin-coated culture dishes.

Embedding VOs into collagen I-Matrigel matrix

Timing: ~5 h

17. Prepare VOs and matrix components.
    a. Determine the number of wells based on aggregate count and target density. Seed 40–60 aggregates per well.

⚠ CRITICAL: Per-well seeding density is important—too high leads to excessive fusion of vascular networks; too low results in inefficient network formation.[5]

b. Calculate total matrix volume. Use 1 mL per well (0.5 mL for layer 1 + 0.5 mL for layer 2). A full 12-well plate requires 12 mL total (6 mL per layer).
c. Thaw Matrigel on ice. Keep all matrix components on ice throughout to prevent premature gelation.
d. Prepare Collagen I solution. For 5 mL batch (enough for one 12-well plate, one layer; adjust accordingly).
   i. Combine the following in a chilled tube—750 μL of 0.1 N NaOH, 313 μL of 10× DMEM, 63 μL of HEPES, 49 μL of 7.5% sodium bicarbonate, 31 μL of GlutaMAX, 460 μL of Ham's F-12, and 3.33 mL of PuroCol (Collagen I).
   ii. Mix gently and confirm pH ~7.4 using indicator strips. Keep the mixture on ice and use promptly.
e. Transfer the prepared Collagen I solution into a fresh 15 mL tube and keep on ice.
f. Add the calculated volume of Matrigel to the Collagen I solution to achieve a 3:1 Collagen: Matrigel ratio. Mix thoroughly by gentle pipetting. Keep on ice.

18. Cast the first matrix layer.
    a. Dispense 0.5 mL of the collagen I–Matrigel solution per well in a 12-well plate.
    b. Swirl gently to ensure even coating of the well bottoms.
    c. Incubate the plate at 37°C for 2 hours to allow matrix polymerization.

Note: Inadequate gelation may indicate insufficient pH neutralization or components not kept cold before casting.

19. Embed VOs in the second matrix layer.
    a. Transfer VOs into a 15 mL tube and allow them to settle by gravity. Carefully aspirate the supernatant.
    b. Add 1 mL of cold StemPro-34 SFM, gently resuspend the pellet, allow settling by gravity, and aspirate.

Note: Use a P200 pipette to remove residual liquid carefully without losing VOs.

    c. Add the required volume of cold collagen I–Matrigel mixture to the VOs and gently resuspend. Keep on ice.
    d. Retrieve the 12-well plate containing the solidified first matrix layer. Add 0.5 mL of the VO-matrix suspension per well.
    e. Gently rock the plate back and forth to distribute VOs uniformly. Place the plate promptly in the incubator.

Note: Gelation begins as the suspension warms. Limit rocking to 30–60 s to avoid disrupting polymerization.

    f. Incubate at 37°C for 2 hours to allow the second layer to solidify.

20. Culture embedded VOs.
    a. Carefully add pre-warmed StemPro-34 SFM complete medium supplemented with 15% FBS, 100 ng/mL VEGF-A, and 100 ng/mL FGF2.

⚠ CRITICAL: Use only pre-warmed medium and avoid fast pipetting directly onto the gel to prevent matrix detachment.

    b. Refresh the StemPro-34 SFM complete medium supplemented with 15% FBS, 100 ng/mL VEGF-A, and 100 ng/mL FGF2 every other day for 5 days.

Table 2. Primary and secondary immunostaining antibodies

Antibody	Vendor	Cat. No.	Dilution
Mouse anti-human CD31	Santa Cruz	RRID:AB_629040 Cat# sc-53411	1:100 in IHC buffer
Rabbit anti-smooth muscle myosin heavy chain 11	Abcam	RRID:AB_2890982 Cat# Ab133567	1:200 in IHC buffer
Donkey anti-mouse IgG AlexaFluor 568	Thermo Fisher Scientific	RRID:AB_11180865 Cat# A-10037	1:500 in IHC buffer
Goat anti-rabbit IgG, AlexaFluor 488	Thermo Fisher Scientific	RRID:AB_2576217 Cat# A-11034	1:500 in IHC buffer
4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	Thermo Fisher Scientific	RRID:AB_2629482 Cat# D1306	1:5000 in IHC buffer

c. Analyze vascular network formation on day 5 using appropriate staining protocols.

Whole-mount CUBIC clearing and immunostaining of gel-embedded VOs

Timing: ~1 week

21. Prepare reagents.
    a. Prepare the following reagents (fresh or thaw just before use)[6,7]:
        i. 4% PFA in PBS for fixation.
        ii. CUBIC R1 buffer: 25% (w/w) urea, 25% (w/w) Quadrol (N, N, N′, N′-Tetrakis(2-hydroxypropyl) ethylenediamine), 15% (w/w) Triton X-100, 35% (w/w) dH_2O.
        iii. R1: dH_2O (1:1 v/v) pre-mix for initial clearing.
        iv. IHC buffer: PBS with 0.1% Triton X-100, 0.1% BSA, 0.01% sodium azide.
        v. CUBIC R2 buffer: 15% dH_2O, 0.1% Triton X-100, 25% urea, 50% sucrose.
22. Fix and wash gel-embedded VOs prior to clearing.
    a. Keep organoids embedded in matrix until fixation.
    b. Under sterile conditions, use 30G needles to gently free each VO, trimming away any remaining matrix.
    c. Immerse VOs in 4% PFA for 24 h at 20°C–25°C with gentle rocking.
    d. Wash VOs 3 × in PBS.
23. Perform CUBIC clearing.
    a. Transfer VOs into R1: dH_2O (1:1) and perform 3 liquid exchanges over 6 h (2 h per exchange) at 20°C–25°C, rocking.
    b. Replace with 100% R1 buffer and incubate for 24h at 20°C–25°C, rocking.
    c. Perform two quick PBS washes to remove residual R1.
24. Perform immunostaining.
    a. Incubate VOs in IHC buffer 3× for 2 h each at 20°C–25°C, rocking.
    b. Replace buffer with primary antibody diluted in IHC buffer. Incubate for 48 h at 20°C–25°C, rocking and protected from light. Detailed antibody information is provided in Table 2.
    c. Wash 3× for 2 h each in IHC buffer at 20°C–25°C, rocking.
    d. Incubate for 12–16 h at 20°C–25°C with secondary antibody in IHC buffer (add DAPI if desired), protected from light.
    e. Wash 3× for 2 h each in PBS, then perform a final PBS wash for 12–16 h.
    f. Immerse stained VOs in CUBIC R2 buffer until optically cleared (typically 6–24 h depending on VO size), rocking gently.
25. Mount cleared VOs for imaging.
    a. Adhere a 9 mm × 1.0 mm imaging chamber to a clean glass slide.

⚠ CRITICAL: Use a spacer thicker than the tallest VO to prevent compression.

    b. Add 40–50 μL of fresh CUBIC R2 buffer to the chamber.

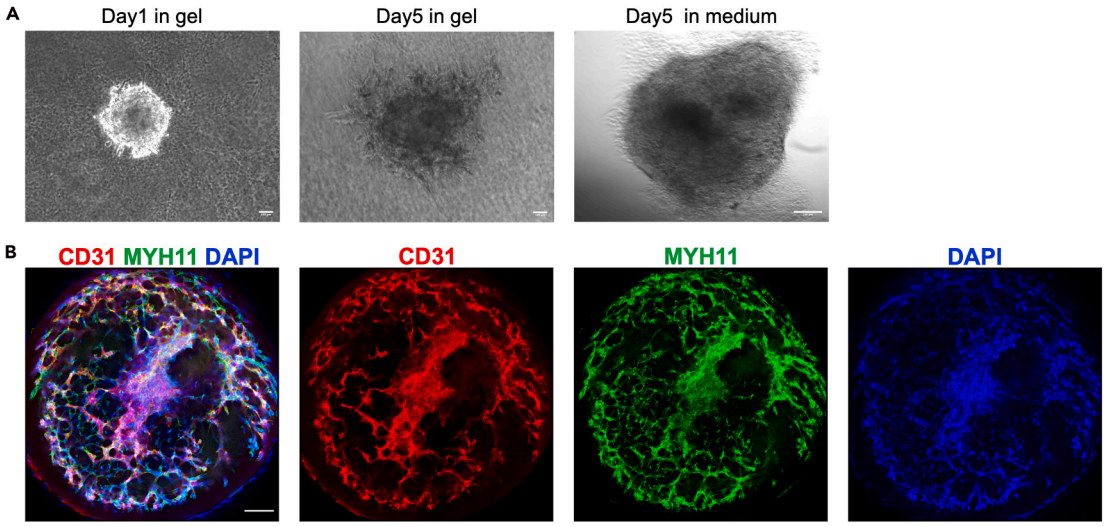

Figure 4. Vascular network maturation in collagen–Matrigel–embedded VOs

(A) Representative bright-field images showing morphological changes of VOs embedded in collagen–Matrigel gels at Day 1 and Day 5, and after retrieval from the matrix and transfer to suspension culture at Day 5. VOs display radial sprouting and increased complexity during gel embedding, while suspension culture preserves the overall 3D structure. Scale bars, 100 m.

(B) Whole-mount immunofluorescence staining of VOs after 5 days in gel reveals extensive vascular network formation composed of $CD31^+$ endothelial cells (red) and $MYH11^+$ smooth muscle cells (green). Nuclei are counterstained with DAPI (blue). Panel B reprinted and adapted with permission from Gong et al., 2025. Scale bars, 100 μm.

c. Transfer 1–3 cleared VOs into the chamber, ensuring they remain fully submerged.
d. Gently arrange VOs with spacing between them. Fill the chamber completely with R2, and remove any visible bubbles at the edges using a fine tip.
e. Cover the chamber with a #1.5H coverslip.
f. Seal the edges with clear nail polish. Once fully dried, proceed with imaging.

EXPECTED OUTCOMES

Successful execution of this protocol yields doxycycline-inducible iPSC lines that enable synchronized expression of the vascular transcription factors ETV2 and NKX3.1. As shown in Figures 1A–1C, Dox-ETV2 and Dox-NKX3.1 iPSCs maintain characteristic pluripotent colony morphology under baseline conditions and exhibit rapid transgene activation upon doxycycline induction. qPCR analysis confirms robust induction of ETV2 and NKX3.1, while core pluripotency markers (OCT4, SOX2, NANOG) remain comparable to parental iPSCs (Figure 1D).

During Stage 1 mesodermal induction, cells transition from compact iPSC colonies to mesenchymal-like progenitors (Figure 3A). By the end of this stage, ~1–2 × 10^6 h-iMePCs can be harvested per well, exhibiting a characteristic spindle-shaped morphology. In Stage 2, aggregation of h-iMePCs under orbital shaking generates compact spherical aggregates within 24 h, with further compaction and growth over the next 3 days (Figure 3B). On average, each well yields ~1,500 VOs, each composed of ~3,000 cells. The VO size distribution centers around ~250 μm in diameter and increases progressively during culture (Figures 3C–3E). Flow cytometry at this stage confirms the emergence of VE-Cadherin$^+$ endothelial cells and PDGFRβ$^+$ mural cells (Figure 3F).

When embedded in a collagen I–Matrigel matrix, VOs generate extensive vascular-like sprouts within 5 days (Figure 4A). A seeding density of ~40–60 aggregates per well of a 12-well plate provides optimal conditions for network formation; excessive density may result in aggregate fusion. Immunofluorescence staining reveals well-formed endothelial tubes ($CD31^+$) with associated mural coverage ($MYH11^+$), recapitulating key features of vessel wall architecture (Figure 4B). It is important

to note that the successful formation of structured vascular organoids requires the combined activation of ETV2 and NKX3.1 to generate iECs and iMCs, respectively. Single-factor conditions (ETV2 alone or NKX3.1 alone) do not support the emergence of organized vascular networks and thus fail to yield the desired morphological or functional outcomes.

In summary, this protocol reproducibly generates inducible VOs from hiPSCs, characterized by a defined size, multicellular composition, and vascular network–forming capacity. This system offers a robust and scalable platform for studying endothelial–mural cell interactions, making it suitable for downstream applications in regenerative medicine, disease modeling, and vascularized tissue engineering, including transplantation-based approaches.

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments were independently repeated at least three times with consistent results. Representative micrographs shown in the figures derive from experiments conducted on three or more independent occasions. Unless otherwise indicated, data are presented as mean ± standard error of the mean (s.e.m.). For statistical comparisons between two groups, unpaired two-tailed Student's *t*-tests were used. For comparisons across multiple groups, one-way ANOVA followed by Bonferroni's post hoc test was applied. No data were excluded from analysis. Statistical analyses were performed using GraphPad Prism (v10), and significance was defined as $P < 0.05$.

LIMITATIONS

This protocol is specifically optimized for the generation of VOs from doxycycline-inducible hiPSC lines engineered to express ETV2 and NKX3.1. Its direct applicability to other hiPSC lines or alternative transcription factor systems may require additional optimization. Differentiation efficiency is highly sensitive to the initial seeding density: low densities can lead to poor cell attachment and loss during medium changes, while overconfluent cultures often exhibit reduced differentiation efficiency.

Suspension culture requires constant agitation at 100 rpm using an orbital shaker housed within a CO_2 incubator. Deviations in shaking speed, incubator vibration, or temperature stability can lead to variable aggregate size and VO yield. Medium exchanges during suspension culture also demand particular care to avoid unintentional aspiration of organoids, which can substantially lower recovery.

Finally, the absence of flow in this culture system precludes exposure of endothelial cells to shear stress, a critical cue for vascular maturation. While this platform effectively models early vasculogenesis, applications requiring more physiologically mature vasculature may benefit from integration with perfusable systems or flow bioreactors.

TROUBLESHOOTING

Problem 1

Cells detach during medium change in early differentiation (Stage 1, Day 0–1).

Potential solution

At this stage, cells are loosely adherent and highly sensitive to mechanical disturbance (Figure 3A). Aspirate medium slowly and at an angle to avoid dislodging cells. When adding fresh medium, dispense gently along the wall of the well to minimize shear. Ensure the initial seeding density is adequate ($0.5–0.6 \times 10^6$ cells/well); low density exacerbates weak attachment. If widespread detachment occurs, restart with increased cell input.

Problem 2

Loss of organoids during medium change in suspension culture (Stage 2).

Potential solution

VOs are free-floating and can be inadvertently aspirated during medium removal (Figure 2). Always aspirate gently from the side of the well and leave a small residual volume to avoid loss. Use wide-bore pipette tips to reduce shear. If organoids are left undisturbed for extended periods (e.g., during imaging), they may settle and adhere to the plate bottom. To prevent this, perform medium changes efficiently, and gently tap the plate to re-suspend aggregates before returning it to the orbital shaker.

Problem 3

Incomplete dissociation of VOs into single cells.

Potential solution

If aggregates remain clumped after TrypLE treatment, extend digestion up to 10 minutes with gentle agitation on the orbital shaker (Figure 2). Avoid prolonged exposure to maintain viability. Triturate gently 5–10 times using a wide-bore pipette tip, and monitor under the microscope. Neutralize digestion immediately once a near-single-cell suspension is achieved.

Problem 4

Inconsistent vascular network formation and matrix instability after VO embedding.

Potential solution

Network formation is sensitive to VO density and matrix handling. Overcrowding (>60 aggregates/well) can lead to fusion and abnormal networks, while under-seeding (<40/well) results in sparse outgrowth (Figure 4A). Target ~40–60 aggregates per well of a 12-well plate, and gently rock the plate during embedding to ensure even distribution.

Use only prewarmed medium during feedings, and add it along the well wall to avoid disturbing the gel. Matrix detachment often results from cold medium or direct pipetting onto the gel surface. Additionally, remove excess Matrigel surrounding VOs prior to embedding, as residual gel can disrupt proper matrix integration. Careful handling ensures stable embedding and reproducible sprouting outcomes.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Juan M. Melero-Martin (juan.meleromartin@childrens.harvard.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Dr. Liyan Gong (liyan.gong@ihm.ac.cn).

Materials availability

Cells and plasmids generated in this study can be shared by the lead contact upon request. This study did not generate new unique reagents.

Data and code availability

This study did not generate new or unique code and did not analyze new or unique datasets.

ACKNOWLEDGMENTS

Some illustrations were partially created with BioRender.com. This work was supported by grants from the National Institutes of Health (R01HL128452, R01HL172968, and R01HL151450 to J.M.M.-M.) and the Breakthrough T1D Foundation (2-SRA-2023-1314-S-B to J.M.M.-M.).

AUTHOR CONTRIBUTIONS

L.G. and J.M.M.-M. conceived and designed the project, discussed and analyzed the data, and wrote the manuscript.

DECLARATION OF INTERESTS

J.M.M.-M. is an inventor on a patent application (Methods of Making and Using iPSC-Derived Mural Progenitor Cells via NKX3.1 Activation; patent number: WO 2025/080915; date of filing: October 11, 2024; filing jurisdiction: PCT [International Application no. PCT/US2024/050882]), filed by the Children's Medical Center Corporation, related to this work.

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