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Generating Blood-Generating Heart-Forming Organoids from human pluripotent stem cells

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We use this protocol and it's working

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Abstract

Our established human pluripotent stem cell (hPSC)-derived heart-forming organoids (HFOs) recapitulate aspects of heart, vasculature, and foregut co-development. Modulating HFO differentiation, we here report the protocol for generating blood-generating (BG)-HFOs; while maintaining a functional ventricular-like heart anlagen, BG-HFOs comprise a mesenchyme embedded hemogenic endothelial layer encompassing multiple hematopoietic derivatives and hematopoietic progenitor cells with erythro-myeloid and lymphoid potential, reflecting aspects of primitive and definitive hematopoiesis. The model enables the morphologically structured co-development of cardiac, endothelial, and multipotent-hematopoietic tissues equivalent to the intra-embryonic hematopoietic region in vivo, promoting research on hematopoiesis in vitro.

Guidelines

Our research complies with all relevant ethical regulations; experiments using hESCs lines were performed under allowance '108 Genehmigung nach dem Stammzellgesetz'; granted by the Robert Koch Institute.

Materials

Materials:

*synthesized by the Institute for Organic Chemistry (Leibniz University Hannover)

MATERIAL	COMPANY
Accutase	Thermo Fisher Scientific, cat. no. A11105-01
Ascorbic acid 2-phosphate	Sigma-Aldrich, cat. no. A8960
B-27 supplement (minus insulin)	Thermo Fisher Scientific, cat. no. A1895601
B-27 supplement (plus insulin)	Thermo Fisher Scientific, cat. no. 17504-044
Basic fibroblast growth factor (bFGF)	PeproTech, cat. no. AF-100-18B
Bone morphogenetic protein 4 (BMP4)	PeproTech, cat. no. 120-05 HeLa
CHIR99021 (CHIR)	*
DMEM/F-12 medium	Thermo Fisher Scientific, cat. no. 11330-057
Erythropoietin (EPO)	PeproTech, cat. no. 100-64
Fms related receptor tyrosine kinase-3 (FLT-3)	PeproTech, cat. no. 300-19
Geltrex LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix	Thermo Fisher Scientific, cat. no. A1413202
Insulin	Sigma-Aldrich, cat. no. I9278
Insulin-like growth factor 1 (IGF-1)	PeproTech, cat. no. 100-11
(Interleukin-3) IL-3	PeproTech, cat. no. 200-03
(Interleukin-6) IL-6	PeproTech, cat. no. 200-06
(Interleukin-11) IL-11	PeproTech, cat. no. 200-11
(Inhibitor of WNT production 2) IWP2	Tocris, cat. no. 3533
Matrigel	Corning, cat. no. 354234; tested lots: no. 100007, 1335001, 1336002, 1013001, 1335001, 1336002, 2033002, 2237001
Phosphate-buffered saline (PBS) (Ca ²⁺ /Mg ²⁺ -free; 10x)	Thermo Fisher Scientific, cat. no. 70011051
Penicillin/ Streptomycin (P/S)	Sigma-Aldrich, cat. no. P0781
Rho kinase inhibitor Y-27632	Tocris Bioscience, cat. no. 1254, or *
RPMI/1640 medium	Thermo Fisher Scientific, cat. no. 21875-091
Sonic Hedgehog (SHH)	PeproTech, cat. no. 100-45
Sodium bicarbonate (NaHCO ₃)	Sigma-Aldrich, cat. no. S5761
Sodium selenite (Na ₂ SeO ₃)	Sigma-Aldrich, cat. no. S5261
Stem Cell Factor (SCF)	PeproTech, cat. no. 300-07
STEMdiff Cardiomyocyte Dissociation Kit	Stemcell Technologies, cat. no. 05025
Tissue-Tek	Sakura Finetek, cat. no. 4583
(Transforming growth factor β) TGF β	PeproTech, cat. no. 100-21C
Thrombopoietin (TPO)	PeproTech, cat. no. 300-18
Transferrin	Sigma-Aldrich, cat. no. T3705

Vascular endothelial growth factor (VEGF)	PeproTech, cat. no. 100-20
Trypan blue solution	Sigma-Aldrich, cat. no. T8154

⊗ Accutase Gibco - Thermo Fisher Scientific Catalog # A1110501

⊗ L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate Merck MilliporeSigma (Sigma-Aldrich) Catalog #A8960



- ✕ B-27™ Supplement, minus insulin **Gibco - Thermo Fisher Scientific Catalog #A1895601**
- ✕ B-27 Supplement **Gibco - Thermo Fisher Scientific Catalog #17504044**
- ✕ Animal-Free Recombinant Human FGF-basic **peprotech Catalog #AF-100-18B**
- ✕ Recombinant Human BMP-4 **peprotech Catalog #120-05**
- ✕ DMEM/F-12, HEPES **Thermo Fisher Catalog #11330057**
- ✕ Recombinant Human EPO **peprotech Catalog #100-64**
- ✕ Recombinant Human Flt3-Ligand **peprotech Catalog #300-19**
- ✕ Geltrex®; LDEV-Free Reduced Growth Factor Basement Membrane Matrix **Thermo Fisher Catalog #A1413202**
- ✕ Insulin solution human **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I9278**
- ✕ Recombinant Human IGF-I **peprotech Catalog #100-11**
- ✕ Recombinant Human IL-3 **peprotech Catalog #200-03**
- ✕ Recombinant Human IL-6 **peprotech Catalog #200-06**
- ✕ Recombinant Human IL-11 **peprotech Catalog #200-11**
- ✕ IWP 2 **Tocris Catalog #3533**
- ✕ Corning® Matrigel® Basement Membrane Matrix, LDEV-free, 10 mL **Corning Catalog #354234**
- ✕ PBS (10X), pH 7.4 **Thermo Fisher Scientific Catalog #70011051**
- ✕ Penicillin-Streptomycin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P0781**
- ✕ Y-27632 dihydrochloride **Tocris Catalog #1254**
- ✕ RPMI 1640 Medium **Thermo Fisher Catalog #21875091**
- ✕ Recombinant Human Sonic Hedgehog **peprotech Catalog #100-45**
- ✕ Sodium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5761**
- ✕ Sodium selenite **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5261**
- ✕ Recombinant Human SCF **peprotech Catalog #300-07**
- ✕ STEMdiff™ Cardiomyocyte Dissociation Kit **STEMCELL Technologies Inc. Catalog #05025**
- ✕ SDS Tissue-Tek® **Sakura Finetek Catalog #4583**
- ✕ Recombinant Human TGF-β1 (CHO derived) **peprotech Catalog #100-21C**
- ✕ Recombinant Human TPO **peprotech Catalog #300-18**
- ✕ Transferrin human **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T3705**
- ✕ Recombinant Human VEGF **peprotech Catalog #100-20**
- ✕ Trypan Blue solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154**

Culture Media:

Medium/buffer	Composition
Essential 8 (E8) medium	DMEM/F-12, HEPES-supplemented with 64 mg/L ascorbic acid 2-phosphate 100 µg/L bFGF 20 mg/L insulin 543 mg/L NaHCO ₃ 14 µg/L Na ₂ SeO ₃ 10.7 mg/L transferrin 2 µg/L TGFβ P/S 1:100
RB-	RPMI/1640 2% B-27 supplement (minus insulin) P/S 1:100
RB+	RPMI/1640 2% B-27 supplement (plus insulin) P/S 1:100

Equipment:

EQUIPMENT	COMPANY, CATALOG NUMBER
Conical tubes, 15 mL	Greiner Bio-One, cat. no. 188271
T25 flasks (TPP, cat. no. 90026)	TPP, cat. no. 90026
U-bottom ultra-low attachment 96-well plates	Nunclon Sphera, cat. no. 174925 FaCellitate cat. no. 202003
Hemocytometer, Neubauer improved	Brand, cat. no. 717805
Pipets (2–20 µL, 20–200 µL, 100–1,000 µL)	Eppendorf
Sterile scissors (heated to at least 180 °C for at least 3 h)	
Centrifuge, Heraeus Multifuge X3R	Thermo Fisher Scientific
Humidified incubator (37 °C, 5% CO ₂)	SANYO
Fluorescence microscope AXIO Observer A1	Zeiss
Inverted microscope Olympus CKX41	Olympus

☒ Conical tube, 15 ml **greiner bio-one** Catalog #188271

☒ Tissue Culture Flasks **Techno Plastic Products (tpp)** Catalog #90026

☒ Nunclon®; Sphera®; Microplates, 96U-Well Plate **Thermo Fisher** Catalog #174925

☒ BRAND® counting chamber BLAUBRAND® Neubauer improved New without clips, double ruled **Merck**
MilliporeSigma (Sigma-Aldrich) Catalog #BR717805



Pre-culture

- 1 Grow the human pluripotent stem cell (hPSC) lines of interest (in our case hES3 NKX2.5-eGFP^{1, 2} and HSC_ADCF_SeV-iPS2³) on irradiated embryonic mouse fibroblasts in the incubator at  37 °C , 5%CO₂.
 - At 80% colony confluence, passage the cells and seed either onto fresh irradiated fibroblasts or, to start organoid differentiation, transfer to Geltrex-coated T25 flasks in Essential 8 (E8) medium supplemented with  10 micromolar (μM) Y-27632.
- 2 Passage the cells every 3-4 days and change the medium daily except the day immediately after the passaging.

Generation of BG-HFOs

2d 0h 56m

3

Note

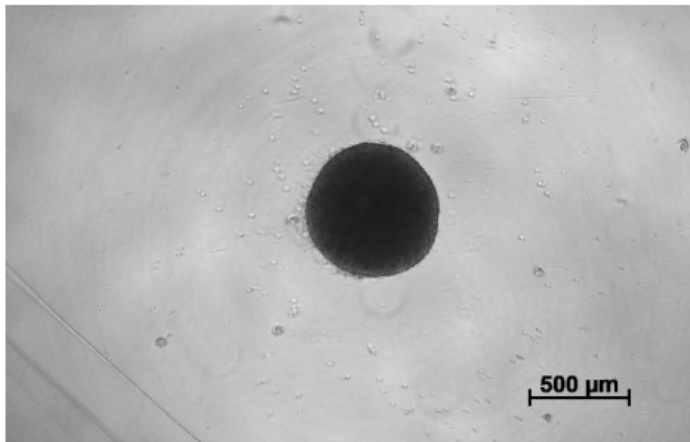
- The protocol for generation of BG-HFOs relies on the published protocol for generating Heart-Forming Organoids (HFOs)^{4, 5}; to have specifics regarding the key protocol passages please refer to the cited manuscript⁴.
- The HFO protocol adaptation in order to generate BG-HFOs consists of the addition of growth factors and cytokines at specific time-points during the differentiation, described as follows:

- 4 On the starting day (day minus 4; d-4), detach hPSCs from Geltrex-coated flasks using Accutase.
- 4.1 Count the cells using a hemocytometer and trypan blue solution; seed 5000 cells per well in a U-shaped ultralow-attachment 96-well plate in E8 medium supplemented with 10 μM Y-27632.
- 4.2 Centrifuge the plate is at  300 x g, 4°C, 00:06:00 and placed in the incubator to let one hPSC aggregate per well form  Overnight .

6m



- 5 After 52 h (d-2), only the hPSC aggregates looking round and with a sharp edge will be further differentiated.
- Embed the selected aggregates in a  20 μL Matrigel droplet. Embedding is performed seeding Matrigel droplets using a p200 pipet set to 20 μL , and in each droplet seed one single aggregate using a p20 pipet set to 3 μL .
 - In order to maintain the integrity of the aggregates, the edge of the 20 μL plastic tip is enlarged by cutting it with a pair of sterilized scissors; for specifics please refer to the HFO protocol⁴.



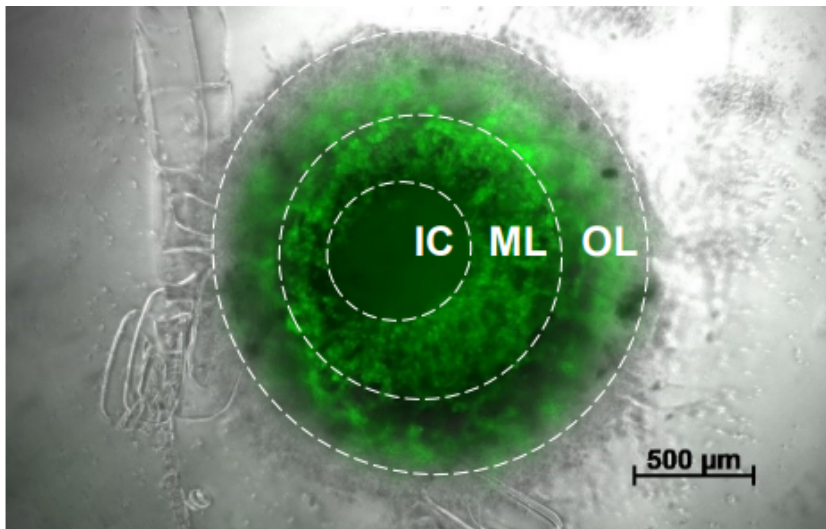
hES3 NKX2.5-eGFP-derived aggregate at d-2 of the protocol with round and sharp edge; such organoids are selected for subsequent Matrigel embedding.

- 6 After embedding, place the plate at  37 °C in the incubator for  00:50:00 to let the Matrigel solidify.
- Afterwards, supplement E8 medium with 10 ng ml⁻¹ BMP4 is added on top of the Matrigel-embedded aggregates.
 - Add  100 μL for each well. Further details are described in our previous publication⁴.
- 7 After 2 days, on d0, replace E8 with  200 μL per well of RB- supplemented with 7.5 μM CHIR 10 ng ml⁻¹ BMP4, and bFGF 5 ng ml⁻¹.
- Remove the medium by using first a P1000 pipet to remove the majority of the medium and then a p200 pipet to remove the remaining medium.

50m

- It is important to do not disturb the Matrigel droplet containing the aggregate during the media change, but gently moving it with the edge of the tip.
 - Perform all the subsequent media changes following this criteria.
- 8 After 24 h, on d1, exchange the medium by RB- supplemented with 50 ng ml⁻¹ VEGF and 10 ng ml⁻¹ bFGF.
- 9 2 days afterwards, on d3, add RB- supplemented with [M] 5 micromolar (μM) IWP2, 50 ng ml⁻¹ VEGF, 10 ng ml⁻¹ bFGF, 100 ng ml⁻¹ SCF, 17 ng ml⁻¹ EPO, 10 ng ml⁻¹ IL-6, 10 ng ml⁻¹ IL-11 and 25 ng ml⁻¹ IGF-1, for ⌚ 48:00:00 .
- 10 After 2 days, on d5, exchange the medium by RB- supplemented with the same molecules of d3 plus 30 ng ml⁻¹ TPO, 10 ng ml⁻¹ FLT3, 30 ng ml⁻¹ IL-3, 10 ng ml⁻¹ BMP4, and 20 ng ml⁻¹ SHH.
- 11 2 days after, from d7 onwards, cultivate BG-HFOs in RB+ supplemented with the same cytokines added on d5.
- 12 After 3 days, on d10, complete BG-HFO differentiation; keep the organoids in culture for downstream analysis until d14.

2d



hES3 NKX2.5-eGFP-derived BG-HFO at d10 of the differentiation. NKX2.5-eGFP, expressed in early cardiomyocytes, allows for the visualization of the organoid's structure; an inner core (IC) NKX2.5-eGFP^{neg}, a myocardial layer (ML) NKX2.5-eGFP^{pos}, and an outer layer (OL) only partially positive for NKX2.5eGFP. Such organoids are used for downstream analyses.



Protocol references

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