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A transgene-free, human peri-gastrulation embryo model with trilaminar embryonic disc-, amnion- and yolk sac-like structures

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Abstract

Peri-gastrulation represents a critical yet experimentally inaccessible stage of human embryonic development. This protocol describes the generation and characterization of a transgene-free peri-gastrulation trilaminar embryonic disc (PTED) embryoid derived exclusively from primed human pluripotent stem cells (hPSCs), enabling modeling of peri-gastrulation development without incorporating trophoderm- or hypoblast-like lineages.

Using this protocol, PTED embryoids are expected to form trilaminar embryonic layers positioned between dorsal amnion-like and ventral definitive yolk sac-like tissues, and followed primitive hematopoiesis and blood cell generation. The model recapitulates key structural and functional features of peri-gastrulation development through coordinated extrinsic control of tissue boundaries and intrinsic self-organization and lineage plasticity of peri-gastrulation-stage cells, offering a tractable and ethically less complex model for investigating the self-organizing properties of human peri-gastrulation development.

Materials

H1 human embryonic stem cell (hESC; WA01, WiCell; NIH registration number: 0043),
H9 hESC (WA09, WiCell; NIH registration number: 0062),
ESI017 hESC (NIH registration number: 0093; a gift from Dr. Aryeh Warmflash),
1196a human induced pluripotent stem cell (hiPSC) line from the University of Michigan Pluripotent Stem Cell Core
mTeSR1 medium (STEMCELL Technologies, cat #85850),
Geltrex (Thermo Fisher Scientific, cat #A1413302)
LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich, cat #MP0035)
Polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning)
glass coverslips (Fisherbrand cat #12541000)
ozone cleaner (Jelight)
DMEM/F12 (Gibco, cat #11-320-082)
BMP4 (R&D System, cat #314-BP-050)
Activin A (R&D System, cat #338-AC-050)
Essential 6™ Medium (E6; Gibco, cat. #A1516401)
Advanced DMEM/F12 (Gibco, cat #12634010)
N2 (Invitrogen, cat#17502048)
B27 (Invitrogen, cat#17504044)
GlutaMAX™ (Gibco, cat #35050061)
HEPES (Gibco, cat #15630080)
penicillin/streptomycin (Gibco, cat #15-140-122)
EGF (PeproTech, cat #AF-100-15).

Cell culture

- 1 hPSC lines used in this study. All cell lines were maintained under feeder-free conditions in mTeSR1 medium (STEMCELL Technologies, cat #85850). Prior to seeding, culture plates were coated with 1% lactate dehydrogenase-elevating virus (LDEV)-free, hESC cell-qualified reduced growth factor basement membrane matrix Geltrex (Thermo Fisher Scientific, cat #A1413302), derived from Engelbreth-Holm-Swarm mouse tumors. To ensure the quality of hPSCs, cells were visually examined at each passage to confirm the absence of spontaneously differentiated, mesenchymal-like cells. All hPSC lines were used prior to passage 70. Authentication of hPSCs was conducted both by the original source and in-house, including immunostaining for pluripotency markers and tri-lineage differentiation potential. Karyotype analysis was conducted by Cell Line Genetics to confirm genomic stability and karyotypic normality. All hPSC cultures were routinely screened for mycoplasma contamination using the LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich, cat #MP0035).

Microcontact printing

- 2 Polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning) stamps featuring circular micropatterns were fabricated using a microstructured silicon mold. PDMS prepolymer, mixed at a 1:20 ratio of curing agent to base polymer, was poured onto the mold and cured at 110°C overnight. Following thermal curing, the PDMS stamps were peeled off the mold, immersed in 1% Geltrex solution (v / v in DMEM/F12), and incubated at 37°C for 1 hr. In parallel, glass coverslips (Thermo Fisher Scientific) were treated with ultraviolet ozone using an ozone cleaner (Jelight) for 7 min. After drying with nitrogen gas, the Geltrex-coated PDMS stamps were brought into conformal contact with the ozone-treated coverslips to transfer the adhesive micropatterns.

PTED embryoid generation

- 3 On Day -2, hPSCs cultured in tissue culture plates were dissociated using Accutase (Millipore-Sigma, cat. #A6964) at 37°C for 8 min. The cells were then centrifuged and resuspended in mTeSR1 medium supplemented with 10 μ M Y27632 (Tocris, cat. #1254) at a concentration of 4×10^6 cells mL⁻¹. A total of 140 μ L of cell suspension was plated onto 12-mm diameter coverslips pre-patterned with Geltrex adhesive islands, resulting in an approximate cell density of 5,000 cells mm⁻². Coverslips were placed into a 24-well plate and incubated for 30 min to initiate attachment and formation of PTED embryoids. Following incubation, unattached cells were removed by gently washing coverslips with DMEM/F12, after which 500 μ L of mTeSR1 containing 10 μ M Y27632 was added to each well.

- 4 On Day -1, culture medium was replaced with 500 μL of mTeSR1 without Y27632.
- 5 On Day 0 and Day 1, culture medium was changed to mTeSR1 supplemented with BMP4 (50 ng mL^{-1} ; R&D Systems, cat. #314-BP-050).
- 6 On Day 2, medium was switched back to mTeSR1 without additional supplements. From Day 3 to Day 8, half of the medium was replaced daily with fresh mTeSR1. Embryoids that spontaneously detached from coverslips between Days 5 and 8 were collected and transferred to a low-attachment 96-well plate, with one embryoid per well. Culture medium in these wells was half-replaced with fresh mTeSR1 daily during this period.
- 7 For extended culture, Day 8 PTED embryoids were bisected manually under a stereomicroscope. The dorsal halves, comprising the amniotic- and embryonic disc-like structures, and the ventral halves, containing yolk sac-like tissues, were transferred to tissue culture plates and maintained separately in gut growth medium or Essential 6™ Medium (E6; Gibco, cat. #A1516401), respectively, both supplemented with 1% Geltrex. Medium was replenished every two days, and tissues were fixed and analyzed on Day 14. The gut growth medium consisted of Advanced DMEM/F12 supplemented with N2 (Invitrogen), B27 (Invitrogen), GlutaMAX™ (Gibco), 10 μM HEPES (Gibco), penicillin/streptomycin, and EGF (100 ng mL^{-1} ; PeproTech, cat. #AF-100-15).

PTED embryoids with enhanced endoderm differentiation

- 8 On Day -1, hPSCs were dissociated in tissue culture plates using Accutase at 37°C for 8 min to obtain single-cell suspensions. Cells were then centrifuged, re-suspended in mTeSR1 containing 10 μM Y27632, and adjusted to a density of 4×10^6 cells mL^{-1} . A 140 μL aliquot of cell solution was dropped onto a 12-mm diameter coverslip pre-coated with Geltrex adhesive islands, resulting in a cell density of approximately 5,000 cells mm^{-2} . Coverslips were then placed into a 24-well plate and incubated at 37°C for 30 min. Unattached cells were removed by washing coverslips multiple times with DMEM/F12. After washing, 500 μL of mTeSR1 containing 10 μM Y27632 was added to replenish the medium.
- 9 On Day 0, culture medium was switched to 500 μL of E6 medium containing 50 ng mL^{-1} BMP4 and 100 ng mL^{-1} Activin A.
- 10 On Days 1 and 2, medium was replenished with 500 μL of E6 medium containing 100 ng mL^{-1} Activin A. Samples were fixed on Day 3 for immunocytochemistry to assess endoderm differentiation.
- 11 For prolonged culture of PTED embryoids with enhanced endoderm differentiation, embryoids were detached from coverslips on Day 3 by mechanically removing them with

pipette tips. Detached cell colonies were transferred to a new 24-well plate, with one coverslip per well. E6 medium supplemented with 1% Geltrex was added.

- 12 On Day 4, culture medium was switched to E6, and fresh E6 medium was replenished daily from Day 4 to Day 14.

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