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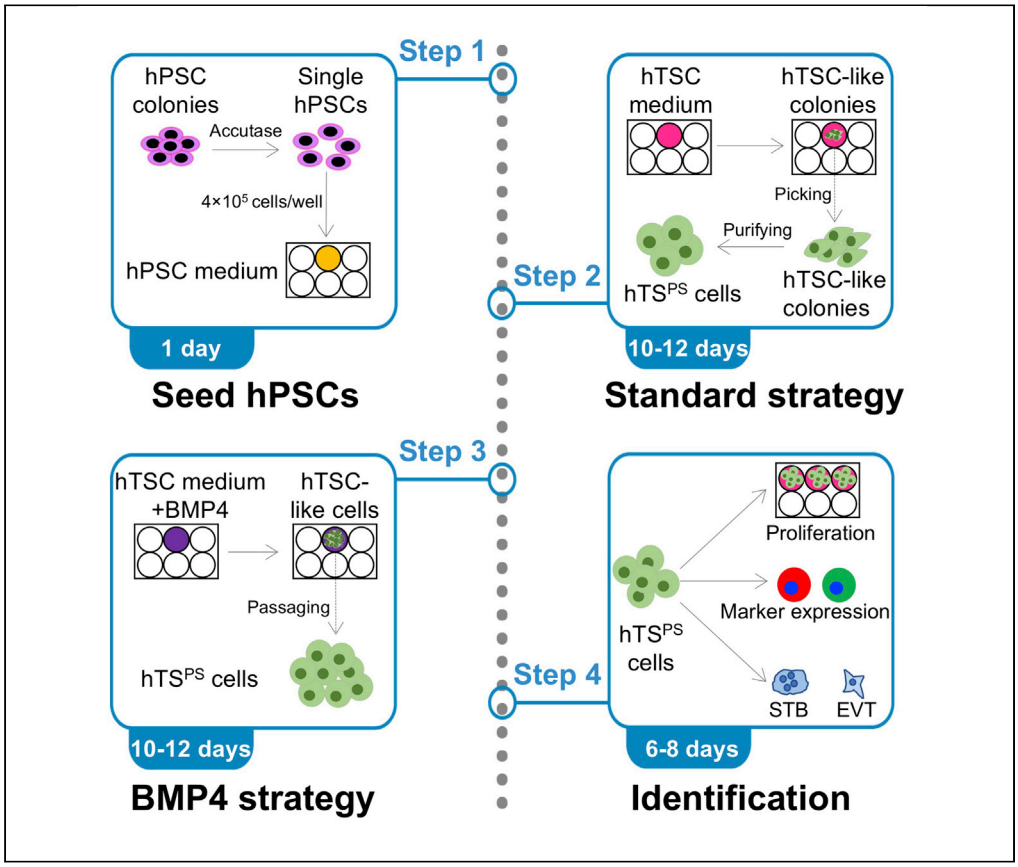
Protocol to derive human trophoblast stem cells directly from primed pluripotent stem cells



Human trophoblast stem cells (hTSCs) are useful for studying human placenta development and diseases, but primed human pluripotent stem cells (hPSCs) routinely cultured in most laboratories do not support hTSC derivation. Here, we present a protocol to derive hTSCs directly from primed hPSCs. This approach, containing two strategies either with or without bone morphogenetic protein 4 (BMP4), provides a simple and accessible tool for deriving hTSCs to study placenta development and disease modeling without ethical limitations or reprogramming process.

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

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Highlights

Protocol includes two strategies for hTSC derivation from primed hPSCs

Standard strategy without BMP4 to derive hTSC from hPSCs

Strategy using BMP4 to promote induction efficiency of hTSCs from hPSCs

Derived hTSCs exhibit typical morphology, gene markers, and ability to differentiate

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Protocol to derive human trophoblast stem cells directly from primed pluripotent stem cells

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SUMMARY

Human trophoblast stem cells (hTSCs) are useful for studying human placenta development and diseases, but primed human pluripotent stem cells (hPSCs) routinely cultured in most laboratories do not support hTSC derivation. Here, we present a protocol to derive hTSCs directly from primed hPSCs. This approach, containing two strategies either with or without bone morphogenetic protein 4 (BMP4), provides a simple and accessible tool for deriving hTSCs to study placenta development and disease modeling without ethical limitations or reprogramming process.

For complete details on the use and execution of this protocol, please refer to Wei et al. (2021).

BEFORE YOU BEGIN

To date, the resources for derivation of human trophoblast stem cells (hTSCs) include blastocysts, early placentas, as well as naïve and expanded pluripotent stem cells (PSCs) (Dong et al., 2020; Gao et al., 2019; Guo et al., 2021; Io et al., 2021; Okae et al., 2018). However, these systems require undergoing ethical approvals or resetting the naïve or expanded state from primed PSCs. Current protocols report that primed PSCs do not support hTSC derivation. On the other hand, the transition of TSCs from PSCs is actively investigated in developmental biology, including in mice. Interestingly, unlike human TSCs, mouse TSCs are not derived directly from embryonic stem cells (ESCs) under culture medium (Peng et al., 2019; Zhang et al., 2022). But, mouse special genetic background, including overexpression (OE) of *Cdx2*, *Hmgn3* OE, knockout (KO) of *Rif1*, or *p53* KO, could facilitate the conversion of TSCs from ESCs (Peng et al., 2019; Zhang et al., 2022). So human PSC-to-TSC transition provides an insight for direct derivation of TSCs from mouse ESCs and a model for uncovering cell fate decisions of early embryonic development. In the present study, we efficiently derive hTSCs from primed hPSCs, including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) (Wei et al., 2021). Here, we describe the detailed step-by-step protocol with and

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without BMP4 for derivation of hTSCs from primed PSCs. Our report shows pictures for the hESC line H1 as an example, but hiPSCs can also be used to derive hTSCs using this protocol.

Preparation for matrigel-coated plates

Timing: 1 h

1. Dilute the hESCs-quality matrigel matrix (Corning, Cat#354277) in cold Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Cat#C11330500BT) (dilution ratio=1:100). The diluted matrigel can be stored at 4°C for up to 1 month.
2. The matrigel coating condition is at 37°C and at least for one hour.
3. Set the cell culture incubator to 37°C and 5% CO_2.

Preparation of human primed pluripotent stem cells

Timing: 3 days

4. hPSCs are cultured in 2 mL/well mTeSR1 (STEMCELL Technologies, Cat#85850), and the fresh medium is changed every day.

   Note: For successful derivation of hTSCs, hPSCs can be maintained across a range of passages from 30 to 70 (hESCs) or from 10 to 40 (hiPSCs).

5. hPSCs are passaged at 80%–90% confluency.
   a. hPSCs are washed in DMEM/F12 and then digested using 1 mL 0.5 mM EDTA (dilution of 0.5 M EDTA in DPBS) for 6 min at 37°C.
   b. Discard EDTA and resuspend the cells with fresh mTeSR1 medium.
   c. Passage the cells at 1:3 ratio on Matrigel-coated plates.
6. Prepare the hPSCs for conversion.
   a. hPSCs are washed in DMEM/F12 and then digested with 600 μL Accutase (Sigma, Cat#A6964) per well for 8 min at 37°C.
   b. Add 600 μL DMEM/F12 into the well.
   c. Transfer the cell suspension to a 1.5 mL centrifuge tube, and centrifuge at 200 *g* for 5 min.
   d. Discard supernatant and resuspend cells with 1.5 mL pre-warmed mTeSR1 medium.
   e. Pipette the cells evenly and count the cell number using the cell count chamber.
   f. Seed 4×10^5 hPSCs per well on the matrigel-coated 6-well plates in mTeSR1 medium plus 10 μM Y27632.

   Note: The cell count chamber requires a cell density of 5×10^5–2×10^6 cells/mL for optimal counting results. Repeat 3 times and calculate the mean as the readout.

   ⚠ CRITICAL: The cells should be healthy and viable. The percentage of live cells must be greater than 90% using trypan blue staining protocols.

Preparation of trophoblast stem cell and their differentiation media

7. hTSC medium.
   a. for TS derivation and culture, TS medium contains DMEM/F12 (Gibco, Cat#11320033), 0.3% bovine serum albumin (BSA), 1% Insulin, Transferrin, Selenium, Ethanolamine Solution (ITS-X), 0.1 mM β-Mercaptoethanol, 0.5% Penicillin-Streptomycin, 0.2% fetal bovine serum (FBS), 1.5 μg/mL L-ascorbic acid, 50 ng/mL EGF, 2 μM CHIR-99021, 0.5 μM A83-01, 1 μM SB431542, 0.8 mM valproic acid (VPA), 5 μM Y27632.
8. EVT differentiation medium.

a. for EVT differentiation, EVT differentiation medium 1 (EVT1) contains DMEM/F12 (Gibco, Cat#11320033), 0.3% BSA, 1% ITS-X, 0.1 mM β-Mercaptoethanol, 0.5% Penicillin-Streptomycin, 7.5 μM A83-01, 20 μM Y27632, 100 ng/mL NRG1, 4% KnockOut Serum Replacement (KSR), 0.2 μM WNT-C59, 1 μM XAV939, 0.5 μM PD0325901, 0.5 μM thiazovivin, 2% Matrigel.
b. for EVT differentiation, EVT differentiation medium 2 (EVT2) contains DMEM/F12, 0.3% BSA, 1% ITS-X, 0.1 mM β-Mercaptoethanol, 0.5% Penicillin-Streptomycin, 7.5 μM A83-01, 2.5 μM Y27632, 4% KnockOut Serum Replacement (KSR), 0.5% Matrigel.

9. STB differentiation medium.
   a. for STB differentiation, STB differentiation medium contains DMEM/F12 (Gibco, Cat#11320033), 0.3% BSA, 1% ITS-X, 0.1 mM β-Mercaptoethanol, 0.5% Penicillin-Streptomycin, 2 μM forskolin, 5 μM Y27632, 50 ng/mL EGF, 4% KnockOut Serum Replacement (KSR), 1 μM cAMP.
10. These media can be stored at 4°C for up to 2 weeks.
11. Order the antibodies found in the key resources table for characterization.

Note: All the chemicals and reagents should be kept sterile. Media and buffers should be prepared under a Class II biological hood. The TS medium is the same as the culture medium for establishing and maintaining the primary TS cell lines, hTS^{blast} and hTS^{CT} (Okae et al., 2018).

KEY RESOURCES TABLE

REGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Matrigel	Corning	Cat#354277
DMEM/F12 basic	Gibco	Cat#C11330500BT
mTeSR1	STEMCELL Technologies	Cat#85850
Accutase	Sigma	Cat#A6964
PBS	GENOM	Cat#GNM20012-2
Dulbecco's PBS (DPBS)	Gibco	Cat#C14190500BT
EDTA	Genstar	Cat#VA17876-500g
DMEM/F12 (for TS medium and differentiation)	Gibco	Cat#11320033
BSA (30% in saline, fatty acid free, aseptically filled)	Sigma	Cat#A9205
ITS-X (100×)	Gibco	Cat#51500056
β-Mercaptoethanol (55 mM)	Gibco	Cat#21985023
Penicillin-Streptomycin (100×)	HyClone	Cat#SV30010
FBS	Gibco	Cat#16000044
L-ascorbic acid	Sigma	Cat#49752
EGF	PeproTech	Cat#AF-100-15
CHIR-99021	Selleck	Cat#S1263
A83-01	Selleck	Cat#S7692
SB431542	Selleck	Cat#S1067
Valproic acid (VPA)	MedChemExpress	Cat#HY-10585
Y27632	Selleck	Cat#S6390
DMSO	Sigma	Cat#D1435
Thiazovivin	Selleck	Cat#S1459
PD0325901	Selleck	Cat#S1036
XAV939	Selleck	Cat#S1180
WNT-C59	Selleck	Cat#S7037
KnockOut Serum Replacement	Gibco	Cat#10828028
NRG1	Cell Signaling Technology	Cat#5218SC
Forskolin	Selleck	Cat#S2449
cAMP	Sigma	Cat#D-0260
BMP4	R&D systems	Cat#314-BP
Serum-free cryopreservation medium	ZENOAQ	Cat#CELLBANKER2

(*Continued on next page*)

Continued		
REGENT or RESOURCE	SOURCE	IDENTIFIER
TrypLE Express	Gibco	Cat#12604021
DAPI	Sigma	Cat#D9542
Experimental models: Cell lines		
Human embryonic stem cell line H1 (WA01)	WiCell	RRID: CVCL_9771
Primary trophoblast stem cell line	Hiroaki Okae Lab	N/A
Antibodies		
KRT7 (1:200 dilution)	ZSGB-BIO	Cat#ZM-0071
GATA3 (1:1000 dilution)	Cell Signaling Technology	Cat#5852
TP63 (1:800 dilution)	Cell Signaling Technology	Cat#13109
TEAD4 (1:500 dilution)	Abcam	Cat#ab58310
OCT-3/4 (1:200 dilution)	Santa Cruz Biotechnology	Cat#sc-5279
HLA-G (1:500 dilution)	Novus Biologicals	Cat#NB500-314
CGB (1:500 dilution)	Abcam	Cat#ab9582
Goat anti-Rabbit IgG (H+L), Alexa Fluor 488 (1:500 dilution)	Thermo Fisher Scientific	Cat#A-11008
Goat anti-Mouse IgG (H+L), Alexa Fluor 568 (1:500 dilution)	Thermo Fisher Scientific	Cat#A-11004
Other		
Culture plates (6 well)	Greiner Bio-One	Cat#657160
Culture plates (12 well)	Greiner Bio-One	Cat#665180
Culture plates (24 well)	Greiner Bio-One	Cat#662160
Disposable Borosilicate Glass Pasteur Pipets	Thermo Fisher Scientific	Cat#13-678-20C
Vertical sharp glass pipet	Lab-made	N/A
Counting chambers	Marienfeld	Cat#0650030
Cryotube	Thermo Fisher Scientific	Cat#377267

MATERIALS AND EQUIPMENT

hTSC medium		
Regent	Final concentration	Amount
DMEM/F12 (Gibco, Cat#11320033)	N/A	484.8 mL
BSA (30% in saline, fatty acid free, aseptically filled)	0.3%	5 mL
ITS-X (100×)	1%	5 mL
β-Mercaptoethanol (55 mM)	0.1 mM	910 μL
Penicillin-Streptomycin (100×)	0.5%	2.5 mL
FBS	0.2%	1 mL
L-ascorbic acid (70 mg/mL)	1.5 μg/mL	10.7 μL
EGF (200 μg/mL)	50 ng/mL	125 μL
CHIR-99021 (15 mM)	2 μM	66.7 μL
A83-01 (5 μM)	0.5 μM	50 μL
SB431542 (10 μM)	1 μM	50 μL
VPA (1 M)	0.8 mM	400 μL
Y27632 (20 mM)	5 μM	125 μL
Total	N/A	**500 mL**

EVT differentiation medium 1 (EVT1)		
Regent	Final concentration	Amount
DMEM/F12 (Gibco, Cat#11320033)	N/A	45.42 mL
BSA (30% in saline, fatty acid free, aseptically filled)	0.3%	0.5 mL
ITS-X (100×)	1%	0.5 mL
β-Mercaptoethanol (55 mM)	0.1 mM	91 μL
Penicillin-Streptomycin (100×)	0.5%	0.25 mL

(*Continued on next page*)

Continued		
Regent	Final concentration	Amount
NRG1 (100 μg/mL)	100 ng/mL	50 μL
Matrigel	2%	1 mL
KnockOut Serum Replacement	4%	2 mL
Thiazovivin (5 μM)	0.5 μM	5 μL
A83-01 (5 μM)	7.5 μM	75 μL
XAV-939 (10 μM)	1 μM	50 μL
PD0325901 (5 μM)	0.5 μM	5 μL
WNT-C59 (1 mM)	0.2 μM	10 μL
Y27632 (20 mM)	20 μM	50 μL
Total	N/A	50 mL

EVT differentiation medium 2 (EVT2)		
Regent	Final concentration	Amount
DMEM/F12 (Gibco, Cat#11320033)	N/A	46.33 mL
BSA (30% in saline, fatty acid free, aseptically filled)	0.3%	0.5 mL
ITS-X (100×)	1%	0.5 mL
β-Mercaptoethanol (55 mM)	0.1 mM	91 μL
Penicillin-Streptomycin (100×)	0.5%	0.25 mL
Matrigel	0.5%	0.25 mL
KnockOut Serum Replacement	4%	2 mL
A83-01 (5 μM)	7.5 μM	75 μL
Y27632 (20 μM)	2.5 μM	6.25 μL
Total	N/A	50 mL

STB differentiation medium		
Regent	Final concentration	Amount
DMEM/F12 (Gibco, Cat#11320033)	N/A	46.58 mL
BSA (30% in saline, fatty acid free, aseptically filled)	0.3%	0.5 mL
ITS-X (100×)	1%	0.5 mL
β-Mercaptoethanol (55 mM)	0.1 mM	91 μL
Penicillin-Streptomycin (100×)	0.5%	0.25 mL
Forskolin (10 mM)	2 μM	10 μL
KnockOut Serum Replacement	4%	2 mL
cAMP (1 mM)	1 μM	50 μL
EGF (200 μg/mL)	50 ng/mL	12.5 μL
Y27632 (20 μM)	5 μM	12.5 μL
Total	N/A	50 mL

Note: These media can be stored at 4°C for up to 2 weeks.

STEP-BY-STEP METHOD DETAILS

Passaging hPSCs for derivation of hTSCs

Timing: 30–60 min

Day -1.

Figure 1 shows the overall approach, including the standard strategy and BMP4 strategy, to derive human trophoblast stem cells (hTSCs) from human pluripotent stem cells (hPSCs). These following steps provide the detailed information to prepare single-cell suspensions of hPSCs from colonies.

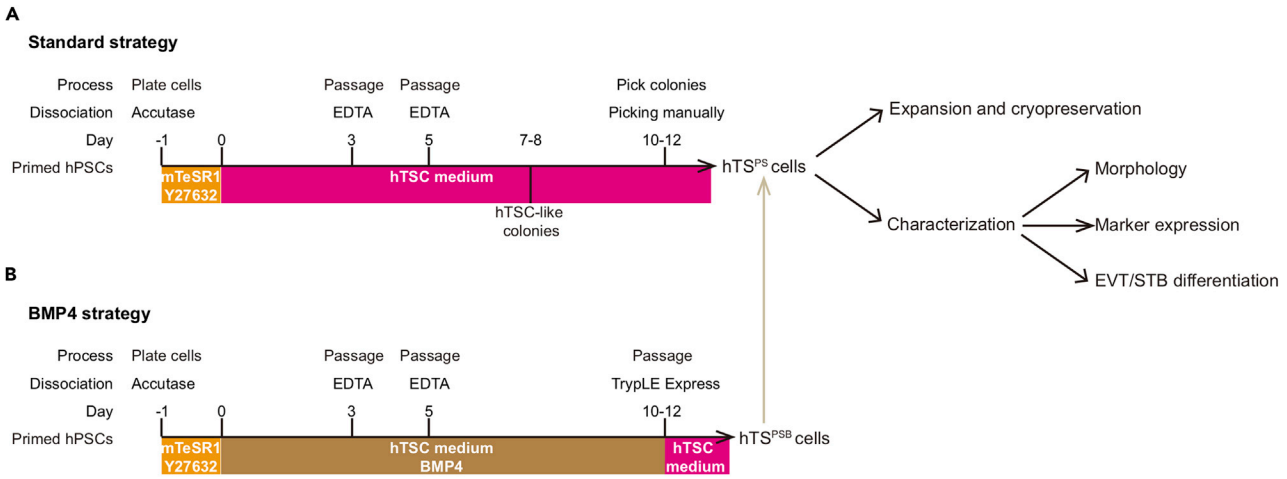

Figure 1. The conversion strategies for derivation of hTS^{PS} cells from primed hPSCs

Overview of conversion strategies to generate and characterize hTSCs (hTS^{PS} cells) from primed PSCs (hPSCs) in the absence (Standard strategy) (A) or presence of BMP4 (BMP4 strategy) (B).

1. Observe the morphology of hPSCs and confirm hPSCs at 80%–90% confluency under the microscope.
2. Remove the medium from culture plate (6 well).
3. Wash hPSCs with 1 mL DMEM/F12 basic medium per well.
4. Remove DMEM/F12 basic medium from the well.
5. Add 600 μL Accutase per well to dissociate into individual cells for 8 min at 37°C and 5% CO_2.
6. 8 min later, observe the hPSCs which are the single formation and floating.
7. Add 600 μL DMEM/F12 basic medium into the well and pipette the cell suspension gently.
8. Transfer the cell suspension to a 1.5 mL centrifuge tube and centrifuge cells at 200 *g* for 5 min.
9. Remove the supernatant and resuspend cells with 1.5 mL mTeSR1 medium 10 μM Y27632.
10. Pipette the cells gently to ensure single-cell suspension.
11. Count the cell number using the cell count chamber using trypan blue.
12. Seed 4×10^5 cells per well of hPSCs in mTeSR1 medium plus 10 μM Y27632 on the matrigel-coated 6-well plates at 37°C and 5% CO_2.

Derivation of hTSCs by the standard strategy

Timing: 10–12 days

These steps describe the derivation of hTSCs by the standard strategy. During the conversion process, the cells are cultured at 37°C and 5% CO_2.

13. One day later (day 0), remove the mTeSR1 medium and wash hPSCs with 1 mL DMEM/F12 basic medium per well.
14. Add 2 mL hTSC medium per well and observe the morphology of hPSCs (Figure 2A).
15. Change the fresh hTSC medium every 2 days.
16. On day 3, confluency of the transdifferentiated cells reaches about 90–100% (Figure 2A).
    a. Remove the medium and add 1 mL per well 0.5 mM EDTA in the well for 8 min at 37°C.
    b. 8 min later, observe the morphology of cells under the microscope, the cell-cell interactions should become loose.
    c. Remove EDTA and add 3 mL fresh TSC medium in the well.
    d. Pipette the cells gently and passage the cells into the new matrigel-coated 6-well plate at 1:3 ratio.

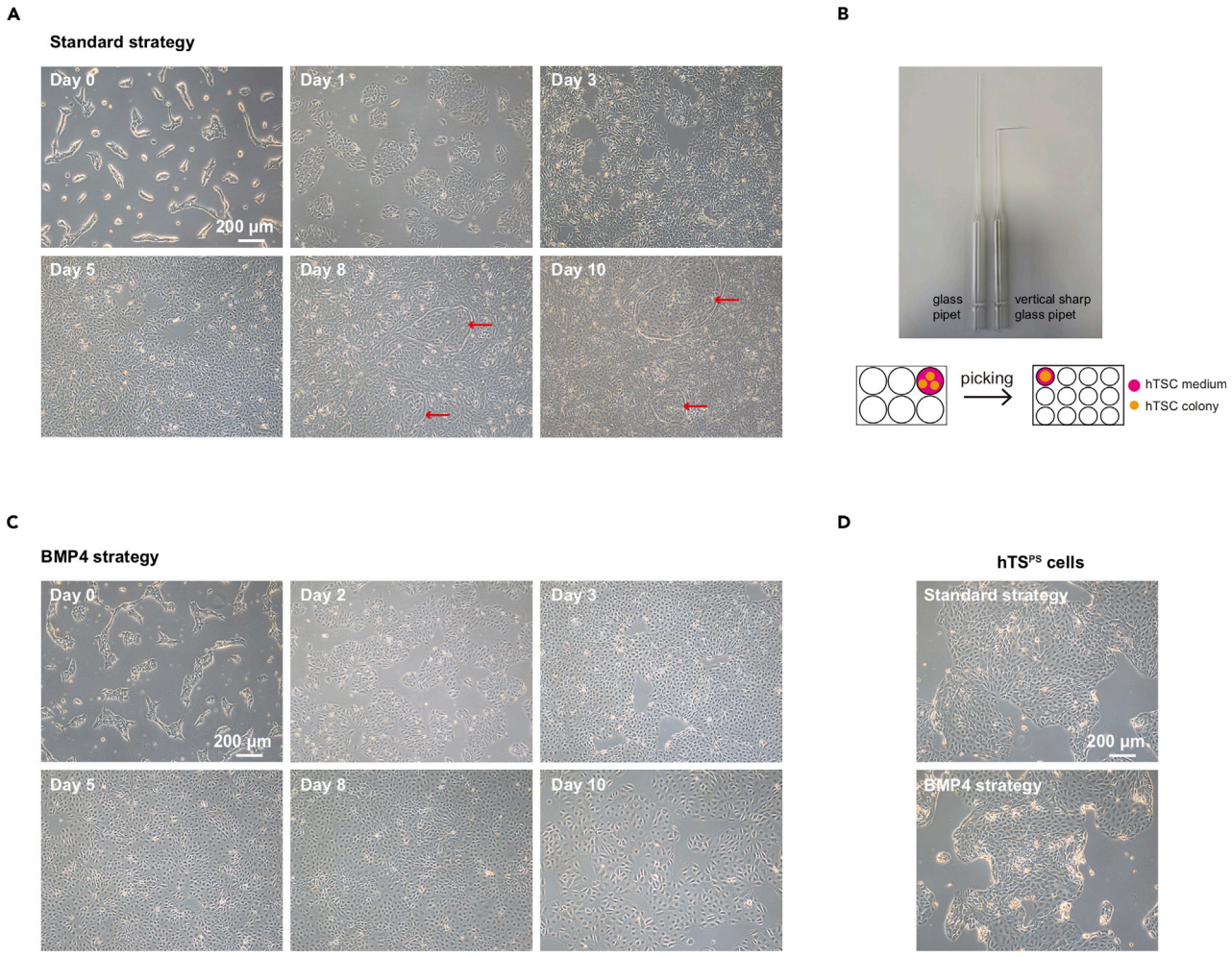

Figure 2. hTS^{PS} cells are efficiently derived from primed hPSCs

(A) Morphology of cells derived from H1 hESCs under TS medium by the standard strategy on the indicated days. Scale bar, 200 μm.

(B) The tool (upper panel) and schematic diagram (bottom panel) of picking hTSC colonies. The glass pipet (Left) and vertical sharp glass pipet (Right).

(C) Morphology of cells derived from H1 hESCs under TS medium plus BMP4 by the BMP4 strategy on the indicated days. Scale bar, 200 μm.

(D) Morphology of hTS cells (named hTS^{PS} cells) derived from H1 hESCs.

e. Add 1 mL per well fresh hTSC medium to every well.

Note: The incubation time with 0.5 mM EDTA is appropriate when cell-cell interactions become loose, but the cells become non-floating.

17. Change the fresh hTSC medium every 2 days.
18. On day 5, repeat step 16 to passage the cells with 0.5 mM EDTA at 1:3 ratio (Figure 2A).

Note: This step describes the passaging of the converted cells, similar to step 16.

19. Change the fresh hTSC medium every 2 days for a week.
20. On day 7–8, observe the morphology of the transdifferentiated cells and confirm appearance of hTSC-like colonies (Figure 2A).

Note: The appearance time of hTSC-like colonies may vary depending on the cell lines, initial cell density, and passaging time point.

21. On day 10–12, the hTSC-like colonies should be observed and can be picked for purification and characterization (Figures 2A and 2B).
    a. Remove the medium from the culture plates and add 1 mL per well DMEM/F12 basic medium to wash the cells.
    b. Remove DMEM/F12 basic medium and add 2 mL fresh hTSC medium.

    Note: Before picking colonies, the microscope, disposable glass pipets, and its surrounding environment are wiped with 75% alcohol, and an alcohol lamp should be lit next to the microscope to maintain sterilization. The glass pipets are burned into a vertical and sharp shape using the alcohol lamp (Figure 2B). New 12-well plates are coated with matrigel for at least 1 h at 37°C.

    c. Burn the glass pipets using alcohol lamp and open the plate.
    d. Observe the hTSC-like colonies under the microscope and gently scrape these colonies from the well along these colonies using the glass pipets.
    e. Shake the culture plate to collect these scraped colonies.
    f. Transfer the cell suspension to the new matrigel-coated 12-well plate at 37°C and 5% CO_2.

    Note: The number of hTSC-like colonies may vary depending on the cell lines. According to the colony number, the cell suspension is transferred at 1:1–1:3 ratio.

    g. Change the fresh hTSC medium every day.
    h. Scrape Some non-hTS cells by the glass pipets.

    Note: There may be some non-hTS cells in these hTSC colonies in the 12-well plate.

    i. Passage the purified hTSC-like cells at 80%–90% confluency with 600 μL TrypLE Express dissociation solution per well for 8–10 min at 37°C.

    Note: Many steps of the cell dissociation using TrypLE Express are similar to accutase. Please see the above description.

    j. 10 min later, collect the cell suspension into 1.5 mL centrifuge tubes and centrifuge cells at 200 *g* for 5 min.
    k. Remove the supernatant and resuspend cells with 1 mL hTSC medium.
    l. Pipette the cells gently and passage the cells in the new matrigel-coated 6-well plate at 1:1 ratio.
    m. Add the fresh hTSC medium to a total of 2 mL per well in 6-well plate and culture the cells at 37°C and 5% CO_2.
    n. Change the fresh hTSC medium every day.
    o. Passage routinely the purified human TSC-like cells using TrypLE Express every three days at 1:3 ratio.

    Note: If these cells grow slowly at the beginning, the culture days can be extended appropriately. The purified human TSC-like cells are named human TS^{PS} cells in the later description.

Derivation of hTSCs by the BMP4 strategy

Timing: 10–12 days

These steps describe the derivation of hTSCs in the presence of BMP4. BMP4 promotes induction efficiency of hTSCs from hPSCs. Thus, some steps for derivation of hTSCs using the BMP4 strategy are different from the steps using the standard strategy. The detailed information for the BMP4

strategy is provided in the following steps. During the conversion process, the cells are cultured at 37°C and 5% CO_2.

22. One day later (day 0), aspirate the mTeSR1 medium and wash hPSCs with DMEM/F12 basic medium.
23. Add 2 mL hTSC medium plus 10 ng/mL BMP4 per well (Figure 2C).
24. Change the fresh hTSC medium plus 10 ng/mL BMP4 every 2 days.
25. On day 3, observe the morphology of the transdifferentiated cells and confirm appearance of hTSC-like colonies (Figure 2C).

Note: The appearance time of hTSC-like cells using the BMP4 strategy is earlier than that of standard strategy.

26. Passage the cells at 90–100% confluency using 0.5 mM EDTA for 8 min at 37°C.
27. Change the fresh hTSC medium plus 10 ng/mL BMP4 every 2 days.
28. On day 5, repeat step 26 to passage the cells with 0.5 mM EDTA at 1:3 ratio.
29. Change the fresh hTSC medium every 2 days for a week.
30. On day 10–12, passage the hTSC-like cells using TrypLE Express for 8 min at 1:3 ratio (Figure 2C).

Note: On day 10, almost all transdifferentiated cells will exhibit hTSC-like morphology. Therefore, these cells do not need to be picked manually and can be directly passaged using TrypLE Express.

31. Add the fresh hTSC medium to a total of 2 mL per well in 6-well plate and culture the cells at 37°C and 5% CO_2.
32. Change the fresh hTSC medium every day.
33. Routinely passage routinely the human TSC-like cells (BMP4 strategy) using TrypLE Express every three days at 1:3 ratio.

Note: If these cells grow slowly at the beginning, the culture days can be extended appropriately. These purified human TSC-like cells are named human TS^{PSB} cells using the BMP4 strategy.

Passaging and cryopreservation of hTS^{PS} cells

Timing: 30 min

hTSC-like cells can be derived from primed hPSCs by the standard and BMP4 strategies. These hTS^{PS} and hTS^{PSB} cells need to be expanded, stored, and characterized as bona fide hTSCs. These steps describe the passaging and cryopreservation of hTS^{PS} and hTS^{PSB} cells.

34. For passaging of hTS^{PS} and hTS^{PSB} cells, confluency of hTS^{PS} and hTS^{PSB} cells should reach 80%–90%.
    a. Remove the medium from the well of hTS^{PS} and hTS^{PSB} cells.
    b. Wash hPSCs with 1 mL DMEM/F12 basic medium per well and remove DMEM/F12 from the well.
    c. Add 600 μL TrypLE Express per well for 8 min at 37°C and 5% CO_2.
    d. 8 min later, shake the plate and add 600 μL DMEM/F12 basic medium into the well.
    e. Pipette the cell suspension gently and transfer the cell suspension to a 1.5 mL centrifuge tube.
    f. Centrifuge cells at 200 *g* for 5 min and remove the supernatant.
    g. Add 1.5 mL hTSC medium and resuspend cells gently.
    h. Seed the cells at 1:3 ratio in a total of 2 mL hTSC medium per well on the matrigel-coated 6-well plate at 37°C and 5% CO_2.
    i. Change the fresh hTSC medium every day.
    j. Passage the hTS^{PS} and hTS^{PSB} cells using TrypLE Express every three days at 1:3 ratio.
35. For cryopreservation of hTS^{PS} and hTS^{PSB} cells, confluency of the cells should reach 80%–90%.
    a. Repeat step 34a-f and add 1 mL Cell Banker 2 to resuspend cells.

b. Transfer the cell suspension to Cryotube vial.
c. Store the vial at −80°C and subsequently liquid nitrogen.

Characterization of hTS^{PS} cells

Timing: 10 min (morphology), 48 h (marker expression)

Bona fide hTSCs maintain hTSC morphology, express hTSC markers (GATA3/KRT7/TEAD4/TP63) (Lee et al., 2016), and differentiate into extravillous cytotrophoblast cells (EVT) and syncytiotrophoblast cells (STB) (Okae et al., 2018). Therefore, the hTS^{PS} and hTS^{PSB} cells should exhibit these characteristics. The following steps describe the characterization of hTS^{PS} and hTS^{PSB} cells.

36. The morphology of hTS^{PS} and hTS^{PSB} cells is captured by the microscope (Figure 2D).
37. The expression of hTSC markers GATA3/KRT7/TEAD4/TP63 and hESC marker OCT4 is validated using immunostaining (Figures 3A and 3B). See key resources table for the antibody information.

Differentiation of hTS^{PS} cells

Timing: 6–8 days

This step describes how to induce differentiation of the hTSCs into extravillous cytotrophoblast cells (EVT) and syncytiotrophoblast cells (STB).

38. For EVT differentiation of hTS^{PS} cells (Figure 3C).
    a. Remove the medium from hTS^{PS} cells on the 6-well plate at 80%–90% confluency and wash with DPBS.
    b. Treat the cells with 600 μL TrypLE Express for 8 min at 37°C.
    c. 8 min later, add 600 μL DMEM/F12.
    d. Transfer the cell suspension to 1.5 mL centrifuge tube, and centrifuge at 200 *g* for 5 min.
    e. Discard supernatant and resuspend cells with 1.5 mL pre-warmed EVT1 medium.
    f. Pipette the cells evenly and count the cell number using the cell count chamber.
    g. Seed 4×10^5 hTS^{PS} cells per well on the matrigel-coated 6-well plate, and add EVT1 medium to a total of 2 mL per well for 3 days at 37°C and 5% CO_2.
    h. After 3 days, Remove EVT1 medium and add EVT2 medium for the next 3 days.
    i. On day 6 to 8, these EVT cells are collected for analysis.
39. For STB differentiation of hTS^{PS} cells (Figure 3D).
    a. Repeat step 38a-d, and resuspend cells with 1.5 mL pre-warmed STB medium.
    b. seed 1×10^5 hTS^{PS} cells per well on the matrigel-coated 6-well plate, and add STB medium to a total of 2 mL per well for 3 days at 37°C and 5% CO_2.
    c. Change the fresh STB medium for the next 3 days.
    d. On day 6, STB cells are collected for analysis.

EXPECTED OUTCOMES

This protocol describes the generation of hTSCs from primed hPSCs using hTSC medium in the presence or absence of BMP4 by successive culturing and passaging (Figure 1). We define the day of medium change from mTeSR1 to TS medium as Day 0. By the standard strategy, the epithelial hTSC-like colonies appear on day 7–8 (Figure 2A). And the hTSC-like colonies enlarge with distinct boundaries (Figure 2A). The hTSC-like colonies need to be picked manually on day 10–12 and transferred to new culture plates for purifying and expanding (Figures 2A, 2B, and 2D). The purified human TSC-like cells are named human TS^{PS} cells. Using the BMP4 strategy, the colony picking step is no longer needed. In detail, BMP4 is added in the TS medium for derivation of hTSCs on day 0 and for next 10 days (Figure 2C). On day 3, the cells exhibit the epithelial hTSC-like morphology (Figure 2C). During the

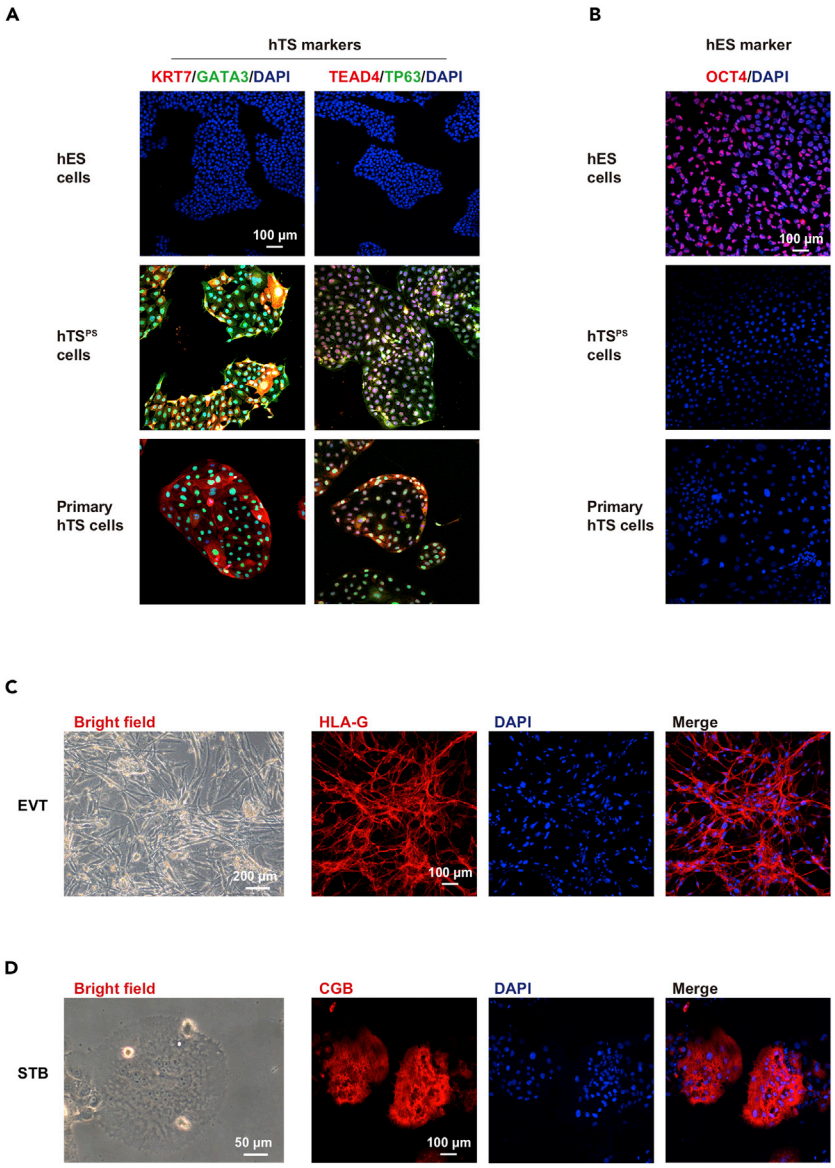

Figure 3. Characterization of hTS^{PS} cells derived from primed hPSCs

(A) Immunostaining of hTSC marker genes GATA3, KRT7, TP63, TEAD4 in hTS^{PS} cells, hES cells, and primary hTS cells. Scale bar, 100 μm.

(B) Immunostaining of hES marker gene OCT4 in hTS^{PS} cells, hES cells, and primary hTS cells. Scale bar, 100 μm.

(C) Left, morphology of EVT derived from hTS^{PS} cells. Scale bar, 200 μm. Right, immunostaining on EVT marker HLA-G for EVT derived from hTS^{PS} cells. Scale bar, 100 μm.

(D) Left, morphology of STB derived from hTS^{PS} cells. Scale bar, 50 μm. Right, immunostaining on STB marker CGB for STB derived from hTS^{PS} cells. Scale bar, 100 μm.

continuous culture process, these cells only need routine passaging to convert to hTSCs (Figures 2C and 2D). The human TSC-like cells are named human TS^{PS} cells. These hTSCs derived from hPSCs are named hTS^{PS} cells and could be maintained for more than 30 passages using each strategy.

These hTS^{PS} cells maintain typical hTSC morphology and highly express TSC markers, such as GATA3, KRT7, TEAD4, TP63 (Figures 2D and 3A). Meanwhile, ESC marker OCT4 is not detected in these hTS^{PS} cells (Figure 3B). Furthermore, these hTS^{PS} cells could differentiate towards downstream trophoblast subtypes, including extravillous cytotrophoblast cells (EVT) and syncytiotrophoblast cells (STB)

(Figures 3C and 3D). These EVT cells derived from hTS^{PS} cells show EVT morphology and express HLA-G. These STB cells derived from hTS^{PS} cells show syncytia and express CGB (Figures 3C and 3D).

LIMITATIONS

We provide two strategies for the derivation of hTSCs from primed hPSCs, including the standard strategy and BMP4 strategy. Firstly, the hTSC medium used by the standard strategy is the same as that previously reported (Okae et al., 2018). However, this strategy leads to an extremely low derivation efficiency of hTSCs from primed hPSCs and requires a colony-picking step (Troubleshooting). If using other cell lines such as other embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), optimization of cell density, passaging days and dissociation methods may be required.

In this report, we introduce a BMP4 strategy to derive hTSCs more efficiently. Furthermore, this strategy only requires routine passaging to gain the long term hTSCs. The results show the importance of BMP4 in hTSCs derivation. However, the mechanisms underlying how the BMP4 signaling pathway regulates the derivation of hTSCs from primed hPSCs requires further investigated.

TROUBLESHOOTING

Problem 1

On day 3 and 5, the cells dissociated with 0.5 mM EDTA are dead or floating, and cell-cell interactions cannot be dissociated (step 16 and 18).

Potential solution

The reasons why the cells are dead and the cells cannot be dissociated may be due to the 0.5 M EDTA or other solvents to changing the osmotic pressure. The preparation of 0.5 mM EDTA solution is dilution of 0.5 mL 0.5 M EDTA in 500 mL DPBS, not ddH_2O.

Moreover, check the morphology of the cells under the microscope for 5 min after incubation, increase the incubation time with 0.5 mM EDTA if necessary. If cell-cell interactions become loose, remove EDTA and add the fresh hTSC medium. If the cells are floating, transfer the cell suspension to 1.5 mL tube to centrifuge and then remove EDTA to passage the cells.

Problem 2

No hTSC-like colonies formation after 7–8 days by the standard strategy (step 20).

Potential solution

Passage the trans-differentiated cells using 0.5 mM EDTA at day 8. Alternatively, optimize the cell density or use the BMP4 strategy to generate hTSCs.

Problem 3

There are other non-hTSC cells growing in the well at the hTSC colony picking step (step 21-h).

Potential solution

These other non-hTSC cells can be manually scraped off using the glass pipets.

Problem 4

Only a few hTSC colonies appear before colony picking or survive after colony picking (step 21).

Potential solution

Firstly, scrap off the other non-hTSC cells around the boundary of the hTSC colonies in the original well and culture these hTSC colonies to grow further. If these colonies grow properly, pick and transfer them to a new matrigel-coated 24-well plate in TS medium.

Problem 5

The hTS^{PS} cells grow slowly or aren't similar to the typical hTSC morphology at the beginning by the the standard strategy and BMP4 strategy (step 21 and 33).

Potential solution

Extend as necessary the number of days in the original culture plate.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yongli Shan (shan_yongli@gibh.ac.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets/code.

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AUTHOR CONTRIBUTIONS

Y.W. and Y.S. initiated and designed the project. Y.W., L.X., and Y.S. performed most experiments and wrote the manuscript. L.M. performed immunostaining. Z.W., L.H., H.L., S.J.L., and G.P. gave suggestions about experiments and the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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