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Current Strategies of Modeling Human Trophoblast Using Human Pluripotent Stem Cells *in vitro*

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We previously established a trophoblast differentiation protocol from primed human pluripotent stem cells (PSC). To induce this lineage, we use a combination of Bone Morphogenetic Protein-4 (BMP4) and the WNT inhibitor IWP2. This protocol has enabled us to obtain a pure population of trophectoderm (TE)-like cells that could subsequently be terminally differentiated into syncytiotrophoblasts (STB) and extravillous trophoblasts (EVT). However, the resulting TE-like cells could only be terminally differentiated to a variable mixture of STB and EVT, with a bias toward the STB lineage. Recently, methods have been developed for derivation and culture of self-renewing human trophoblast stem cells (TSC) from human embryos and early gestation placental tissues. These primary TSCs were further able to differentiate into either STB or EVT with high efficiency using the lineage specific differentiation protocols. Based partly on these protocols, we have developed methods for establishing self-renewing TSC-like cells from PSC, and for efficient lineage-specific terminal differentiation. Here, we describe in detail the protocols to derive and maintain PSC-TSC, from both embryonic stem cells (ESC) and patient-derived induced pluripotent stem cells (iPSC), and their subsequent terminal differentiation to STB and EVT. © 2023 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Trophoblast Differentiation into TE-like Cells
Basic Protocol 2: Conversion of PSC-Derived TE-like Cells to TSC
Basic Protocol 3: Passaging PSC-Derived TSC in iCTB Complete Medium
Basic Protocol 4: STB Differentiation from PSC-derived TSC
Basic Protocol 5: EVT Differentiation from PSC-derived TSC
Support Protocol 1: Geltrex-coated tissue culture plate preparation
Support Protocol 2: Collagen IV-coated tissue culture plate preparation
Support Protocol 3: Fibronectin-coated tissue culture plate preparation

Keywords: extravillous trophoblast • human trophoblast stem cell • human pluripotent stem cell • syncytiotrophoblast • trophoblast differentiation

INTRODUCTION

A significant portion of our current knowledge of the cellular and molecular mechanisms of trophoblast differentiation comes from the use of mouse trophoblast stem cells (TSC) (Tanaka et al., 1998) and tumor-derived or immortalized human trophoblast cell lines (Cerneus & van der Ende, 1991; Kohler & Bridson, 1971), due to the difficulties in deriving *bona fide* human trophoblast stem cells (TSC). However, the in-depth comparison of mouse and human placental development has revealed significant differences, particularly in early gestation (Blakeley et al., 2015; Soncin et al., 2018). Therefore, it is necessary to develop a model to enable studies of normal human trophoblast differentiation. Over the past two decades, multiple groups have shown that human pluripotent stem cells (PSC)—both embryonic (ESC) and induced pluripotent stem cells (iPSC)—can be differentiated into trophoblast and used to model both normal development and disease phenotypes (Aghajanova et al., 2012; Alici-Garipcan et al., 2020; Amita et al., 2013; Castel et al., 2020; Das et al., 2007; Erb et al., 2011; Horii et al., 2016; Horii et al., 2019; Horii et al., 2021; Kobayashi et al., 2022; Krendl et al., 2017; Kurek et al., 2015; Li et al., 2013; Sheridan et al., 2019; Sudheer et al., 2012; Telugu et al., 2013; Warmflash et al., 2014; Wu et al., 2008; Xu et al., 2002; Yabe et al., 2016; Yang et al., 2015). The use of patient-derived iPSC provides the added opportunity of exploring specific mutation-associated imprinting disorders in addition to genetic or epigenetic abnormalities associated with specific pregnancy and/or neonatal adverse outcomes (Alici-Garipcan et al., 2020; Horii et al., 2016; Horii et al., 2021; Sheridan et al., 2019). This is particularly important for studying placenta-based pregnancy disorders because they are known to be epigenetically affected by maternal lifestyle and the uterine environment (Apicella et al., 2019; Maccani & Maccani, 2015; Workalemahu et al., 2020). However, such previously established protocols result in a mixed population of terminally-differentiated syncytiotrophoblasts (STB) and extravillous trophoblasts (EVT), and are generally biased towards the STB lineage (Horii et al., 2019). The variability of a mixed culture is not ideal for determining the individual contribution of EVT and STB to a cellular or molecular phenotype, as characterization will be based on the balance of mixed culture in a dish. Further, the minor EVT population limits the types of assays we can use to further examine this cell type.

In 2018, Okae et al. established a method to derive and culture *bona fide* TSCs from human pre-implantation-stage embryos and early first trimester placenta (Okae et al., 2018). The *in vitro* maintenance and subsequent differentiation of TSC in a lineage-specific manner into EVT and STB has allowed the field to make tremendous progress towards understanding the molecular mechanisms of human trophoblast differentiation. However, the use of pre-implantation embryos and early trimester placentas holds multiple challenges due to both unknown pregnancy outcomes, and ethical, legal, and funding barriers associated with these tissues. Applying the Okae culture conditions to derive TSC from PSCs avoids the latter challenges. Importantly, iPSC-based models have the added benefit of known patient information, including pregnancy outcome and placental pathology.

Recently, new protocols for TSC derivation have been reported from both naïve (pre-implantation) and primed (post-implantation) pluripotent states (Cinkornpumin et al.,
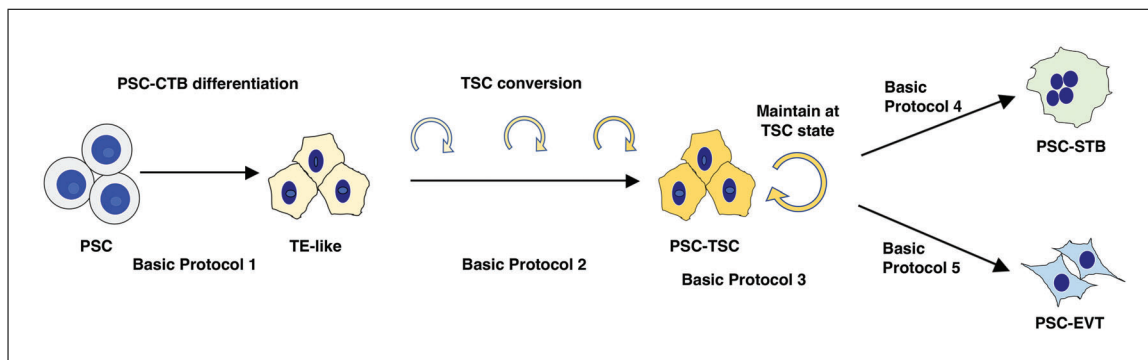
Figure 1 An illustration of the trophoblast differentiation from PSC leading to formation of trophectoderm (TE)-like cells, further adaptation to TSC medium for complete conversion to PSC-TSC, and terminal differentiation to STB and EVT with each step showing corresponding protocol numbers.

2020; Dong et al., 2020; Io et al., 2021; Jang et al., 2022; Li et al., 2019; Mischler et al., 2021; Soncin et al., 2022; Wei et al., 2021). TSC derived from both naïve and primed PSC have trophoblast characteristics, such as the capacity for terminal differentiation into multinucleated human chorionic gonadotropin (hCG)-secreting STB and invasive human leukocyte antigen-G$^+$ (HLA-G$^+$) EVT, with appropriate gene expression profiles, as well as E74 like ETS transcription factor 5 (ELF5) promoter hypomethylation and lack of expression of classical human leukocyte antigen (HLA) molecules (Io et al., 2021; Soncin et al., 2022). Naïve PSC-derived TSC also share one additional feature with early gestation trophoblast, which is high expression of chromosome 19 miRNA cluster (C19MC) genes (Io et al., 2021; Kobayashi et al., 2022). However, converting PSC to the naïve state is accompanied by a global loss of DNA methylation, which includes loss of imprinting throughout the genome (Theunissen et al., 2016). Differentiation of naïve PSC into TSC appears to lead to the re-emergence of at least some of the placenta-associated DNA methylation pattern (Cinkornpumin et al., 2020); however, the capacity of these cells for modeling trophoblast-associated defects underlying pregnancy disorders, particularly those associated with an epigenetic basis, remains unknown.

Here, we provide a detailed protocol for derivation of TSC from primed PSC, which is improved from the original version (Horii et al., 2019), and suited for modeling lineage-specific trophoblast differentiation (Soncin et al., 2022). This manuscript describes protocols for the induction of trophectoderm (TE)-like cells using a simple defined medium (Basic Protocol 1); conversion of these cells to TSC-like cells using "iCTB" medium, developed in the Kessler laboratory, for reprogramming of term CTB into TSC-like cells (Bai et al., 2021) (Basic Protocol 2); maintenance of PSC-derived TSC (PSC-TSC) (Basic Protocol 3); terminal differentiation of these PSC-TSC into STB (Basic Protocol 4); terminal differentiation of these PSC-TSC into EVT (Basic Protocol 5); Geltrex coated plate preparation (Support Protocol 1); Collagen IV coated plate preparation (Support Protocol 2); and Fibronectin coated plate preparation (Support Protocol 3) in a step-by-step method (Fig. 1).

CAUTION: All cell culture must be done in a suitable class II laminar flow hood to avoid contamination. All of the protocols described here require the proper use of aseptic technique.

CAUTION: Follow all appropriate guidelines and regulations for the use of human pluripotent stem cells at each individual institution/country (i.e., approval from an Embryonic Stem Cell Research Oversight (ESCRO) committee).

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of

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laboratory animals. Appropriate informed consent is necessary for obtaining and use of human study material.

TROPHOBLAST DIFFERENTIATION INTO TE-LIKE CELLS

This protocol describes differentiation of PSCs into TE-like cells. Once the PSC are ready, it requires 5 days to make TE-like cells. This section is partially abstracted from Horii et al. (2019), which originally referred to the resulting cells as "CTB-like," and expanded upon to provide a clear description of this protocol (see Background Information section below for details on the new designation of these as "TE-like" cells). Successful completion of this protocol will result in TE-like cells with a "cobblestone" morphology and over 80% EGFR positivity by flow cytometry.

Materials

PSC line(s)
> *NOTE:* We have used both hESC (including WA01/H1, WA09/H9) and several of our own iPSC lines (derived from human dermal fibroblast or human umbilical cord mesenchymal stem cells) in this protocol. Prior to differentiation, cells should be adapted to StemFlex media (ThermoFisher) or mTeSR Plus (STEMCELL Technologies), following the manufacturer's protocol.

StemFlex medium (ThermoFisher, cat. no. A3349401)
EDTA, 0.5 mM (see recipe)
TrypLE express (ThermoFisher, cat. no. 12604021)
Y27632 (see recipe)
PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)
Knockout serum replacement (KSR) (ThermoFisher, cat. no. 10828-028)
Trophoblast induction medium (see recipe and Table 8)

Geltrex coated 6-well tissue culture plates (see Support Protocol)
5-ml Serological pipettes
10-ml Serological pipettes
25-ml Serological pipettes
15-ml Conical tubes
50-ml Conical tubes
Hemocytometer or automated cell counter
Centrifuge (ThermoFisher, cat. no. 75004261 or equivalent)
Cell culture incubator
Flow cytometer and additional materials for cell staining and analysis as described in Horii et al, 2019

1. Use newly prepared Geltrex coated tissue culture plates (see Support Protocol 1). Culture PSCs in StemFlex until sub-confluent (∼80%). Cells are recommended to be passaged by clump passaging using a gentle dissociation buffer such as 0.5 mM EDTA every 2 to 3 days to expand the cells. Passaging details can also be found in Horii et al., 2019.

   NOTE: It is essential to have good starting material. Ideal passage number for the use of ESC and iPSC are passage numbers below 50 for ESC, and passage number below 20 for iPSC. If ESC or iPSC are not growing well, consider thawing a new vial, or changing to new batches of StemFlex or Geltrex. For PSC medium, other regularly used medium (i.e., StemPro, E8, or mTeSR Plus) will also work for this protocol. We have found passaging at split ratios between 1:6 and 1:9 for H9 ESC, and 1:3 for our iPSC works well; however, splitting ratio varies between cell lines, so we recommend optimizing the split ratio beforehand.
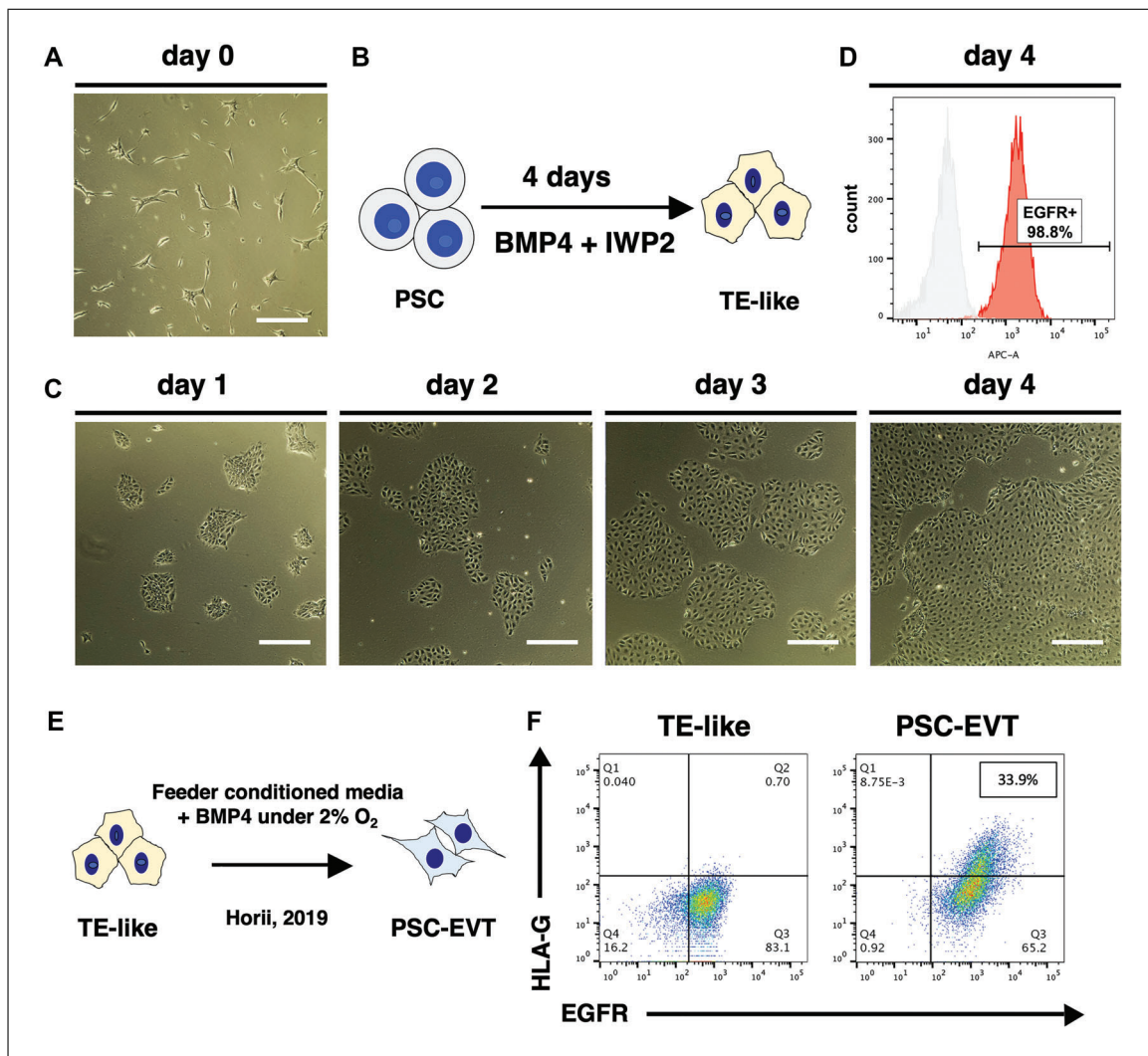
Figure 2 (**A**) Representative bright phase image (10×) of PSC one day after plating for trophoblast induction (day 0). The cell density shown is appropriate to begin trophoblast induction. Scale bar: 100 μm. (**B**) An illustration of the induction into trophectoderm (TE)-like cells. (**C**) Representative bright phase images (10×) of daily morphologic changes during trophoblast induction. Scale bar: 100 μm. (**D**) Representative flow cytometric analysis of EGFR expression (with isotype control) at day 4 of TE induction. At day 4, successful induction to TE-like cells will be marked by over 90% EGFR$^+$ cells. Gray peak indicates AIisotype control. (**E**) An illustration of the EVT differentiation from Horii et al. (2019). Following the 4-day induction with BMP4+IWP2, cells were re-plated in feeder-conditioned media with 10 ng/ml BMP4. (**F**) Representative flow cytometric analysis of EGFR/HLA-G double staining before and after differentiation using the protocol from Horii et al. (2019). EVT differentiation resulted in (at most) 30% HLA-G positive cells from this protocol.

2. On day -1, seed cells into a new Geltrex-coated 6-well plate (see Support Protocol 1). Pre-warm new Geltrex-coated plate (either a freshly-made or stored plate) at 37°C for at least 30 min. Aspirate the medium from sub-confluent well of PSCs and add 1 ml of room temperature TrypLE express for 3 min at 37°C to dissociate to single cells. Harvest cells, placing them in a conical tube. Wash wells with 2 to 5 ml of warm StemFlex media to collect as many cells as possible. Centrifuge 5 min at 200 × *g*, room temperature to pellet the PSC. Re-suspend and count the PSC.

3. Remove Geltrex from the well and seed 60,000 cells per well in StemFlex with 5 μM Y27632. Shake the plate gently by hand to spread the cells, allowing them to attach evenly on the plate (Fig. 2A).

   NOTE: 60,000 cells per well of a 6-well plate is optimal for WA09/H9 ESC. However, seeding density will need to be optimized for each ESC/iPSC line. For our iPSC, we

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plate 100,000 cells per well of a 6-well plate. At these seeding densities, cells could grow up to roughly 1 million per well of a 6-well plate by day 4 of TE induction. It is very important to plate the cells sparsely at the beginning of the TE induction. If the PSC colonies are too big at the start, the center of the colony will not differentiate well. This is one reason to dissociate the cells using TrypLE express instead of EDTA. Daily cell culture work for TE induction should be done during a similar time in the day to ensure collection of samples and media changes are done every 24 hr. For subsequent conversion to TSC (Basic Protocol 2), you will need around 500,000 cells/well of a 6-well plate.

4. On day 0, check that the cells have attached evenly and formed small and sparse colonies (Fig. 2A). If the PSC morphology and density look good, change medium to Trophoblast Induction medium (Fig. 2B). Change the medium every day for 4 days. Cells will gradually acquire an epithelial (cobblestone-like) morphology (Fig. 2C).

5. On day 4, check cell surface EGFR expression using the flow cytometer. Using one well from the differentiation, remove the media and wash the cells with 1 ml of PBS. Aspirate PBS and add 1 ml of TrypLE express. Incubate at 37°C for 3 min. Add 1 ml of 10% Knockout Serum Replacement (KSR) in PBS onto the wells containing the TrypLE express, then wash the well and collect the cells in the TrypLE Express/10% KSR in PBS mixture in a 15 ml conical tube. Centrifuge 5 min at $200 \times g$, room temperature. Stain cells for EGFR expression following previously published protocol described in Horii et. al., 2019.

   NOTE: Without the KSR, the cells will not form a dense pellet and there is a higher risk to lose the cells during aspiration of the supernatant.

6. If flow cytometric analysis shows over 80% of cells are EGFR$^+$, proceed to the re-plating step in Basic Protocol 2. If the proportion of EGFR$^+$ cells is less than 80%, change the medium on the remaining cells with Trophoblast Induction Medium and let the induction proceed for another day prior to measuring surface EGFR expression again by flow cytometry.

7. At the end of this step, successful induction should result in over 80% of the cells positive for surface EGFR expression by flow cytometry (Fig. 2D). Terminal differentiation of the TE-like cells generated by this protocol, previously referred to as CTB-like cells, using feeder-conditioned medium plus 10 ng/ml BMP4, as previously described by Horii et al., 2019, resulted in differentiation into mostly STB, with a small proportion (around 30%) of HLA-G$^+$ EVT (Fig. 2E, F). EVT differentiation is significantly improved by adaptation to PSC-TSC (Basic Protocol 2) and subsequent use of Basic Protocol 5.

CONVERSION OF PSC-DERIVED TE-LIKE CELLS TO TSC

This protocol describes conversion of PSC-derived TE-like cells to TSC (PSC-TSC). Once the PSC-derived TE-like cells are confirmed to be over 80% EGFR positive by flow cytometry, proceed to Basic Protocol 2 to re-plate the TE-like cells onto Collagen IV-coated plate. Collagen IV-coated plates could be prepared on day 3 of the Basic Protocol 1. This step is essential to derive self-renewing PSC-TSC, and for subsequent successful terminal trophoblast differentiation.

Materials

TrypLE express (ThermoFisher, cat. no. 12604021)
PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

iCTB complete medium (see recipe and Table 5)
Collagen IV-coated plate (see Support Protocol 2)

    5-ml Serological pipettes
    10-ml Serological pipettes
    25-ml Serological pipettes
    15-ml Conical tubes
    50-ml Conical tubes
    Hemocytometer or automated cell counter
    Centrifuge (ThermoFisher, cat. no. 75004261 or equivalent)
    Cell culture incubator

1. Before you begin, prepare the collagen IV-coated plates (see Support Protocol 2).

2. Aspirate media from the well containing PSC-derived TE from Basic Protocol 1 and add 1 ml TrypLE express to dissociate the cells to single cells. Incubate the plate at 37°C for 3 min.

3. Without removing TrypLE express, add 2 to 3 ml PBS to the well using a 5 ml pipette and collect the cells into a conical tube.

4. Centrifuge 5 min at 200 × g, room temperature. Aspirate the supernatant and re-suspend in iCTB complete medium to make a cell suspension of 250,000 cells per ml.

   NOTE: If the cells do not form a dense pellet, add KSR for a final concentration of 10% to the cell suspension and centrifuge again.

5. Remove collagen IV from the well and wash once with PBS. Seed 500,000 cells per well in a 6-well plate, for a total volume of 2 ml per well. Agitate the plate to have the cells spread and attach evenly on the plate.

   NOTE: Up to 1 million TE-like cells are generated in one well of a 6-well plate at the end of Basic Protocol 1. If there are 4 wells of a 6-well plate at the end of Basic Protocol 1, we recommend coating 4 to 6 wells of a 6-well plate with collagen IV.

6. Change iCTB medium every other day until the cells are confluent.

PASSAGING PSC-DERIVED TSC IN ICTB COMPLETE MEDIUM

BASIC PROTOCOL 3

This protocol describes how to passage PSC-derived TSC. This step is essential to establish self-renewing PSC-TSC, and terminal trophoblast differentiation. Once the PSC-derived TE-like cells are replated onto a collagen IV-coated plate, EGFR expression level by flow cytometry will diminish during the first couple of passages. If completed successfully, EGFR expression will be regained and be expressed by over 90% of the cells by passage 5. This protocol typically takes up to 2 to 3 weeks to complete and will establish self-renewing PSC-TSC.

Materials

    PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)
    TrypLE express (ThermoFisher, cat. no. 12604021)
    iCTB complete medium (see recipe and Table 5)

    Collagen IV-coated plate (see Support Protocol 2)
    5-ml Serological pipettes
    10-ml Serological pipettes
    25-ml Serological pipettes
    15-ml Conical tubes
    50-ml Conical tubes
    Hemocytometer or automated cell counter
    Centrifuge (ThermoFisher, cat. no. 75004261 or equivalent)
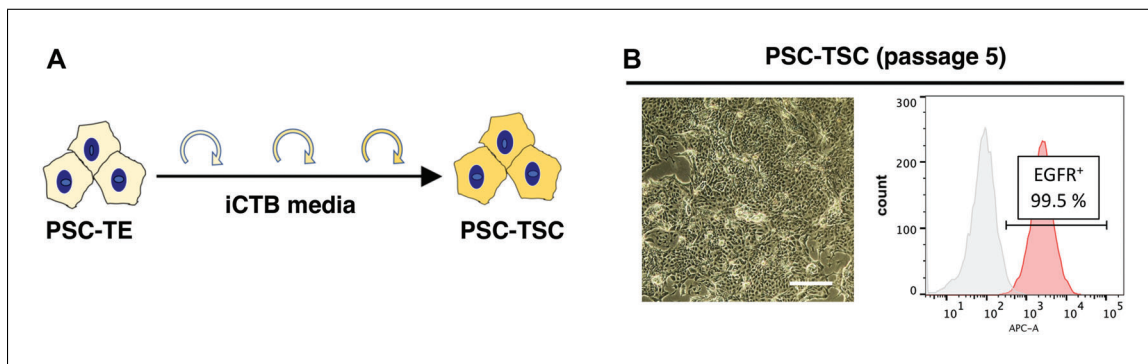   Cell culture incubator

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Figure 3 (**A**) An illustration of TSC conversion from trophectoderm (TE)-like cells in iCTB medium. Cells undergo 4 to 5 passages in this medium before they are fully converted into PSC-derived TSC (PSC-TSC). (**B**) Left panel shows a representative bright phase image (10×) of established PSC-TSC. Scale bar: 100 μm. Right panel shows representative flow cytometric analysis after 5 passages in iCTB medium, confirming that cells are over 90% EGFR$^+$. Gray peak indicates APC-isotype control.

1. Coat the appropriate number of wells with collagen IV before passaging (see Support Protocol 2). We recommend preparing enough wells to passage at a 1:3 split ratio. Therefore, for 1 well of PSC derived TSC, prepare 3 wells with collagen IV).

2. When the cells are 80% confluent, passage the cells at a 1:3 split ratio.

   NOTE: Split ratio varies between the cell lines, so optimizing the split ratio beforehand is recommended. We typically use a 1:2 to 1:4 split ratio among different ESC- and iPSC-derived TSC lines.

3. Aspirate iCTB medium and add 1 ml of TrypLE express per well of a 6-well plate. Incubate at 37°C for 10 to 15 min.

4. Collect cells in 15 ml conical tube using PBS or iCTB basal medium.

5. Centrifuge 5 min at 200 × *g*, room temperature.

6. Aspirate the supernatant and then re-suspend cells in appropriate volume of iCTB complete medium for a split ratio of 1:3.

7. Aspirate collagen IV from the wells that are intended for plating and wash wells once with PBS.

8. Plate cells with appropriate split ratio in 2 ml medium for each well. Agitate the plate to allow the cells to spread out and attach evenly.

9. Passage cells every 2 to 3 days, or when 80% confluent.

10. Following 4 to 5 passages in this media (Fig. 3A), the cells should be over 90% EGFR$^+$ when examined by flow cytometry (Fig. 3B). At this point, cells can be frozen down. After thawing, PSC-TSC can be passaged and differentiated in a lineage-specific manner into either STB or EVT.

    NOTE: It takes about 1 week to prepare the cells for the initial TE induction (Basic Protocol 1) and an additional 2 to 3 weeks to complete the TSC derivation (Basic Protocols 2 and 3). During the adaptation phase into iCTB media, a drop in EGFR cell surface expression has been observed and is expected (as low as 10% EGFR$^+$ cells). Over a few passages, cells will re-acquire EGFR expression as their morphology becomes more similar to primary TSC (Soncin et al., 2022). Conversion time to PSC-TSC varies between cell lines and is usually completed by passage 8 for most cell lines. After establishing PSC-derived TSC, cells can be passaged multiple times (we have successfully maintained them for up to 20 passages). Similar to primary

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TSC, it is important to passage PSC-derived TSC at sub-confluency (<80% confluency), rather than over-confluent, to avoid differentiation and acquisition of cell surface HLA-G. However, once a high proportion of cells acquire over 20% HLA-G, it is difficult to recover cells to the TSC stage (EGFR$^+$, HLA-G$^-$). In this case, thawing earlier passage cells or re-derivation of PSC-TSC is recommended.

STB DIFFERENTIATION FROM PSC-DERIVED TSC

This protocol describes STB differentiation from PSC-derived TSC. Correct confluency (∼30%) of PSC-TSC on day 0 is essential for successful STB differentiation. If the cells are under- or over-confluent, they will detach from the plate before differentiation is complete. It will take around 8 days to complete the protocol (days -1 to day 6). If successful, resulting cells at day 6 of the protocol will exhibit STB-associated function, such as hCG secretion. Cellular assays such as ELISA for hCG and cell-cell fusion assays can be conducted on day 6. An example of positive results is shown in Figure 4C and D. The usage of these assays is described in detail in previous publications (Horii et al., 2016; Horii et al., 2021; Soncin et al., 2022).

Materials

TrypLE express (ThermoFisher, cat. no. 12604021)
PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)
iCTB complete medium (see recipe and Table 5)
STB differentiation medium (see recipe and Table 6)
Collagen IV-coated plate (see Support Protocol 2)
5-ml Serological pipettes
10-ml Serological pipettes
25-ml Serological pipettes
15-ml Conical tubes
50-ml Conical tubes
Hemocytometer or automated cell counter
Centrifuge (ThermoFisher, cat. no. 75004261 or equivalent)
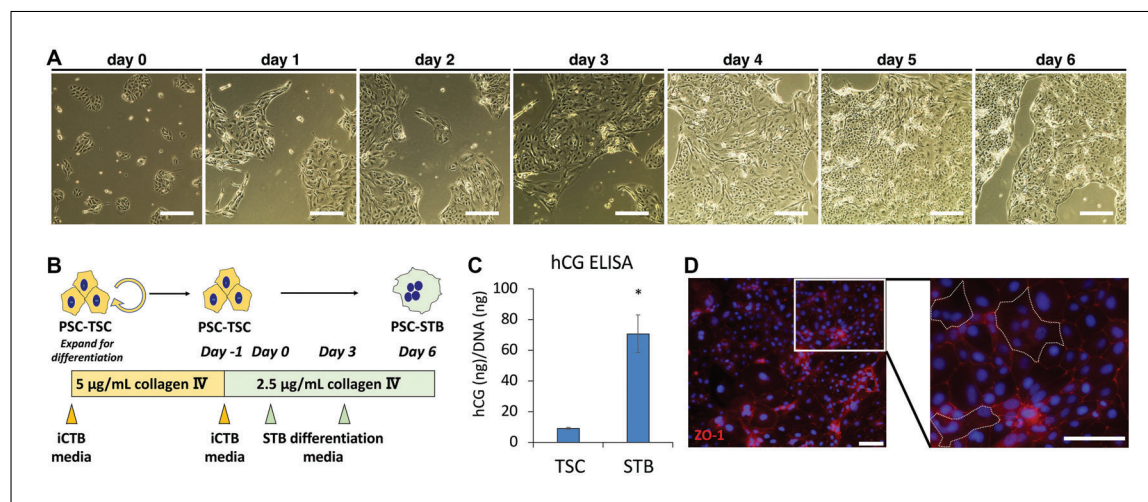Cell culture incubator

Figure 4 (**A**) Representative bright phase images (20×) of daily changes during STB differentiation. The confluence should be ∼30% on day 0 to initiate STB differentiation. Scale bar: 100 μm. (**B**) An illustration of the STB differentiation protocol starting with PSC-TSC. Changes in matrices and media are noted and highlighted with different colors. Arrowheads correspond to time-points requiring media change. (**C**) Representative hCG ELISA data measured on day 6 STB, compared to the starting TSC state (on day 0). $^*p < .05$, student's t-test. (**D**) Representative immunofluorescence image of day 6 STB with cell membrane marker ZO-1 (red). Perforated white line outlines areas with cell fusion. Appropriate cell plating density is necessary for measuring cell-cell fusion. Scale bar 100 μm.

1. Prepare plates coated with 2.5 µg/ml collagen IV in PBS (see Support Protocol 2).

2. Day -1: prior to differentiation, check the cells by flow cytometry to ensure the PSC-TSC population is over 80% EGFR$^+$ and less than 20% HLA-G$^+$.

3. Day -1: for STB differentiation, plate PSC-TSC at a density of 100,000 to 150,000 cells per well of a 6-well plate. Using PSC-TSC from Basic Protocol 3, roughly 300,000 cells are expected from one well of a 6-well plate.

4. Aspirate iCTB medium from the well that is going to be passaged, then add 1 ml of TrypLE express. Incubate at 37°C for 10 to 15 min.

5. Collect cells in 15 ml conical tube using PBS or iCTB basal medium. Count the cells.

6. Centrifuge 5 min at 200 × g, room temperature.

7. Aspirate the supernatant, then re-suspend cells in appropriate volume of iCTB complete medium.

8. Aspirate collagen IV solution from wells and wash with 1 ml of PBS.

9. Remove PBS from the well and plate 100,000 to 150,000 cells in 2 ml of iCTB complete medium into one well of a 6-well plate. Incubate cells for 1 to 2 days, until the cells are 30% confluent (see Fig. 4A).

10. Day 0: when the cells are 30% confluent (Fig. 4A), change medium to STB differentiation medium with 2 ml per well of a 6-well plate (Fig. 4B).

    NOTE: Due to differences in growth rate, it may be required to optimize the seeding density of PSC-TSC to achieve 30% confluence at day 0. Alternatively, if the cells are less than 30%, wait until the PSC-TSC reach 30% of confluence before switching to STB differentiation media. This will avoid significant cell death throughout the differentiation and will perform better cell-cell fusion.

11. On day 3, change medium using freshly prepared STB differentiation medium (Fig. 4B).

12. On day 6, collect cells for analysis of STB function (Fig. 4B-D).

EVT DIFFERENTIATION FROM PSC-DERIVED TSC

This protocol describes EVT differentiation from PSC-TSC, which takes 8 days to complete (days -1 to day 6). It is important that the PSC-TSC are undifferentiated (over 80% EGFR$^+$ and less than 20% HLA-G$^+$) at the start of the protocol. If completed successfully, cells should be over 60% positive for EVT marker, HLA-G, at the end of day 6. This assay is also described in our previous publications (Horii et al., 2016; Horii et al., 2021; Soncin et al., 2022).

Materials

PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)
TrypLE express (ThermoFisher, cat. no. 12604021)
iCTB complete medium (see recipe and Table 5)
EVT differentiation medium-I (see recipe and Table 2)
EVT differentiation medium-II (see recipe and Table 3)

Fibronectin-coated plate (see Support Protocol 3)
5-ml Serological pipettes
10-ml Serological pipettes
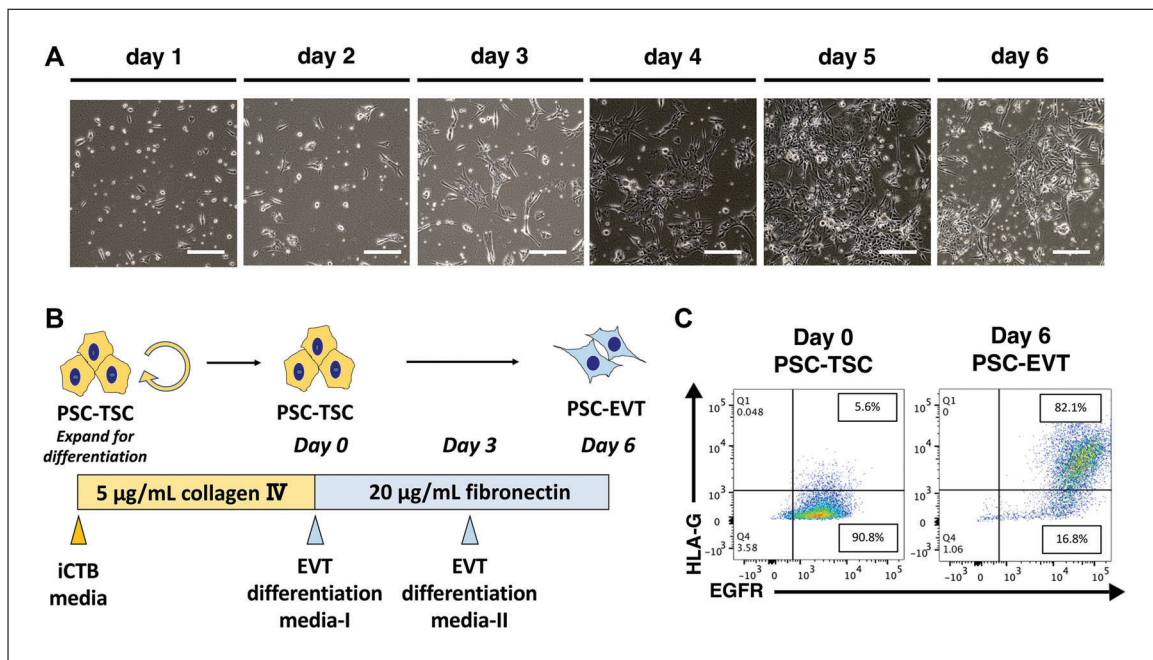25-ml Serological pipettes

Figure 5 (**A**) Representative bright phase images (20×) of daily morphologic changes during EVT differentiation. Scale bar: 200 μm. (**B**) An illustration of the modified EVT differentiation protocol starting with PSC-TSC. Changes in matrices and media are noted and highlighted with different colors. Arrowheads correspond to time-points requiring media change. (**C**) Representative flow cytometric data prior (day 0) and after (day 6) EVT differentiation modified from Okae et al. (2018). Over 90% of PSC-TSC express EGFR and only a very small percentage (~5% in this example) express HLA-G. After EVT differentiation, over 80% of PSC-EVT are positive for surface expression of HLA-G. Differentiation of EVT from PSC-TSC has a significantly better yield of HLA-G⁺ EVT than our previous two-step trophoblast differentiation protocol (compare to Fig. 2F).

15-ml Conical tubes
50-ml Conical tubes
Hemocytometer or automated cell counter
Centrifuge (ThermoFisher, cat. no. 75004261 or equivalent)
Cell culture incubator

1. Prepare plates coated with 20 μg/ml fibronectin in PBS (see Support Protocol 3).

2. Day -1 or 0: prior to differentiation, check the cells by flow cytometry to ensure the PSC-TSC population is over 80% EGFR⁺ and less than 20% HLA-G⁺.

3. Day 0: for EVT differentiation, plate cells at a density of 200,000 to 250,000 cells per well of 6-well plate. We expect to obtain about 300,000 PSC-TSC per confluent well of a 6-well plate at the end of Basic Protocol 3.

4. Thaw Matrigel on ice. Once Matrigel is thawed, prepare EVT differentiation medium-I (see recipe) and allow medium to reach room temperature before use.

5. Aspirate iCTB medium, then add 1 ml of TrypLE express. Incubate at 37°C for 10 to 15 min.

6. Collect cells in 15 ml conical tube using PBS or iCTB basal medium. Count the cells.

7. Centrifuge 5 min at 200 × g, room temperature.

8. Aspirate supernatant, then re-suspend cells in EVT differentiation medium-I.

9. Aspirate fibronectin solution from wells and wash with 1 ml of PBS per well.

10. Aspirate PBS and plate 200,000 to 250,000 cells in 2 ml of EVT differentiation medium-I for each well of a 6-well plate.

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11. On day 3, prepare EVT differentiation medium-II, and change medium (Fig. 5A, B).

12. On day 6, collect cells for further analysis (Fig. 5C).

GELTREX-COATED TISSUE CULTURE PLATE PREPARATION

This protocol describes how to prepare Geltrex coated plates in a step-by-step manner for expansion of PSC and differentiation into TE like cells.

Materials

Geltrex (Thermo Fisher, cat. no. A14133-02)
DMEM/F-12, HEPES (Thermo Fisher, cat. no. 11330-032)

Tissue culture treated 6-well plates (Corning, cat. no. 3506)
5-ml Serological pipettes
10-ml Serological pipettes
25-ml Serological pipettes
15-ml Conical tubes
50-ml Conical tubes
Parafilm
Cell culture incubator

1. Thaw Geltrex on ice. Wait for 30 to 45 min until completely thawed (no ice remains). Prepare 20 ml of ice-cold DMEM/F-12, HEPES in a 50-ml conical tube and add 100 µl Geltrex to make a 1:200 dilution as recommended by the manufacturer. Mix gently.

2. Add medium/Geltrex mixture to the plate (2 ml per well for 6-well plate, and 1 ml per well for 12-well plate). Incubate the plate at 37°C for 30 min for immediate use. For preparation for later use, incubate for 2 hr in either room temperature or 37°C incubator. After the incubation, cover with Parafilm and store the plate at 4°C for up to 4 weeks. Do not use if the well is dry. When using the plate, simply aspirate the medium/Geltrex mixture and add stem cell culture media (i.e., StemFlex) containing cells.

COLLAGEN IV-COATED TISSUE CULTURE PLATE PREPARATION

This protocol describes how to prepare Collagen IV-coated plates in a step-by-step manner for derivation and passaging of PSC-derived TSC as well as PSC-TSC differentiation into STB.

Materials

Collagen IV (MilliporeSigma, cat. no. C0543-1VL)
PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

Tissue culture treated 6-well plates (Corning, cat. no. 3506)
5-ml Serological pipettes
10-ml Serological pipettes
25-ml Serological pipettes
15-ml Conical tubes
50-ml Conical tubes
Cell culture incubator

1. If collagen IV is frozen, thaw aliquot on ice prior to use. Once thawed, collagen IV can be stored in 4°C for up to 3 months, or according to the manufacturer's recommendation.

In a sterile environment, add 5 μl of 1 mg/ml collagen IV into 1 ml of PBS to make a 5 μg/ml solution for TSC derivation and maintenance (Basic Protocols 2 and 3). Mix gently.

2. Add diluted collagen IV mixture (5 μg/ml) to the plate (1 ml per well of a 6-well plate, 500 μl per well of a 12-well plate). Incubate the plate at 37°C for at least 2 to 3 hr at 37°C (optimal attachment occurs with overnight incubation at 37°C). The plate could be kept at 37°C up to a week. Do not use if the well is dry.

NOTE: Instead of 5 μl of 1 mg/ml collagen IV into 1 ml of PBS, add 2.5 μl of 1 mg/ml collagen IV into 1 ml of PBS to make a 2.5 μg/ml solution for STB differentiation (Basic Protocol 4).

FIBRONECTIN-COATED TISSUE CULTURE PLATE PREPARATION

This protocol describes how to prepare fibronectin coated plates in a step-by-step manner for EVT differentiation of PSC-TSC.

Materials

Fibronectin (MilliporeSigma, cat. no. F1141)
PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

Tissue culture created 6-well plates (Corning, cat. no. 3506)
5-ml Serological pipettes
10-ml Serological pipettes
25-ml Serological pipettes
15-ml Conical tubes
50-ml Conical tubes
Cell culture incubator

1. In a sterile environment, add 20 μl of fibronectin into 1 ml of PBS to make a 20 μg/ml solution. Mix gently.

2. Add fibronectin mixture to the plate (1 ml per well for 6-well plate, 500 μl per well for 12-well plate). Incubate the plate at 37°C for 1 hr for immediate use. For preparation for use the next day, incubate the plate overnight at 37°C. Do not use if the well is dry. Rinse well with 1 ml of PBS prior use.

REAGENTS AND SOLUTIONS

Prepare all reagents and solutions in a sterile environment suitable for cell culture. Sterile filter as indicated.

A 83-01, 5 mM

A 83-01, 10 mg (Tocris, cat. no. TB2939-RMU

DMSO (MilliporeSigma, cat. no. D2650)

Add 4.74 ml DMSO to 10 mg A 83-01 to make 5 mM (w/v) stock. Make 80 μl aliquots. Store at −80°C for up to a month.

Basal trophoblast induction medium

DMEM/F-12 50/50 (Corning, cat. no. 10-092-CV)

ITS (see recipe)

L-ascorbic acid (MilliporeSigma, cat. no. A8960)

Sodium bicarbonate (NaHCO$_3$), 7.5% solution (ThermoFisher, cat. no. 25080094)

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Table 1 Basal Trophoblast Induction Medium

Component	Stock concentration	Final concentration	Volume for 50 ml
DMEM/F-12 50/50	–	–	45.7 ml
ITS	100×	1×	500 µl
L-ascorbic acid	33.3 mg/ml	64 µg/ml	96 µl
NaHCO$_3$	75 mg/ml (7.5%)	543 µg/ml	362 µl
BSA, 30% (w/v) solution	30%	2%	3.3 ml

BSA, 30% (w/v) solution (Gemini Bio-Products, cat. no. 700-110)

Prepare medium, referring to Table 1 for volumes and final concentrations. Sterile filter and sore at 4°C. Use within a week.

Bone morphogenetic protein 4 (BMP4), 10 µg/ml

BSA, 30% (Gemini Bio-Products, cat. no. 700-110)

PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

BMP4, 50 ug (R&D Systems, cat. no. 314-BP)

Prepare 0.1% (w/v) BSA in PBS. To make a 10 µg/ml BMP4 solution, combine 5 ml of 0.1% (w/v) BSA in PBS solution to 50 µg of BMP4. Gently pipette up and down to reconstitute the protein. Thoroughly mix and prepare 100 µl aliquots. Store at −80°C for up to a year. Once thawed, keep in 4°C. Avoid freeze-thaw cycles.

CHIR99021, 3 mM

CHIR99021 (MilliporeSigma, cat. no. SML1046)

DMSO (MilliporeSigma, cat. no. D2650)

Add 3.58 ml DMSO to 5 mg CHIR99021 to prepare a 3 mM stock. Make 533 µl aliquots. Store at −80°C for up to a year.

Collagen IV, 1 mg/ml

HCl, 1 M (MilliporeSigma cat. no. H9892)

PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

Collagen IV (MilliporeSigma, cat. no. C0543)

Dilute 1 M HCl with PBS to 0.05 M. Add 750 µl of 0.05 M HCl to 750 µg of collagen IV. Make 100 µl aliquots of 1 mg/ml collagen IV. Store at −80°C for up to a year. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

EDTA, 0.5 mM

EDTA, 0.5 M (ThermoFisher, cat. no. 15575020)

PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

Add 50 µl of 0.5 M EDTA to 50 ml PBS. Sterile filter and store at room temperature.

EVT differentiation medium-I

Terminal differentiation medium (see recipe)

Y-27632, 10 mM (Selleck Chemicals, cat. no. S1049)

Knockout serum replacement (KSR) (ThermoFisher, cat. no. 10828028)

Table 2 EVT Differentiation Medium-I

Component	Stock concentration	Final concentration	Volume for 12 ml
Terminal differentiation medium	–	–	11.2 ml
Y-27632	10 mM	2.5 μM	3 μl
KSR	100%	4%	480 μl
A 83-01	5 mM	7.5 μM	18 μl
NRG1	100 μg/ml	100 ng/ml	12 μl
Matrigel	100%	2%	240 μl

A 83-01, 5 mM (Tocris, cat. no. 2939)

NRG1 100 μg/ml (Abcam, cat. no. ab50227)

Matrigel (Corning, cat. no. 356231)

Prepare EVT differentiation medium-I fresh prior to use. Matrigel will solidify and clump if added to warm media, so add ice-cold liquid Matrigel to ice-cold medium. The complete medium should be warmed up before feeding the cells. See Table 2 for volumes and concentrations.

EVT differentiation medium-II

Terminal differentiation medium (see recipe)

Y-27632, 10 mM (Selleck Chemicals, cat. no. S1049)

Knockout serum replacement (KSR) (ThermoFisher, cat. no. 10828028)

A 83-01, 5 mM (Tocris, cat. no. 2939)

Matrigel (Corning, cat. no. 356231)

Prepare EVT differentiation medium-II fresh prior to use. Matrigel will solidify and clump if added to warm media, so add ice-cold liquid Matrigel to ice-cold medium. The complete medium should be warmed up before feeding the cells. See Table 3 for volumes and concentrations.

Forskolin, 10 mM

Forskolin, 10 mM (Selleck Chemicals, cat. no. S2449)

Aliquot the 10 mM forskolin in DMSO into 50 μl aliquots. If forskolin is in powder form, add appropriate amount of DMSO to dissolve the powder to a final concentration of 10 mM. Make 50 μl aliquots. Keep aliquots in -80°C for 2 years in liquid form. Keep powder stocks in -20°C for up to 3 years.

Table 3 EVT Differentiation Medium-II

Component	Stock concentration	Final concentration	Volume for 12 ml
Terminal differentiation medium	–	–	11.4 ml
Y-27632	10 mM	2.5 μM	3 μl
KSR	100%	4%	480 μl
A 83-01	5 mM	7.5 μM	18 μl
Matrigel	100%	0.5%	60 μl

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Geltrex, rowth factor-reduced

Geltrex, growth factor-reduced (ThermoFisher, cat. no. A1413302)

Thaw growth factor-reduced Geltrex on ice or overnight at 4°C. Using a cooled tip, make 100 μl aliquots on ice. We recommend putting the whole tip box in −20°C freezer for 5 min before use. Keep aliquots at −80°C for up to a year.

iCTB basal medium

Advanced DMEM/F-12 (ThermoFisher, cat. no. 12634028)

N-2 supplement, 100× (ThermoFisher, cat. no. 17502001)

B-27 supplement, 50× (ThermoFisher, cat. no. 17504001)

GlutaMax supplement, 100× (ThermoFisher, cat. no. 35050061)

1-Thioglycerol, 11.5 M (MilliporeSigma, cat. no. M1753)

BSA (30% (w/v) solution (Gemini Bio, cat. no. 700-110-100)

Knockout serum replacement (KSR) (ThermoFisher, cat. no. 10828028)

Combine ingredients according to volumes and concentrations in Table 4. Sterile filter. Use within 2 weeks.

iCTB complete medium

iCTB basal media (see recipe and Table 4)

TSC inhibitor cocktail, 1000× (see recipe and Table 9)

Y-27632, 10 mM (Selleck Chemicals, cat. no. S1049)

Valproic acid sodium salt, 300 mM, 50 mg/ml (MilliporeSigma, cat. no. 676380)

rhFGF2, 100 μg/ml (BioPioneer, cat. no. HRP-0011)

rhEGF, 100 μg/ml (R&D Systems, cat. no. 236-EG)

rhNoggin, 20 μg/ml (R&D Systems, cat. no. 6057-NG)

rhHGF, 100 μg/ml (STEMCELL Technologies, cat. no. 78019.2)

Prepare iCTB complete medium fresh prior to feeding. See Table 5 for volumes and concentrations.

Insulin-transferrin-sodium selenite (ITS)

Insulin-transferrin-sodium selenite (ITS), 100× (MilliporeSigma, cat. no. I1884)

Water, sterile, cell culture-grade

Table 4 iCTB Basal Medium

Component	Stock concentration	Final concentration	Volume for 250 ml
Advanced DMEM/F-12	–	–	237 ml
N-2 supplement	100×	1×	2.5 ml
B-27 supplement	50×	1×	5 ml
GlutaMax supplement	100×	1×	2.5 ml
1-Thioglycerol	11.5 M	150 μM	3.3 μl
BSA	30%	0.05%	417 μl
KSR	100%	1%	2.5 ml

Table 5 iCTB Complete Medium

Component	Stock concentration	Final concentration	Volume for 12 ml
iCTB basal media (Table 4)	–	–	11.9 ml
TSC inhibitor cocktail (Table 9)	1000×	1×	12 µl
Y-27632 (Selleck Chemicals, cat. no. S1049)	10 mM	5 µM	6 µl
Valproic acid sodium salt (MilliporeSigma, cat. no. 676380-5GM)	50 mg/ml (300 mM)	130 µg/ml (0.8 mM)	31.4 µl
rhFGF2	100 µg/ml	100 ng/ml	12 µl
rhEGF	100 µg/ml	50 ng/ml	6 µl
rhNoggin	20 µg/ml	20 ng/ml	12 µl
rhHGF	100 µg/ml	50 ng/ml	6 µl

Add 50 ml of sterile cell culture-grade water to the ITS vial. Mix well and make 1 ml aliquots. Store at −20°C for up to 1 year. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

IWP-2

IWP-2 (Selleck Chemicals, cat. no. S7085)

Prepare a 2 mM IWP-2 stock solution in DMSO and make 100 µl aliquots. Store at −80°C for up to 2 years. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

L-ascorbic acid 2-phosphate

L-ascorbic acid 2-phosphate (MilliporeSigma, cat. no. A8960)

Advanced DMEM/F-12 (ThermoFisher, cat. no. 12634028)

Add 6 ml of DMEM/F-12 to 200 mg of L-ascorbic acid to make 33.3 mg/ml L-ascorbic acid reagent. Sterile filter the solution and prepare 1 ml aliquots. Store at −20°C for up to 6 months. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

Recombinant human epidermal growth factor (rhEGF)

Recombinant human epidermal growth factor (rhEGF) (R&D Systems cat. no. 236-EG)

BSA (Gemini Bio-Products, cat. no. 700-110)

PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

First, prepare 0.1% (w/v) BSA in PBS solution. To make 100 µg/ml rhEGF, combine 10 ml of 0.1% (w/v) BSA in PBS solution to 1 mg of rhEGF. Gently pipette up and down to reconstitute the protein. Prepare 100 µl aliquots. Store at −80°C for up to 6 months. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

Recombinant human basic fibroblast growth factor 2 (bFGF-2)

Recombinant human basic fibroblast growth factor 2 (FGF-2) (BioPioneer, cat. no. HRP-0011)

BSA (Gemini Bio-Products, cat. no. 700-110)

PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

First, prepare 0.1% (w/v) BSA in PBS solution. To make 100 µg/ml bFGF-2, combine 500 µl of 0.1% (w/v) BSA in PBS solution to 50 µg of bFGF-2. Gently pipette up and down to reconstitute the protein and prepare 100 µl aliquots. Store at −20°C for up to 6 months. Once thawed, keep at 4°C. Avoid freeze-thaw cycles.

Recombinant human hepatocyte growth factor (rhHGF)

Recombinant human hepatocyte growth factor (rhHGF) (STEMCELL Technologies, cat. no. 78019.2)

PBS without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

To prepare 100 µg/ml rhHGF, combine 10 ml of sterile PBS to 1 mg of rhHGF. Gently pipette up and down to reconstitute protein and prepare 100 µl aliquots. Store at −80°C. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

Recombinant human neuregulin 1 (NRG1)

Recombinant human neuregulin 1 (NRG1) (Abcam, cat. no. ab50227)

Water, sterile, cell culture-grade

Reconstitute 50 µg of recombinant human NRG1 with 500 µl of cell culture-grade water to make a concentration of 100 µg/ml. Prepare 50 µl aliquots and store at −80°C for 3 years.

Recombinant human noggin (rhNoggin)

Recombinant human noggin (rhNoggin) (R&D Systems, cat. no. 6057-NG)

BSA (Gemini Bio-Products, cat. no. 700-110)

PBS without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

First, prepare a 0.1% (w/v) BSA in PBS solution. To make 20 µg/ml rhNoggin, combine 5 ml of 0.1% (w/v) BSA in PBS solution to 100 µg of rhNoggin. Gently pipette up and down to reconstitute the protein and prepare 100 µl aliquots. Store at −80°C. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

SB431542

SB431542 (MilliporeSigma, cat. no. 616464)

DMSO (MilliporeSigma, cat. no. D2650)

Thaw SB431542 at room temperature. Once thawed, spin down the sample to ensure no residual stock has remained on the wall of the tube. Dilute the vial to make 10 mM SB431542 (i.e., if the vial is at 100 mM [5 mg in 119 µl DMSO], add 1.071 ml DMSO). Mix well. Make 80 µl aliquots. Store at −80°C for up to a year.

STB differentiation medium

Terminal differentiation medium (see recipe and Table 7)

Y-27632, 10 mM (Selleck Chemicals, cat. no. S1049)

Knockout serum replacement (KSR) (ThermoFisher, cat. no. 10828028)

Forskolin, 10 mM (see recipe)

Prepare STB differentiation media fresh prior to feeding. See Table 6 for volumes and concentrations.

Table 6 STB Differentiation Medium

Component	Stock concentration	Final concentration	Volume for 12 ml
Terminal differentiation medium	–	–	11.5 ml
Y-27632	10 mM	2.5 μM	3 μl
KSR	100%	4%	480 μl
Forskolin	10 mM	2 μM	2.4 μl

Table 7 Terminal Differentiation Medium

Component	Stock concentration	Final concentration	Volume for 50 ml
DMEM/F-12 no HEPES	–	–	48.9 ml
BSA 30% (w/v) solution	30%	0.3%	500 μl
ITS-X	100×	1×	500 μl
2-Mercaptoethanol	55 mM	100 μM	90 μl

Table 8 Trophoblast Induction Medium

Component	Amount for 1 ml	Stock concentration	Final concentration
Basal trophoblast induction medium	1 ml	–	–
BMP-4	1 μl	10 μg/ml	10 ng/ml
IWP-2	1 μl	2 mM	2 μM

Terminal differentiation medium

DMEM/F-12 no HEPES (ThermoFisher cat. no. 11320-033)

BSA 30% (w/v) solution (Gemini Bio-Products, cat. no. 700-110-100)

ITS-X, 100× (ThermoFisher, cat. no. 51500056)

2-Mercaptoethanol (ThermoFisher, cat. no. 21985-023)

Combine ingredients using volumes and concentrations as listed in Table 7. Sterile filter and store at 4°C. Use within 2 weeks.

Trophoblast induction medium

Basal trophoblast induction medium (see recipe and Table 1)

BMP-4, 10 μg/ml (R&D Systems, cat. no. 314-BP-050)

IWP-2, 2 mM (Selleck Chemicals, cat. no. S7085)

Prepare trophoblast induction medium with a final concentration of 10 ng/ml BMP-4 (1:1000 from the stock solution) and 2 μM IWP-2 (1:1000 from the stock solution). 1.5 ml of media is required for one well of a 6-well plate. Prepare fresh. Filtering is not recommended. See Table 8 for volumes and concentrations.

TSC inhibitor cocktail

CHIR99021, 3 mM (see recipe)

A 83-01, 5 mM (see recipe)

SB431542, 10 mM (see recipe)

DMSO (MilliporeSigma, cat. no. D2650)

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Table 9 TSC Inhibitor Cocktail

Component	Stock concentration	Final concentration	Volume for 800 μl
CHIR99021	3 mM	2 mM	533 μl
A 83-01	5 mM	0.5 mM	80 μl
SB431542	10 mM	1 mM	80 μl
DMSO	–	–	107 μl

Thaw CHIR99021, A 83-01, and SB431542 aliquots that were prepared as listed above. Combine ingredients according to Table 9 using volumes and concentrations as indicated. This will make 800 μl of the inhibitor cocktail. Mix well. Make 100 μl aliquots. Store at −80°C. Once thawed, store at 4°C. (H. Okae, personal communications).

Valproic acid sodium salt

Valproic acid sodium salt (MilliporeSigma, cat. no. 676380)

PBS, without calcium and magnesium (Corning, cat. no. 21-040-CV, or equivalent)

Add 20 ml sterile PBS to 1 g of valproic acid sodium salt. This will make a 50 mg/ml sodium valproate in PBS solution. Sterile filter the solution and make 500 μl aliquots. Store at −80°C. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

Y-27632, 10 mM

Y-27632 (Selleck Chem, cat. no. S1049)

Cell culture grade water (Corning, cat. no. 25-055-CV)

Prepare a 10 mM stock solution and store in 100 μl aliquots. Store at −80°C for up to 2 years. Once thawed, store at 4°C. Avoid freeze-thaw cycles.

COMMENTARY

Background Information

Our initial work started with the establishment of a stepwise differentiation protocol of PSCs using BMP4 only, first into TE-like cells, which were previously described as CTB-like cells (Horii et al., 2016; Li et al., 2013). Recent single cell transcriptomic-based characterization found that these cells have a TE-like transcriptome signature (Soncin et al., 2022). We have therefore updated the terminology in this manuscript to reflect the TE-like nature of these cells. We previously terminally differentiated these TE-like cells directly into STB- and EVT-like cells (Fig. 6A), using PSC derived from both normal and diseased pregnancy (Horii et al., 2016). Specifically, we showed that, similar to primary CTB, hypoxia enhances differentiation of PSC-derived TE-like cells toward the EVT lineage. We also showed that T21-affected PSC recapitulate defects in STB differentiation, as seen in T21-affected placentas and primary CTB (Gerbaud et al., 2011), showing that PSC can be used to model trophoblast differentiation in normal

and diseased settings. This protocol was further improved by the addition of IWP2 in the first step of the trophoblast differentiation protocol, which increased the purity of the TE-like population to over 80% (Fig. 6B). TE-like cells generated using this protocol could subsequently be differentiated by treatment with feeder-conditioned media (FCM) + BMP4. If differentiation was performed under 21% oxygen, most of the cells would become STB, while differentiation under 2% oxygen could increase the proportion of EVT (Horii et al., 2019).

Using the 2019 improved protocol for trophoblast differentiation of PSCs, we established a "disease-in-a-dish" model to study molecular pathogenesis of the hypertensive disorder of pregnancy, called preeclampsia (Horii et al., 2019; Horii et al., 2021). However, a limitation of our study was the inefficient generation of EVT, which is a key cell type underlying PE pathophysiology. To overcome this limitation, we applied a modified media, named iCTB, developed in the Kessler
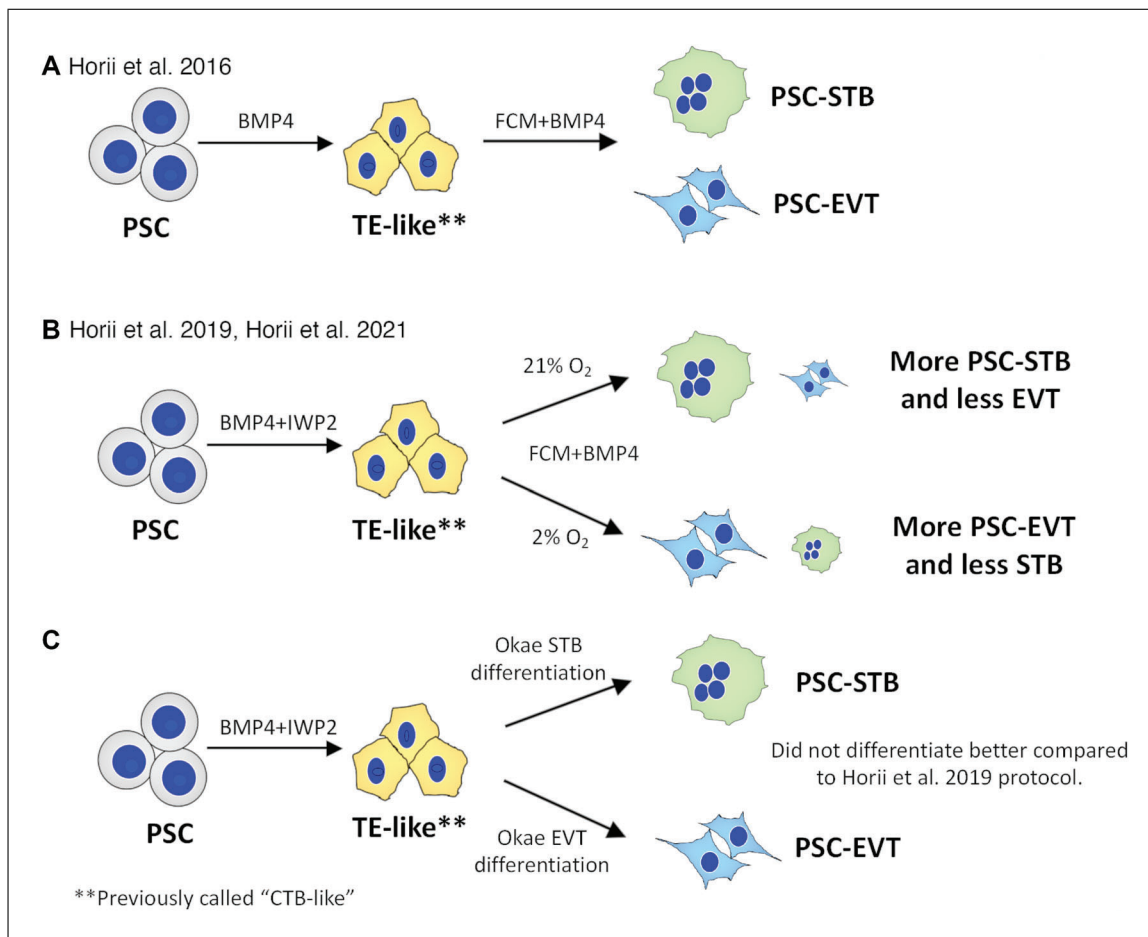
Figure 6 (**A**) An illustration of the original two-step trophoblast differentiation from PSC used for Horii et al. (2016). (**B**) An illustration of the improved two-step trophoblast differentiation with addition of IWP2 in the first step differentiation, and change in oxygen tension at the second step differentiation, directing to either EVT lineage by 2% O_2 or STB lineage by 21% O_2. (**C**) An illustration of the trophoblast differentiation protocol applying Okae's STB and EVT differentiation protocol to the TE-like cells.

lab for reprogramming of CTB from term placenta into TSC-like cells (Bai et al., 2021), to PSC-derived TE-like cells (Basic Protocol 1), to generate stable PSC-TSC (Basic Protocol 2). In our hands, the iCTB media is superior to the Okae TSC media for generation and maintenance of both primary and PSC-derived TSC (Bai et al., 2021; Soncin et al., 2022). Subsequent lineage-specific differentiation of PSC-TSC into EVT and STB lineages was further improved by adapting the medias and protocols established by Okae et al. (2018). In addition, we would also like to point out that we have attempted to directly perform Okae's STB and EVT differentiation protocol on the PSC-derived TE-like cells; however, this did not improve terminal differentiation of these cells, over and above the Horii et al. (2019) protocol (Fig. 6C). Therefore, TSC conversion of these TE-like cells (Basic Protocol 2) is required prior to terminal trophoblast differentiation (Fig. 1). These im-

provements are reflected in this updated protocol, and are currently employed among our three groups (Horii, Soncin, and Parast) to study trophoblast lineage specification, as well as preeclampsia-associated trophoblast phenotypes.

Both our protocol (using primed-PSC) and others' (using naïve-PSC) include step-wise differentiation, starting with derivation of a TE-like cell, continuing to a TSC-like stage, and followed by terminal differentiation into EVT or STB through lineage-specific protocols (Io et al., 2021; Soncin et al., 2022). Our TE-like cells (from Basic Protocol 1) and naïve PSC-derived TE-like cells (Io et al., 2021) have a similar transcriptome profile and clustered together by RNAseq analysis (Soncin et al., 2022). Both protocols produce TSC, which have the ability to generate STB and EVT (with appropriate gene expression profiles), and show appropriate ELF5 promoter hypomethylation and lack of classical

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Table 10 Troubleshooting Guide

Problem	Possible cause(s)	Solution(s)
Poor attachment of the PSCs on Geltrex-coated plate	Matrix issues	Check matrix. Be sure to plate Geltrex in a cold environment. Keep on ice until plating, as it gels in 5-10 min at temperatures above 15°C.
	No Y27632	PSCs attach less efficiently when they are single cells. Include Y27632 when plating.
PSCs detach from the plate	Matrix issues	Check matrix. Be sure to plate Geltrex in a cold environment. Keep on ice until plating, as it gels in 5-10 min at temperatures above 15°C. Heating the Geltrex could result in premature formation of gel.
Low EGFR at the induction of TE-like cells from PSC	Overseeding	Re-start the experiment. Seed at a lower concentration if necessary.
	Cells are in big clusters	When treating cells with TrypLE, try to break up the cells into single cells. Use a P1000 to pipette up and down, harshly. Alternatively, incubate cells for a longer time to better dissociate the cells into single cells.
	Reagent issues	Check differentiation factors with a positive control to ensure that reagents are working.
	High passage number	Thaw earlier passage PSCs.
Cells do not attach well on collagen IV coated plate	Matrix issue	Prepare plates and incubate overnight at 37°C. Longer incubation has shown better cell attachment.
PSC-derived TSC shows high HLA-G expression	High passage number	Thaw earlier passage PSC-derived TSC.
	Cells grown to over-confluence	Thaw earlier passage PSC-derived TSC.
Overgrown STB differentiation	High seeding density	Reduce the number of cells plated on day -1 to reach 30% confluency on day 0 before starting the differentiation.

HLA (HLA-A and -B) expression (Io et al., 2021; Soncin et al., 2022). However, naïve-PSC derived TSC share one additional similarity to early gestation trophoblast, which is high expression of C19MC compared to primed PSC-derived TSC (Io et al., 2021; Soncin et al., 2022). The advantage of our protocol is that no additional step is required to convert primed-state PSC (regular hESC or reprogrammed iPSC) into naïve-PSC; therefore, there is no global loss of DNA methylation, including loss of imprinting throughout the genome (Theunissen et al., 2016). It is unclear whether this loss of DNA methylation which accompanies conversion to naïve pluripotency could negatively impact utility of patient-derived iPSC to model trophoblast development in the setting of placenta-based pregnancy disorders, such as pre-eclampsia, particularly as the latter is driven, at least in part, by environmental insults. The differences between TSC/trophoblast derived from naïve- and primed-PSC have recently been reviewed

in depth (Morey et al., 2022; Roberts et al., 2022).

Critical Parameters

It is essential to have good starting material. If ESC or iPSC are not growing well, consider thawing a new vial, or changing to new batches of StemFlex or Geltrex. For PSC medium, other regularly-used media (i.e., StemPro, E8 or mTeSR Plus) besides StemFlex will also work for this protocol. Successful terminal differentiation to EVT requires undifferentiated (HLA-G <20%, EGFR >80%) PSC-TSC.

Cell attachment is improved if the collagen IV-coated plate is prepared a day before and kept in the incubator overnight. It is also acceptable to use the plate incubated for at least 2 to 3 hr on the day of plating but expect reduced cell attachment.

Troubleshooting

Following Basic Protocol 1 at day 4, TE-like cells express over 80% EGFR by flow

Table 11 List of Abbreviations

Abbreviation	Definition
BMP4	Bone morphogenetic protein 4
BSA	Bovine serum albumin
CTB	Cytotrophoblast
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ESC	Embryonic stem cells
EVT	Extravillous trophoblast
FCM	Feeder conditioned media
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
HLA-G	Human leukocyte antigen G
iPSC	Induced pluripotent stem cells
ITS	Insulin-transferrin-selenium
KSR	Knockout Serum Replacement
NRG1	Neuregulin 1
PSC	Pluripotent stem cell
PSC-TSC	PSC-derived TSC
STB	Syncytiotrophoblast
TE	Trophectoderm
TSC	Trophoblast stem cells

cytometry. Do not proceed to Basic Protocol 2 if the cells show lower EGFR expression, but consider restarting the protocol and implementing the additional considerations listed in Table 10. Once the PSC-TSC are derived, consider expanding and freezing down for future experiments. After multiple passages, PSC-TSC may acquire HLA-G, and become difficult to recover as undifferentiated TSC. Cultures in which over 30% of the cells have acquired HLA-G by flow cytometry should be tossed, and younger passage cells should be thawed and expanded. All the reagents are used only from the listed manufactures; therefore, it will be necessary to test the differentiation efficiency if using reagents from different sources. A detailed troubleshooting guide is listed in Table 10.

Understanding Results

The main part of this protocol uses TSC derivation and differentiation methods developed by Okae et al. (2018), and maintenance media (iCTB media) developed by Bai et al. (2021). Terminal differentiation protocols to STB and EVT were modified from Okae et al. (2018) (Morey et al., 2021; Soncin et al., 2022); in particular, coating tissue culture plates with fibronectin rather than collagen IV for EVT differentiation was adapted from primary EVT culture/differentiation protocols (Knofler & Pollheimer, 2013; Plessl et al., 2015; Wakeland et al., 2017), which is based on the decidual stromal composition of the EVT niche (Aplin et al., 1988; Earl et al., 1990). In the initial step of trophoblast induction (Basic Protocol 1), the colonies begin to show differentiation within a day, and by day 4, uniformly-flattened (epithelial) morphology appears throughout the culture (Fig. 2C). After 4 days of BMP4+IWP2 treatment, cells show over 80% EGFR positivity (Fig. 2D). This is important for the successful TSC derivation at the subsequent steps in Basic Protocol 2.

Using the previous protocol described by Horii et al. (2019), terminal differentiation of these TE-like cells (referred to as CTB-like cells in the original manuscript) using FCM plus 10 ng/ml BMP4 showed mixed differentiation into mostly STB, with a small proportion (less than 30%) of HLA-G$^+$ EVT at the end of differentiation when cultured under 2% oxygen (Fig. 2E, F). However, after the development of TSC and lineage specific STB

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and EVT differentiation protocols (Okae et al., 2018), our group has applied the media to derive PSC-TSC from TE-like cells and was able to directly differentiate PSC-TSC to STB or EVT using lineage specific differentiation protocol (Fig. 1).

Notably, differentiation into EVT has had the greatest improvement. The PSC-TSC derived using Basic Protocols 1 to 3 (Fig. 3A, B), results in better yield of EVT showing over 60% HLA-G positive cells at the end of the Basic Protocol 5 (Fig. 5A-C) compared to 30% HLA-G positive EVT using our previous protocol (Fig. 2E, F). We can also specifically generate STB with high purity resulting in multinucleated cells with STB-associated gene expression (Soncin et al., 2022), high secretion of hCG (Fig. 4C), and cell-cell fusion (Fig. 4D). These assays are useful to study detailed mechanisms of trophoblast differentiation. A summary of abbreviations used can be found in Table 11.

Time Considerations

It takes about 1 week to expand and maintain the cells for the initial TE induction (Basic Protocol 1) and an additional 2 to 3 weeks to complete the TSC derivation (Basic Protocol 2). After establishing PSC-derived TSC, cells can be passaged multiple times (we have successfully maintained them for up to 20 passages). However, once a high proportion of cells acquire over 30% HLA-G, it is difficult to recover cells to the TSC stage (EGFR$^+$, HLA-G$^-$). In this case, thawing earlier passage cells may be required. For the STB differentiation (Basic Protocol 4), it takes about 8 days to complete the differentiation, and for EVT differentiation (Basic Protocol 5), it takes about 8 days to complete the differentiation. All time considerations include plate-coating steps.

Author Contributions

Virginia Cheung: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; visualization; writing original draft; writing review, and editing. **Tony Bui:** Conceptualization; data curation; investigation; methodology; writing review, and editing. **Francesca Soncin:** Conceptualization; methodology; funding acquisition; writing review, and editing. **Tao Bai:** Methodology; writing review, and editing. **Jack Kessler:** Methodology; writing review, and editing. **Mana Parast:** Conceptualization; funding acquisition; investigation; methodology; writing review, and editing. **Mariko Horii:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; visualization; writing original draft; writing review, and editing.

Conflict of Interest

M.M.P., F.S., and M.H. have filed a provisional patent for the protocol described. V.C.C., T.B., T.B., and J.A.K. declare no competing interests.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Key References

Soncin et al., 2022. See above.

In this paper, we have shown four important points. First, the initial trophoblast induction of primed human PSCs (using BMP2+IWP2) resulted in cells showing a TE-like signature by transcriptome profile. Second, we showed that these TE-like cells can convert to TSC by applying iCTB media, which resulted in a transcriptome and ELF5 promotor DNA methylation profile similar to primary TSC. Third, primed PSC-derived TSC can further differentiate into STB or EVT by using both in vitro lineage-specific differentiation protocols and in vivo engraftment assay. Finally, PSC-derived TE and TSC showed similar transcriptome profile to naïve (pre-implantation state)-PSC derived TE and TSC. These data showed that primed human ESC/iPSC can in fact become functional TSC, and further give rise to STB or EVT through lineage-specific differentiation.