11

Mouse Trophoblast Stem Cells

Jennifer Quinn, Tilo Kunath, and Janet Rossant

Summary

The trophectoderm is one of the earliest cell types to differentiate in the forming mammalian embryo. It is responsible for the initial implantation and the formation of the trophoblast components of the placenta, an organ essential for nutrient and waste exchange between the fetus and its mother. The trophoblast can be modeled in vitro using trophoblast stem cells. Trophoblast stem cells require fibroblast growth factor (FGF)4, heparin, and contact with embryonic fibroblasts, or fibroblast-conditioned medium. They grow as tight epithelial colonies, which express markers of the early trophectoderm and have been shown to contribute to all of the components of the placenta through chimera studies. These cells can be passaged indefinitely and can be differentiated by removal of FGF4 and fibroblasts and will express genetic markers of later placental cell types. This chapter will discuss the initial derivation of trophoblast stem cells from the blastocyst stage, maintenance, differentiation, flow cytometry and transfection techniques that can be used with these cells.

Key Words: Trophoblast stem cell; trophectoderm; derivation; culture maintenance; differentiation; flow cytometry; transfection.

1. Introduction

In a mouse blastocyst at embryonic day (E) 3.5, the specification of the trophectoderm and the inner cell mass is the first differentiation to occur. By E 4.5, there are three cell types: the primitive endoderm, which will form the visceral and parietal endoderm; the primitive ectoderm, which will form the embryo proper; and the trophectoderm, which will produce all the trophoblast tissues ***(1,2)***. The trophoblast is essential for survival of the mammalian conceptus because it mediates implantation and ultimately creates the placenta, which allows nutrient and waste exchange between the fetus and its mother ***(3)***.

The outer cells of the blastocyst—the trophectoderm—can be divided into two distinct components: polar and mural ***(4)***. The mural trophectoderm is comprised of the cells that are most distal to the inner cell mass. These cells will differentiate into primary trophoblast giant cells. Giant cells undergo

From: *Methods in Molecular Medicine, Vol. 121: Placenta and Trophoblast: Methods and Protocols, Vol. 1*
Edited by: M. J. Soares and J. S. Hunt © Humana Press Inc., Totowa, NJ

endoreduplication, which results in large polyploid cells ***(5)***. The mural trophectoderm and its resulting primary giant cells are important for the initial implantation of the blastocyst ***(3)***. This differentiation continues laterally toward the border of the inner cell mass ***(4)***. The polar trophectoderm is located in direct contact with the inner cell mass ***(4)***. These cells remain diploid and continue to divide, giving rise to the trophoblast lineage. This includes the extra-embryonic ectoderm and the ectoplacental cone and, eventually, the components of the mature chorioallantoic placenta—the spongiotrophoblast, labyrinth and giant cell layer ***(3)***. This chapter discusses trophoblast stem cells as an in vitro model of the trophoblast cell lineage.

Embryonic stem cells from the primitive ectoderm are a well-established in vitro model ***(6–8)***. These cells can be genetically manipulated and have provided insight into the development of the embryo and essential genes involved in this process ***(9)***. Trophoblast stem (TS) cells may be used in a similar fashion to elucidate the mechanism of differentiation and the role of genes and cell types in the development of the placenta.

Trophoblast stem cells are diploid, permanent, and self-renewing when they are maintained in stem cell conditions. They express markers of the trophectoderm, extra-embryonic ectoderm, and ectoplacental cone (*see* **Note 1**). TS cells can be derived from E 3.5 blastocysts, the extra-embryonic ectoderm from E 6.5 conceptuses, and the chorionic ectoderm from E 7.5 to E 10 embryos ***(10–12)***. Specific mutant TS cell lines can be developed if the gene in question is not required for stem cell initiation or maintenance. TS cells require: fibroblast growth factor (FGF)4 , heparin, and embryonic fibroblasts (EMFIs) or embryonic fibroblast-conditioned medium (FCM) to maintain their stem cell morphology of tight adherent epithelial colonies. These cells have been shown to contribute to all trophectoderm derivates through chimera experiments and can be maintained in culture indefinitely ***(10)***.

When stem cell factors are removed, TS cells differentiate and show an increase in expression of genetic markers for the spongiotrophoblast, labyrinth, and giant cells and decrease in expression of genes from the blastocyst, extra-embryonic ectoderm, and ectoplacental cone. Ultimately, these cells become terminally differentiated giant cells with large cytoplasm and high ploidy ***(10)***.

This chapter describes methods for TS cell derivation from blastocysts, maintenance, differentiation, fluorescence-activated cell sorting (FACS), and transfection.

2. Materials

2.1. Embryonic Fibroblasts

1. Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, Cat. Nos. D2650 and D5879).

2. Trypsin/ethylenediamine tetraacetic acid (EDTA) (Invitrogen Life Technologies, Carlsbad, CA, cat. no. 25200-056).
3. DMEM + 10% fetal bovine serum (FBS).
4. Mitomycin C (Sigma, cat. no. M0503).
5. 0.45-µm filter (Corning, Acton, MA, cat. no. 430945).
6. TS cell medium: to 500 mL RPMI 1640 + antibiotics (penicillin/streptomycin at 50 µg/mL each final concentration; Invitrogen, cat nos. 61870 or 11875), add the following:
   a. 130 mL FBS, final concentration 20% (Invitrogen).
   b. 6.5 mL, 100 m*M* sodium pyruvate (final concentration 1 m*M*) (Invitrogen).
   c. 6.5 mL, 10 m*M* β-mercaptoethanol (final concentration 100 µ*M*) (Sigma).
   d. 6.5 mL, 200 m*M* L-glutamine (final concentration 2 m*M*) (Invitrogen).
7. 2X Freezing medium. 50% FBS, 30% TS medium, 20% dimethylsulfoxide (DMSO); cool to 4°C.
8. 1000X FGF4 human recombinant FGF4 (Sigma, cat. no. F2278), 25 µg
   a. Resuspend lyophilized FGF4 in its vial with 1.0 mL of phosphate-buffered saline (PBS)/0.1% w/v fraction V bovine serum albumin (BSA).
   b. Mix well with P200 and make 10 aliquots of 100 µ*M* into 1.5-mL microfuge tubes and freeze at –70°C.
   c. Thaw each aliquot as needed and store at 4°C; do not re-freeze. 10 mL PBS/0.1% (w/v) BSA is prepared by dissolving BSA (Sigma, cat. no. A3311) in PBS without Ca^{2+}/Mg^{2+}; filter through a 0.45-µL syringe filter, and make 1-mL aliquots in microfuge tubes; store at –70°C and thaw one tube when a vial of FGF4 must be reconstituted.
9. 1000X Heparin (Sigma, cat. no. H3149 10,000 U):
   a. Resuspend heparin in PBS to a final concentration of 1.0 mg/mL (1000X).
   b. Make nine aliquots of 1.1 mL in 1.5 microfuge tubes.
   c. Store at –70°C.
   d. Thaw aliquots as needed and store at 4°C.
   e. Heparin can also be prepared as a 10,000X (10 mg/mL) stock in PBS without Ca^{2+}/Mg^{2+} and stored at –70°C. This can be used multiple times to make batches of 1000X heparin.
10. 70% FCM + F4H. 70% FCM, 30% TS medium, 1/1000 FGF4, 1/1000 heparin.
11. TS Medium + F4H. TS medium, 1/1000 FGF4, 1/1000 heparin.
12. PBS without calcium and magnesium.
13. Tissue culture equipment.
14. Hemocytometer to count cells.
15. Isopropanol freezing container.

2.2. TS Cell Derivation

1. M2 Medium (Specialty Media, Phillipsburg, NJ, MR-015-D).
2. KSOM Medium (Specialty Media, MR-121-D).
3. Pulled glass pipet (12-in.).

4. Mouth pipetting equipment (tubing [one-thirty-second of an inch wall, VWR Scientific Products, West Chester, PA, cat. no. 62996-350], p1000 pipet tip, cotton plug, aspirator mouth piece).
5. TS cell culture medium.
6. PBS without calcium and magnesium.
7. 70% FCM + F4H. 70% FCM, 30% TS medium, 1/1000 FGF4, 1/1000 heparin.
8. TS Medium + F4H. TS medium, 1/1000 FGF4, 1/1000 heparin..
9. Tissue culture equipment.
10. Hemocytometer to count cells.

2.3. TS Cell Maintenance

1. TS cell culture medium.
2. PBS without calcium and magnesium.
3. 70% FCM + F4H. 70% FCM, 30% TS medium, 1/1000 FGF4, 1/1000 heparin.
4. TS Medium + F4H. TS medium, 1/1000 FGF4, 1/1000 heparin.
5. Tissue culture equipment.
6. Hemocytometer to count cells.
7. 2X Freezing medium: 50% FBS, 30% TS medium, 20% DMSO, cool to 4°C.
8. Isopropanol freezing container.

2.4. TS Cell Differentiation

1. TS cell culture medium.
2. PBS without calcium and magnesium.
3. 70% FCM + F4H. 70% FCM, 30% TS medium, 1/1000 FGF4, 1/1000 heparin.
4. TS Medium + F4H. TS medium, 1/1000 FGF4, 1/1000 heparin.
5. Tissue culture equipment.
6. Hemocytometer to count cells.

2.5. Flow Cytometry

1. 70% Ethanol.
2. Propidium iodide (PI; Molecular Probes, Eugene, OR), 1 mg/mL in water.
3. Triton-X 100, 0.1% final concentration (Sigma).
4. RNase A.
5. PI/Triton X-100 staining solution with RNase A: to 10 mL of 0.1% Triton X-100 in PBS, add 2 mg DNase-free RNase A and 200 µL of 1 mg/mL PI.
6. Polypropylene or polystyrene tubes (5 mL).
7. Hoechst 33342, 1 mg/mL in water.
8. 70-µm Cell Strainer (BD Biosciences, Bedford, MA, Falcon cat. no. 352350).
9. Flow cytometer with 488-nm argon laser.

2.6. Transfection

1. LipofectAMINE PLUS (Gibco-BRL, Gaithersburg, MD, cat. no. 10964-013): RPMI culture medium, PLUS reagent, lipofectamine.

2. GenePulser cuvet (BioRad, Hercules, CA, cat. no.165-2088).
3. Linearized DNA.
4. PBS.
5. Drugs for selection, e.g., G418 (200 µg/mL), puromycin (1 µg/mL), hygromycin (150 µg/mL).

3. Methods

The methods detailed in this chapter describe the (1) the isolation and culturing of mouse embryo fibroblasts, (2) derivation of TS cells from blastocysts, (3) maintenance, and (4) differentiation of TS cells, as well as (5) protocols to perform flow cytometry to sort cells and analyze DNA content and (6) to perform DNA transfection.

3.1. Embryonic Fibroblasts

The protocol for isolating and culturing mouse embryo fibroblasts is based on the procedure previously described ***(9)***.

3.1.1. Isolation and Expansion of EMFI Cultures

1. Dissect one litter of E 15.5 to E 16.5 embryos into PBS.
2. Remove embryos' limbs, brains, and internal organs.
3. Place the carcasses into 50-mL Falcon tubes with PBS.
4. Rinse with DMEM three times.
5. Aspirate the medium.
6. Mince the embryos into small pieces.
7. Add 10 mL Trypsin/EDTA to minced pieces in 50-mL tube.
8. Add 5 mL of sterile glass beads and a stir bar.
9. Incubate at 37°C for 30 min while stirring.
10. Repeat **steps 8–10**.
11. Split cell suspension into two 50-mL Falcon tubes, each containing 3 mL FBS.
12. Wash the original Falcon tube twice with DMEM + 10% FBS and add to tubes from **step 11**.
13. Centrifuge at 200*g* for 5 min.
14. Aspirate supernatant.
15. Resuspend pellet in 50 mL of DMEM + 10% FBS.
16. Use trypan blue to quantify viable nucleated cells.
17. Plate 5×10^6 cells per 15-cm tissue culture dish containing DMEM + 10% FBS.
18. Change the medium the next day.
19. Continue feeding every 2 d until cells reach confluency.
20. Split cells 1:6.
21. Continue to feed every 2 d until cells reach confluency.
22. Freeze in chilled 2X Freezing medium (follow steps outlined under **Subheading 3.3.**).

3.1.2. EMFI Feeders

1. Thaw one vial of EMFIs in five 15-cm tissue culture dishes containing DMEM + 10% FBS.
2. When cells are confluent (approx 3 d), treat with 100 µL mitomycin C (1 mg/mL) in DMEM + 10% FBS.
3. Use as feeders.
4. Mitomycin treated EMFIs can be frozen for later use.

3.1.3. Fibroblast-Conditioned Medium

FCM is used to culture TS cells in the absence of EMFIs.

1. Plate mitomycin-treated primary EMFIs in 100-mm dishes (2×10^6 cells; 2×10^5 cells/mL).
2. Culture in approx 11 mL TS medium.
3. Incubate for 72 h.
4. Lift TS media into 14-mL Falcon tubes.
5. Spin at 200*g* for 4 min to remove floating cells and debris.
6. Filter through 0.45-µm filter.
7. Aliquot.
8. Store at –20°C.
9. Thaw each aliquot as needed and store at 4°C; do not re-freeze.
10. Follow **steps 2–8** to prepare two more batches of FCM.
11. Discard the cells. EMFIs are only used up to 10 d after the mitomycin treatment.

3.2. TS Cell Derivation

The initial derivation of trophoblast stem cells is described under **Subheadings 3.2.1.–3.2.5.** These sections cover the methods for obtaining, isolating, and culturing blastocysts as well as the early culturing techniques required for TS cell derivation and maintenance. The time lines indicated are generalized and can differ between genotypes and even between blastocysts from the same litter. It is common to observe the cells every 2 d throughout the process of derivation and maintenance.

3.2.1. Obtaining Blastocysts

TS cells arise from the trophectoderm layer of the blastocyst. Thus, the first step involved in deriving TS cells is to obtain blastocysts to culture (*see* **Notes 2** and **3**).

1. Day 0. Place female mice in estrus with preteased males mid-afternoon. By 1000 h the following morning, check for a plug to ensure that the mice have mated. This is E 0.5. It is assumed that the mating took place at 2400 h. Continue to house the pregnant female with adequate food and water on a 12-h light/dark cycle for three more days.

2. E 3, AM. In a four-well tissue culture dish, plate 0.5 mL mitomycin-treated EMFIs at a density of 5×10^4 cells/mL in TS medium + F4H in each well. Place in a standard incubator at 37°C/5% CO_2. A mouth pipet is needed to collect and transfer the blastocysts once they are removed from the mother. To prepare a mouth pipet, insert mouthpiece into one end of 15 cm of tubing (diameter one-thirty-second inch), and at the opposite end insert a p1000 pipet tip, filled with cotton ***(9)*** (*see* **Note 4**)
3. E 3, PM. Prepare a pipet with a suitable diameter to maneuver the blastocysts. A 12-in, glass pipet must be heated at the junction where the glass thickens until it becomes slightly pliable. At this point, pull the pipet in one fluid motion until it is about 50 cm long. Break off so that about 10 cm of the thin diameter glass remains ***(9)***. Store pipet with thin end up in a clean location. The pipet can be used for several flushings, but should be made fresh each day.

3.2.2. Collecting Blastocysts

The following steps describe the process of removing blastocysts from the mother on E 3, PM, so that they can be plated in order to derive TS cells.

1. Set up dissection area with a cloth/diaper for the initial dissection, dissection tools (scissors, forceps), and spray bottle with 70% ethanol.
2. Place KSOM in syringe in 37°C/5%CO_2 incubator.
3. Sacrifice female mouse by cervical dislocation ***(9)***.
4. Spray abdomen with ethanol to wet fur.
5. Use scissors to cut through skin and body wall.
6. Remove uterus by cutting below oviduct on both horns and above cervix.
7. Place uterine horns in 10-cm dish and view with dissecting microscope.
8. Insert 1-cc syringe with a 20-gauge needle filled with M2 into oviduct end of the uterus.
9. Flush with approx 0.3 cc M2, then flush same horn from cervix end (*see* **Note 5**).
10. Repeat for second uterine horn.
11. Use mouth pipet with small amount of M2 and a few bubbles to control fluid flow in order to collect and transfer the blastocysts through four drops of M2 (in a 60-mm tissue culture dish).
12. Use mouth pipet with small amounts of M2 to transfer blastocysts through four drops of KSOM heated to 37°C (in 60-mm tissue culture dish).
13. Use mouth pipet to place all embryos in center of an organ culture dish in KSOM.
14. Use mouth pipet to transfer one blastocyst per well onto EMFIs with TS med + F4H (*see* **Note 6** and **Fig. 1**).
15. Place in incubator. This is day 0 of the blastocyst outgrowth stage. It is normal for the blastocyst to remain floating in the medium for awhile (*see* **Note 7**).

3.2.3. Blastocyst Outgrowth and First Disaggregation

Within 24–36 h after the initial plating, the blastocyst should attach to the plate and hatch from the zona pellucida. By the third day, in TS cell conditions,

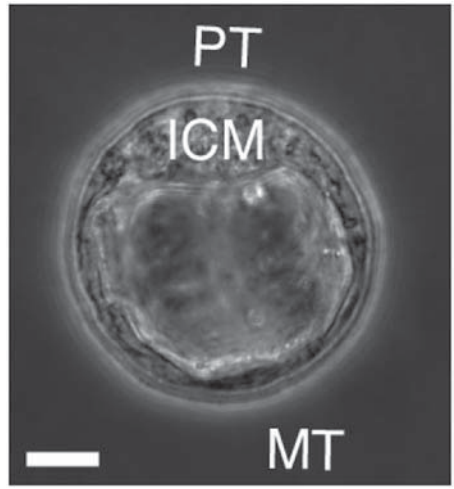

Fig. 1. Fully expanded blastocyst collected by uterine flushing which can be used to derive trophoblast stem cells. The polar trophectoderm (PT) overlying the inner cell mass (ICM) will give rise to most of the trophectoderm derivatives and the mural trophectoderm (MT) will mediate implantation and give rise to primary giant cells. Phase contrast, scale bar 50 μm.

a blastocyst outgrowth should form. When this outgrowth is disaggregated, it will allow the formation of stem cell colonies. If there is no outgrowth, or the blastocyst has not yet attached, the culture still requires fresh media by the third day after initial plating (*see* **Note 7**).

3.2.3.1. Day 3 (After Initial Plating) Feeding

1. Remove media by aspiration.
2. Feed with 500 μL TS med + F4H (1/1000 FGF4 stock, 1/1000 heparin stock).
3. Continue every 48 h until outgrowth has reached an appropriate size for disaggregation (**Fig. 2**).

3.2.3.2. Days 4–5 (After Initial Plating) Disaggregate

The blastocyst outgrowth should be formed by this time point (*see* **Note 8**). Disaggregation must occur before the outgrowth becomes too large. **Figure 2** outlines an appropriate outgrowth size to harvest TS cells efficiently. Beyond this point, the TS cells will not be derived as efficiently and an endoderm-like cell type may form in the cultures ***(11)*** (**Fig. 3**) (*see* **Note 9**).

1. Select an appropriate outgrowth for disaggregation (between days 3–8).
2. Aspirate media.
3. Rinse plate with 500 μL PBS.

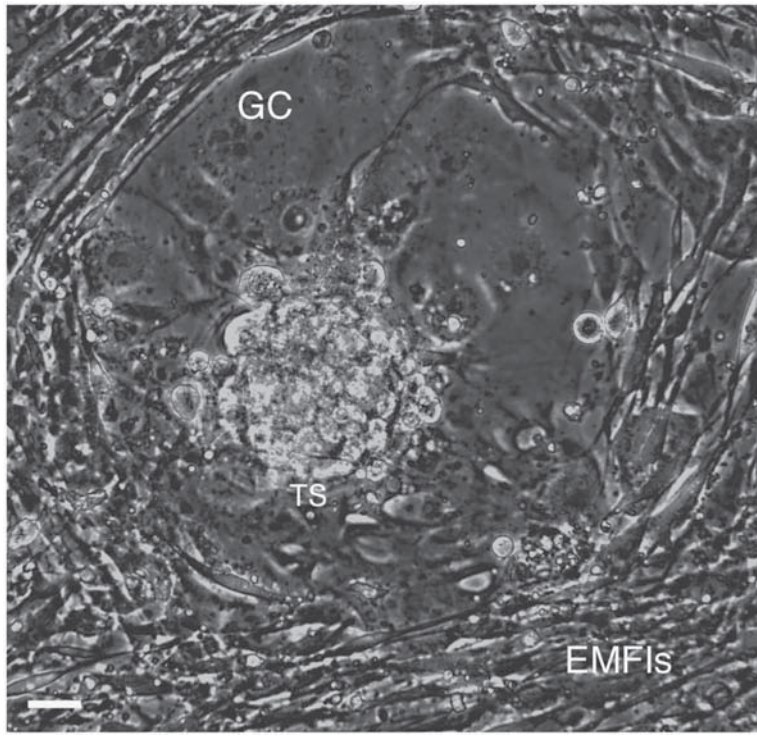

Fig. 2. Blastocyst outgrowth 4 d after initial plating. This outgrowth is an appropriate size for dissaggregation. Note the field of giant cells (GC) surrounding the outgrowth (trophoblast stem [TS] cells) and the embryonic fibroblasts (EMFIs) covering the plate. Phase contrast, scale bar 50 µm.

4. Add 100 µL trypsin.
5. Place in the incubator for 5 min at 37°C/5% CO_2.
6. Stop this reaction with 400 µL 70% FCM + 1.5X F4H.
7. Pipet the contents of the well to near-single cell suspension.
8. Return to the incubator.

3.2.3.3. Day 6 (After Initial Plating/48 h After Disaggregation) Feeding

Between 6 and 10 d after the disaggregation, TS cell colonies begin to form. TS cell colonies grow as tight, flat epithelial sheets and are present in the culture along with giant cells, which are differentiated TS cells (**Fig. 4**). These cells require fresh 70% FCM + 1.5X F4H every 48 h until the plate becomes approx 50% confluent.

1. Aspirate the media.
2. Replace with 500 µL of 70% FCM + 1.5X F4H.

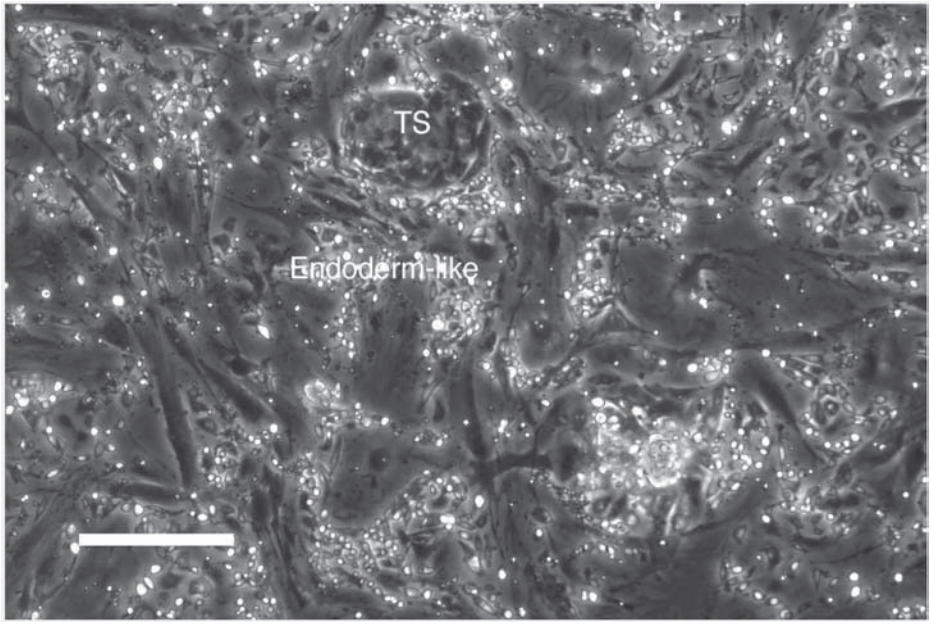

Fig. 3. Early trophoblast stem (TS) cell colony surrounded by endoderm-like cells. These round refractile cells are present in TS cell cultures if the blastocyst outgrowth is allowed to get too large prior to disaggregation. The endoderm-like cells are difficult to alleviate since they do not require fibroblast-conditioned medium or fibroblast growth factor-4 to continue to grow. Phase contrast, scale bar 250 μm.

3. This process needs to be repeated every 48 h until the TS cells reach approximately 50% confluent and require passaging.

3.2.4. First Passage

The colonies that have arisen from the disaggregated outgrowths require their first passage when they are approx 50% confluent. This typically occurs between 15 and 25 d after initial disaggregation. The first passage of TS cells is the most likely time that these cells can differentiate (*see* **Note 10**). For each of the disaggregated outgrowths that require passaging:

1. Prepare a four-well dish (one well per disaggregated outgrowth) by plating EMFIs (5×10^4 cells/mL) with fresh TS medium + 1.5X F4H (400 μL).
2. Place new plates in a incubator for at least one h to condition the medium and limit the amount of TS cell differentiation.
3. Remove media from original well by aspirating.
4. Rinse the well with 500 μL PBS.
5. Aspirate PBS.

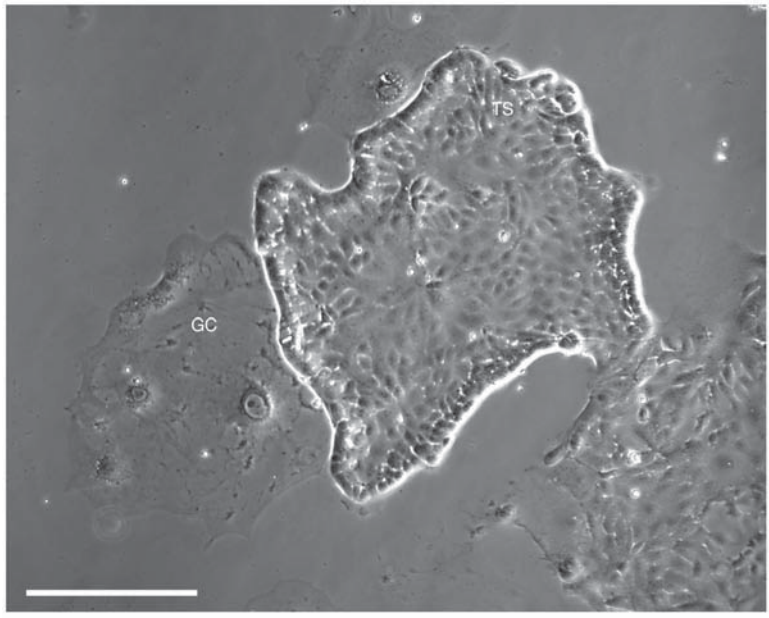

Fig. 4. Trophoblast stem (TS) cell colony grown on plastic tissue culture dish in TS cell conditions. The stem cell colony has tight epithelial borders. Giant cells (GC) can be seen at the edge of the colony. Phase contrast, scale bar 250 µm.

6. Add 100 µL of trypsin/EDTA.
7. Place in the incubator for 5 min.
8. Add TS medium + 1.5X F4H (400 µL) from new plate to stop trypsinization.
9. Pipet up and down to prepare a near single cell suspension.
10. Transfer all of the cells in suspension to a new well in a four-well plate with EMFIs.
11. Change the medium approx 8 h after this passage.
12. Continue to feed cells every 2 d with 500 µL TS med + 1.5X F4H.

3.2.5. Early Passages (Passages 2–7)

TS cells are still vulnerable to differentiation throughout the early passages. Allow the cells to become at least 70% confluent between passages (*see* **Note 10**).

1. Repeat **steps 1–9** as listed under **Subheading 3.2.4.**
2. Transfer cell suspension to a 14-mL Falcon tube.
3. Spin at 200*g* for 3 min.
4. Aspirate supernatant.
5. Resuspend in 1 mL 70% FCM + F4H.

6. If the majority of the TS cells have differentiated as a result of passaging or the culture dish is less than 70% confluent and the cells have stopped expanding, passage to new well (1:2) (proceed to **step 9**).
7. If the cells have maintained their stem cell morphology and are growing rapidly, split into four new wells (1:4).
8. Ensure that each well has a total volume of 500 µL 70% FCM + F4H.
9. Continue to feed cultures every 48 h with fresh medium (*see* **Note 10**).
10. Passage as required.
11. By passage 7, it is possible to expand the line to a six-well dish (35 mm), which can be maintained as described under **Subheading 3.3.**

3.3. TS Cell Maintenance

Subheadings 3.3.1.–3.3.5. cover protocols required for the maintenance and storage of a stable TS cell line, including feeding, passaging, plating, and freezing and thawing techniques, which can be used on stable TS cell lines (**Fig. 4**).

A stable line can be defined as one in which at least 70% of the cells maintain typical stem cell morphology. It is normal to have some giant cells in any TS culture, but if they make up the majority of the population of cells, the culture is not stable and it may not be possible to recover TS cells through a freeze/thaw cycle. It is also important to note that, because TS cells and giant cells are adherent, cells floating in the medium are indicative of dead or dying cells. The protocols in this section can be applied to cells grown on EMFIs or to cells grown directly on tissue culture plates. They do not require gelatin as previously demonstrated ***(10)***.

3.3.1. Feeding

In order to maintain TS cells, fresh medium and growth factors are required every 48 h. TS cells plated on EMFIs tend to recover more rapidly from a thaw or other detrimental conditions. For TS cell analysis, it is beneficial to have a pure population without EMFIs. Pure populations of TS cells grown on plastic require 70% feeder conditioned medium (*see* **Note 11**).

3.3.1.1. Culturing TS Cells on EMFIs

EMFIs can effectively condition the TS medium for 10 d; thereafter, it is best to passage the TS cells onto fresh EMFIs. When these cells are on EMFIs, they require TS med + F4H. If the cells have not reached 70% confluency on the original EMFIs beyond 10 d, begin feeding with 70% FCM + F4H as described under **Subheading 3.3.1.2.**

1. Prepare fresh TS medium + F4H.
2. Aspirate old medium.
3. Feed TS cells on EMFIs with new TS med + F4H.

4. Place in a standard tissue culture incubator, 37°C/5% CO_2.
5. Replace with fresh TS medium + F4H every 48 h up to 10 d after the initial plating of EMFIs.

3.3.1.2. Culturing TS Cells on Tissue Culture Plastic

TS cells grow well on standard tissue culture dishes if the medium is supplemented with 70% FCM + F4H. This is an ideal condition for analysis of DNA content, RNA isolation, or for visualizing the cells with immunohistochemistry techniques, because it provides a pure population of TS cells. These steps must also be followed in the TS cells grown on EMFIs that are more than 10 d old.

1. Prepare 70% FCM + F4H (e.g.,10 mL of 70% FCM +F4H = 7 mL FCM, 3 mL TS medium, 10 µL 1000X FGF4 stock, 10 µL 1000X heparin stock).
2. Aspirate old medium.
3. Add fresh medium.

3.3.2. Passaging

When the cells reach approx 80–90% confluency, they must be passaged to a new plate so they can continue to grow and expand. Stable TS cells can be passaged at 1:20 every 5–7 d. TS cells that differentiate or are slow growing can be passaged at 1:5. Each line is unique and requires some level of optimization. To expand the line to a larger surface area, passage no less than 1:7 of the total cells. Cells can be passaged directly onto plastic tissue culture dishes or they may be plated onto EMFIs after they have adhered or co-plated with the EMFIs at the time of passaging.

Density has an effect on cell growth and differentiation. Cells grown or passaged at too high a density can become overcrowded. This results in cell death and differentiation (*see* **Note 12**). Cells plated at too low a density have high rates of differentiation even when maintained in stem cell feeding conditions.

1. Aspirate media.
2. Rinse with PBS.
3. Aspirate PBS.
4. Add trypsin (one-half volume of medium) (*see* **Note 11**).
5. Incubate at 37°C/5% CO_2 for 5 min.
6. Tap plate and cells should lift into suspension. If the cells do not lift, continue to incubate for another 2–5 min.
7. Stop trypsinization with TS medium (same volume as trypsin).
8. Pipet vigorously to attain near single cell suspension.
9. Lift all media to 14-mL Falcon tube.
10. Spin at 200*g* for 3 min.
11. Aspirate supernatant.
12. Resuspend pellet of cells with 1 mL TS medium.
13. Plate into new dishes with 70% FCM at an appropriate volume (*see* **Note 11**).

3.3.3. Differential Plating

This technique can be used for two main purposes. The first is to remove EMFIs from TS cell cultures to provide a pure population of TS cells. Second, differential plating can enrich either stem cells or giant cells.

3.3.3.1. Removing EMFIs From TS Cell Cultures

When switching from EMFI cells to plastic dishes with 70% FCM, it may be desirable to get rid of the EMFI cells immediately. The different adherence rates of EMFI cells (fast) and TS cells (slow) can be used to obtain a pure TS cell population. Because some TS cells do settle along with the EMFIs, the desired passage density may be increased a bit (e.g., 1:14 instead of 1:16).

1. Passage cells to a new plate following the steps under **Subheading 3.3.2.**
2. Incubate the culture for 1.5 h at 37°C/5% CO_2.
3. Remove the supernatant and plate onto another dish.

3.3.3.2. Enriching for Giant Cells or TS Cells

Giant cells can be removed from a culture based on their different adherence rates. Giant cells adhere more quickly and more strongly than stem cells to the culture dishes. The supernatant should have a reduced population of giant cells compared with the initial cell population, whereas the cells remaining on the plate should have an increased proportion of giant cells.

1. Follow the passaging protocol under **Subheading 3.3.2.**
2. Incubate for 15–30 min at 37°C/5% CO_2.
3. Remove supernatant and passage onto another dish.

3.3.4. Freezing/Thawing

TS cells can be frozen for indefinite periods and then later thawed for use. This allows a certain level of security, because it is not necessary to derive new TS cells every time one wants a new plate, and it is possible to expand and store lines of interest for extended periods of time.

3.3.4.1. Freezing

1. Obtain cells in suspension (1 mL) using the protocol outlined under **Subheading 3.3.2.**
2. Add 1 vol of 2X Freezing medium cooled to 4°C.
3. Place 1 mL in a freezing vial.
4. If freezing several lines, keep those in the 2X freezing medium on ice until all can go in the freezer.
5. Place tubes in an isopropanol slow-freeze container.
6. Slowly freeze in –70°C Freezer for at least 48 h.
7. Transfer to liquid nitrogen.

3.3.4.2. Thawing

To recover a cell line from a frozen vial, culture and passage freshly thawed cells at least twice to ensure they have recovered sufficiently before beginning any experiments. After the initial thaw, it is normal to have a large number of floating cells, some giant cells and some stem cell colonies (*see* **Note 12**). If the plate appears confluent (it may require aspirating the media and rinsing with PBS to see through the floaters), the cells require a passage. Otherwise, continue to feed the cells until they reach 80% confluency then follow the protocol for passaging. Cells often recover more rapidly when they are thawed onto EMFIs.

1. Remove the vial of cells from liquid nitrogen or –70°C freezer.
2. Warm in 37°C water bath until just thawed, approx 3 min.
3. Use a 1 mL pipet to transfer contents of vial to a 14-mL Falcon tube containing 1 mL TS medium.
4. Spin at 200*g* for 3 min.
5. Aspirate to remove DMSO contained in the freezing medium.
6. Resuspend into an appropriate medium depending on if they are on EMFIs or on plastic.
7. Plate all cells from vial onto a surface area, which is smaller than the original plate.
8. 24 h after initial plating aspirate medium (expect many floaters).
9. Rinse with PBS.
10. Add appropriate fresh medium.

3.4. TS Cell Differentiation

As TS cells differentiate in vitro, markers of later cell types of the trophectoderm lineage show an increased expression whereas markers of the blastocyst, extra-embryonic ectoderm, and ectoplacental cone show a decrease (*see* **Note 1**). The changes in gene expression are associated with changes in cell morphology and DNA content. These changes are indicative of cells that are changing from tight epithelial TS cell colonies to intermediate cell types and finally to terminal giant cells with expansive cytoplasm and large polyploid nuclei. Most TS cell cultures will differentiate to predominantly giant cells by the sixth day of differentiation although some genotypes require a longer period of time (**Fig. 5**).

The rate of differentiation is influenced by several factors, including genotype and cell density. It is important to select a consistent density to work with. TS cell plated at a very low density differentiate very rapidly to giant cells and do not show an increased expression of intermediate markers of spongiotrophoblast and labyrinth cells. Typically, a density that results in the culture becoming nearly confluent by day 6 of differentiation is used (~2×10^5/60-mm plate). A protocol for the induction of differentiation is presented.

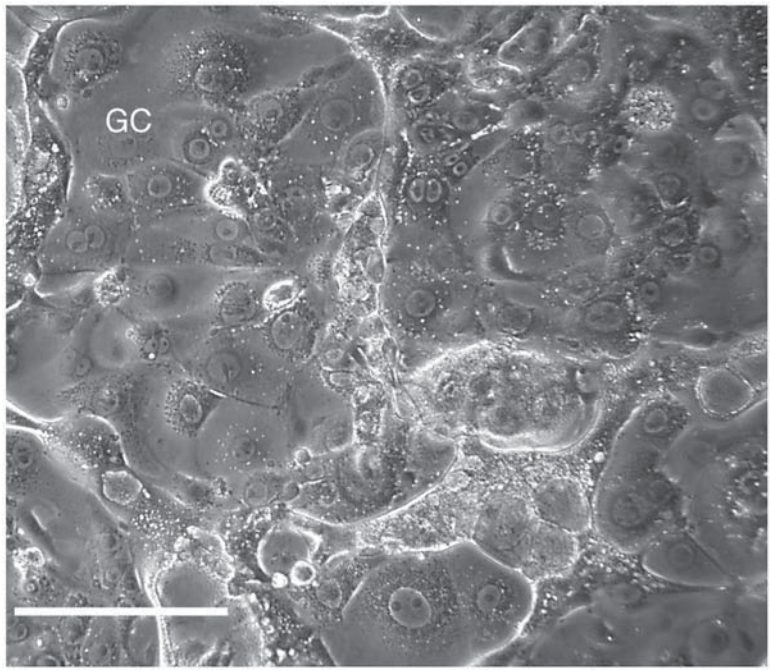

Fig. 5. Differentiated trophoblast stem cells grown on plastic dishes. These cells have been without fibroblast-conditioned medium + F4H for 6 d and show a characteristic giant cell morphology with a large cytoplasm and nucleus. Phase contrast, scale bar 250 μm.

1. Establish the number of plates required for selected time points.
2. Using the same density, plate all initial dishes with TS cells with 70% FCM + F4H.
3. Incubate for 24 h.
4. After 24 h collect the day 0 culture.
5. Prepare day 0 sample for further analysis.
6. Initiate differentiation of remaining plates by removing 70% FCM + F4H (*see* **Note 13**).
7. Rinse briefly with PBS.
8. Aspirate.
9. Add PBS for 5 min.
10. Aspirate.
11. Replace with TS medium.
12. Incubate.
13. Follow **steps 7–12** every 2 d throughout the course of the differentiation experiment.
14. Collect plates at time points throughout differentiation (*see* **Note 14**).

3.5. Flow Cytometry Analysis and Sorting

FACS sorting by flow cytometry is a method used to measure ploidy (*see* **Subheading 3.5.1.**) and to sort cells by DNA content or other markers (*see* **Subheading 3.5.2.**). This procedure can be done on living or fixed samples. It can be used to measure the percentage of diploid, tetraploid, and cells with a ploidy >8 N to quantify the percentage of giant cells in a population (**Fig. 5**). It is also an efficient method to sort for diploid TS cells or green fluorescent protein (GFP)-positive cells to isolate and expand these populations. The initial steps of these methods are outlined in this section. These methods have been adapted from procedures described by Darzynkiewicz and Juan ***(13)***.

3.5.1. To Analyze Ploidy

1. Collect cells at selected time points throughout differentiation by trypsinizing entire plate following protocol to passage. If giant cells are abundant at later time points in differentiation, use a cell scraper to ensure all giant cells are lifted (*see* **Note 15**).
2. Stop trypsinization with TS medium.
3. Lift all cells to a 14-mL Falcon tube. Ensure that all cells have been collected by rinsing plate with an additional 2 mL TS medium.
4. Count and record cell number.
5. Spin cells at 200*g* for 4 min.
6. Remove supernatant.
7. Resuspend cells in 500 µL PBS (*see* **Note 16**).
8. Add 6 mL 70% ethanol to fix the cells.
9. Store in –20°C freezer until ready to use.
10. When ready to analyze, make fresh PI/Triton X-100 staining solution with RNase A.
11. Spin cells at 500*g* for 5 min.
12. Remove ethanol.
13. Resuspend pellet in 5 mL PBS.
14. Let stand for 1 min.
15. Centrifuge cells for 5 min at approx 200*g*.
16. Remove supernatant.
17. Suspend cell pellet, 1×10^6 cells/mL based on cell count in **step 4** in PI/Triton X-100 staining solution with RnaseA.
18. Keep at room temperature for 30 min or in incubator at 37°C for 15 min.
19. Filter cells through a 70-µm filter into 5 mL polypropylene or polystyrene test tubes.
20. Set up and adjust the flow cytometer for excitation with an argon ion laser (488 nm) and detection of PI emission using a 675 band pass filter.
21. Measure cell fluorescence using pulse peak–pulse area signal to discriminate between G2 cells and cell doublets.
22. Analyze the ploidy of cells using DNA content frequency histogram (**Fig. 6**).

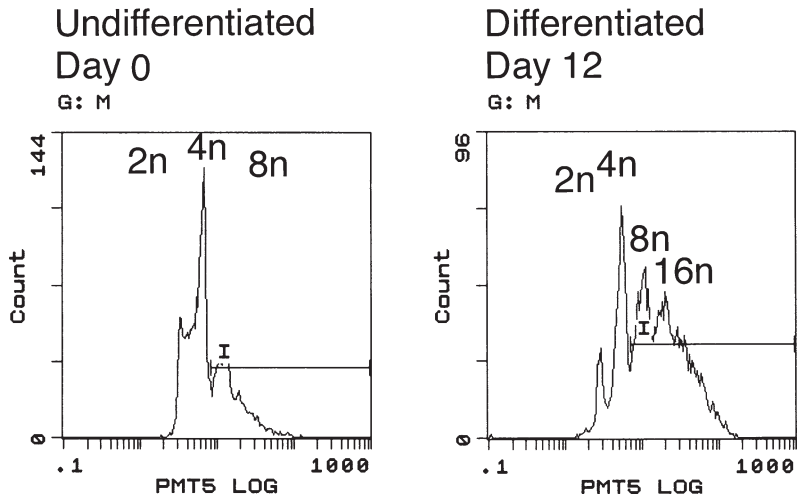

Fig. 6. Fluorescence-activated cell sorting profile of undifferentiated trophoblast stem (TS) cells (day 0) and differentiated TS (day 12 in TS medium without embryonic fibroblasts, fibroblast growth factor-4, and Heparin). These data have been collected from a gated channel to exclude cell doublets. The first peak indicates diploid cells; the second peak shows tetraploid cells. The peaks included within line I are giant cells with a ploidy greater than or equal to 8 N. There is an increase in total percentage of high ploidy cells (>8 N) by day 12 of differentiation indicating an increased percentage of giant cells.

3.5.2. To Sort Live Cells

A flow cytometer can sort TS cells for GFP expression and for diploid DNA content, which is indicative of stem cells.

1. Put cells into single cell suspension following the cell passage protocol.
2. Count cells using hemocytometer.
3. Spin at 200*g* for 3 min.
4. Remove supernatant.
5. Resuspend cells in TS medium (1×10^6 cells/mL).
6. Add Hoechst 33342 staining solution to cell solution for a final dye concentration of 2–5 μg/mL.
7. Incubate at 37°C for 20–90 min.
8. Strain cells through 70 μm filter into 5 mL polypropylene or polystyrene tubes.
9. Set up and adjust flow cytometer for ultraviolet excitation at 340–380 nm to detect Hoechst 33342 and GFP; enhanced green fluorescent protein (EGFP) can be detected at 488 nm.
10. Measure cell fluorescence using pulse width–pulse area signal to discriminate between G2 cells and cell doublets.

11. Set a gate to sort out cell doublets.
12. Set a gate to sort out cells with DNA ploidy >8 N (giant cells).
13. Single diploid TS cells will be collected in collection tube.
14. Plate cells on EMFIs + TS med + F4H or on to plastic dishes with 70% FCM + F4H.
15. Incubate and continue to maintain as outlined under **Subheading 3.3.**

3.6. DNA Transfection

DNA transfection can be used to alter the genome of TS cells by introducing a specifically designed DNA fragment. The new DNA is only incorporated into a subpopulation of the cells treated. A positive selectable marker gene is needed to detect successfully transfected cells. Two methods have been used to transfect TS cells: lipofectamine (*see* **Subheading 3.6.1.**) and electroporation (*see* **Subheading 3.6.2.** and **Note 17**).

3.5.1. Transient Transfections With Lipofectamine

1. Obtain a plate of subconfluent cells (1 d after passage) in a six-well dish.
2. Combine circular plasmid carrying gene of interest (1 µg) and plasmid containing reporter gene (e.g., GFP) (0.2 µg) with RPMI 1640 (200 µL without antibiotics) and PLUS reagent (12 µL) (*see* **Note 18**).
3. Incubate for 15 min at room temperature.
4. Add RPMI 1640 (190 µL) and Lipofectamine (10 µL) mixture to the DNA/PLUS.
5. Incubate for 15 min at room temperature.
6. Wash TS cells in PBS.
7. Add 800 µL RPMI 1640.
8. Add DNA/PLUS/Lipofectamine solution.
9. Incubate at 37°C/5% CO_2 for 3 h.
10. Add 2 mL FCM with 50 ng/mL FGF4 and 2 µg/mL heparin.
11. Incubate for 16 h.
12. Change the medium to 70% FCM with F4H.
13. Incubate 24 h.
14. Assay for reporter gene activity.

In transient transfections the GFP reporter construct has been shown to start expressing GFP 24 h after the transfection. This expression peaks by 48 h and the amount of protein shows a dramatic decrease by the fifth day after transfection.

3.5.2. Stable Transfections Using Electroporation

1. Obtain a plate of near confluent cells (5×10^6).
2. Prepare plasmid by isolating DNA using a DNA preparation kit (e.g., Qiagen).
3. Linearize vector DNA with a restriction enzyme digest.
4. Switch on electorporation apparatus and set it to electroporate at 0.25V and a capacitance of 500 uFD.
5. Follow passaging protocol to pellet cells and remove supernatant.

6. Resuspend in PBS (0.8 mL) (*see* **Note 19**).
7. Transfer to GenePulser cuvet (0.4 cm electrode).
8. Add linearized DNA (4–25 µg).
9. Electroporate at 0.25 V and a capacitance of 500 uFD.
10. Incubate cells on ice for 20 min.
11. Plate cells on 100 mm plate with 10 mL 70% FCM +F4H.
12. Incubate for 24 h.
13. Start suitable drug selection depending on drug resistance gene on plasmid: G418 (200 µg/mL), puromycin (1 µg/mL), hygromycin (150 µg/mL).
14. Incubate.
15. Feed cells with 70% FCM + F4H + selectable marker every 48 h.
16. Pick colonies 12 d later (*see* **Note 20**).

4. Notes

1. TS cells show regulation of different genetic markers throughout differentiation. **Table 1** outlines a few of these genes as well as some genes that can be used to screen colonies for endoderm-like cells, which might be contaminating a culture.
2. The protocols outlined in this chapter are for deriving TS cells from E 3.5 blastocysts. Embryos flushed at E 2.5 and cultured overnight can be used to plate on EMFIs for TS cell derivation.
3. TS cells have not been successfully derived from C57BL6 mice. Naturally mated ICR mice can carry 8–15 embryos.
4. Mouth pipets or finger pipets are required to manipulate blastocysts. Pulling pipets is a delicate process—practice first! To control the blastocysts within the pipet, ensure that there are at least three air bubbles before attempting to pick up. In order to keep the mouthpiece clean, place the cap of a 14-mL Falcon tube (with a hole in it) on the tubing below the mouthpiece. The mouth pipet can be covered when not in use with a cap of a 14-mL Falcon and the main body of the Falcon tube. Further directions for mouth pipetting can be found on page 177 in **ref. *9***.
5. When flushing blastocysts, the uterine horn will bulge when KSOM is added and a slightly cloudy liquid will emerge from opposite end. It is important not to squeeze or puncture uterine horn.
6. EMFIs can "condition" the medium for approx 10 d; after that point 70% FCM is required.
7. If adding new medium before the blastocysts have fully attached, be careful not to dislodge or aspirate them.
8. Ensure that each blastocyst and subsequent TS line are kept separate from all others to avoid contamination.
9. Primitive endoderm-like cells are round and highly refractile. They can be found in TS cultures if the blastocyst outgrowth becomes too large before the initial dissociation. These cells grow well in TS medium with or without F4H and are very difficult to remove.

Table 1
Genes Used to Characterize TS Cell Cultures Throughout Differentiation

Gene Name	Expression	TS cell expression profile	Reference
Cdx2	Trophectoderm E 3.5	TS cell marker	***14,15***
Eomesodermin	Trophectoderm E 3.5 Extra-embryonic ectoderm (Exe)	TS cell marker	***10,16,17***
Errβ	Exe E 5.5 Chorion E 8.5	TS cell marker	***10,18***
Esx1	Exe Chorion E 8.5 Labyrinth E 9.5	Chorion and labyrinth marker	***19–21***
Wnt7b	Exe E 7.5 Chorion E 8.5	Chorion marker	***22***
4311	Ectoplacental cone E 6.5 Spongiotrophoblast E 8.5–18.5	Spongiotrophoblast marker	***23,24***
Nodal	Spongiotrophoblast E 10	Spongiotrophoblast marker	***25***
Gcm1	Chorion E 7.5–9.5 Labyrinth E 9.5–E 17.5	Chorion and labyrinth marker	***26–29***
Placental lactogen 1	Primary giant cells E 5–E 12	Giant cell marker	***30–32***
Placental lactogen II	Secondary giant cells E 12–term	Giant cell marker	***30–34***
α Fetoprotein	Visceral and parietal endoderm	Endoderm marker	***35***
Indian hedgehog	Visceral endoderm	Endoderm marker	***36***

10. **Table 2** provides guidelines for the appropriate dish, amount of medium, and passage requirements for various stages throughout the derivation of TS cell lines.
11. **Table 3** provides the area and requirements of commonly used tissue culture dishes in the maintenance and differentiation of TS cells.
12. Cultures that have a high rate of floating cells should be rinsed thoroughly. Aspirate the media, rinse with room temperature PBS, and aspirate. Add PBS for 5 min, aspirate, and feed cells with fresh medium.
13. Cells cannot be differentiated on EMFIs, because they will condition the media and will inhibit differentiation.
14. Giant cells are very adherent and are difficult to trypsinize. If after 5 min of trypsinization the cells remain attached, try to dislodge cells by pipetting up and down in trypsin only before stopping the reaction with TS medium. If the cells still remain attached, use a cell scraper to dislodge the rest of the cells.
15. Ensure that all cells are in suspension, especially giant cells, which adhere very strongly to the plate.

Table 2
A Guideline for Passaging at Different Stages Throughout Derivation

Stage	Size of dish	Amount of medium	Amount to passage
Blastocyst plating	Four-well + feeders	500 µL	Not applicable
Disaggregation	Same four-well	500 µL	All
First passage	Four-well + feeders	500 µL	
	All or 1:2		
Passage 2–7	Four-well + feeders	500 µL	1:2–1:10
Passage 8 + to expand	Four-well–six-well		
	Six-well + or – feeders	2 mL	1:4–1:7
Six-well (60-mm)	60-mm	5 mL	1:7
Maintaining on 60-mm	60-mm	5 mL	1:10–1:30

Table 3
Tissue Culture Dishes and Conditions

Well	Area cm^2	Volume to feed	Volume of trypsin
Four-well		0.500 mL	0.100 mL
Six-well (35 mm)	9.62	2 mL	1 mL
60-mm	28.27	5 mL	2.5 mL
100-mm	78.54	10 mL	5 mL

16. Cells used in FACS sorting must be in a single cell suspension or they will be lost in the filtration step.
17. Transient transfections in TS cell occurs with a success rate of approx 1%.
18. In transient transfections, the ratio of reporter plasmid to gene of interest must be optimized; often, higher concentrations of DNA are helpful.
19. Approximately 50% cell death is expected with optimal transfection efficiency when PBS is used.
20. On a 10-cm plate, there are often approx 100 drug-resistant colonies present after 12 d.

References

1. Gardner, R. L. (1982) Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. *J. Embryol. Exp. Morphol.* **68,** 175–198.
2. Snell, G. D. and Stevens, L. C. (1966) Early embryology, in *Biology of the Laboratory Mouse* (Green, E. L., ed.). McGraw-Hill, New York: pp. 205–245.

3. Rossant, J. and Cross, J. (2002) Extraembryonic lineages, in *Mouse Development; Patterning, Morphogenesis and Organogenesis* (Rossant, J. and Tam, P. P., eds.). Academic, San Diego: pp. 155–180.
4. Dickson, A. D. (1963) Trophoblastic giant cell transformation of mouse blastocyts. *J. Reprod. Fertil.* **169,** 465–466.
5. Barlow, P. W. and Sherman, M. I. (1972) The biochemistry of differentiation of mouse trophoblast: studies on polyploidy. *J. Embryol. Exp. Morphol.* **27,** 447–465.
6. Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309,** 255–256.
7. Evans, M. J. and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154–156.
8. Martin, G. R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78,** 7634–7638.
9. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. R. (2003) *Manipulating the Mouse Embryo, 3rd Ed.* (Inglis, J. and Cuddihy, J., eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
10. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A., and Rossant, J. (1998) Promotion of trophoblast stem cell proliferation by FGF4. *Science* **282,** 2072–2075.
11. Kunath, T. (2003) PhD thesis in *Medical and Molecular Genetics*, University of Toronto, Toronto
12. Uy, G. D., Downs, K. M., and Gardner, R. L. (2002) Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse. *Development* **129,** 3913–3924.
13. Darzynkiewics, Z. and Juan, G. (1997) Nucleic acid analysis, in *Current Protocols in Cytometry*, John Wiley and Sons, New York: pp. 7.5.1–7.5.23.
14. Beck, F., Erler, T., Russell, A., and James, R. (1995) Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. *Dev. Dyn.* **204,** 219–227.
15. Chawengsaksophak, K., James, R., Hammond, V. E., Kontgen, F., and Beck, F. (1997) Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature* **386,** 84–87.
16. Ciruna, B. G. and Rossant, J. (1999) Expression of the T-box gene eomesodermin during early mouse development. *Mech. Dev.* **81,** 199–203.
17. Russ, A. P., Wattler, S., Colledge, W. H., et al. (2000) Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature* **404,** 95–99.
18. Luo, J., Sladek, R., Bader, J. A., et al. (1997) Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. *Nature* **388,** 778–782.
19. Li, Y., Lemaire, P., and Behringer, R. R. (1997) Esx1, a novel X chromosome-linked homeobox gene expressed in mouse extraembryonic tissues and male germ cells. *Dev. Biol.* **188,** 85–95.
20. Li, Y. and Behringer, R. R. (1998) Esx1 is an X-chromosome-imprinted regulator of placental development and fetal growth. *Nat. Genet.* **20,** 309–311.

21. Cross, J. C. (2000) Genetic insights into trophoblast differentiation and placental morphogenesis. *Semin. Cell. Dev. Biol.* **11,** 105–113.
22. Parr, B. A., Cornish, V. A., Cybulsky, M. I., and McMahon, A. P. (2001) Wnt7b regulates placental development in mice. *Dev. Biol.* **237,** 324–332.
23. Lescisin, K.R., Varmuza, S., and Rossant, J. (1988) Isolation and characterization of a novel trophoblast-specific cDNA in the mouse. *Genes. Dev.* **2,** 1639–1646.
24. Deussing, J., Kouadio, M., Rehman, S., Werber, I., Schwinde, A., and Peters, C. (2002) Identification and characterization of a dense cluster of placenta-specific cysteine peptidase genes and related genes on mouse chromosome 13. *Genomics* **79,** 225–240.
25. Ma, G. T., Soloveva, V., Tzeng, S. J., et al. (2001) Nodal regulates trophoblast differentiation and placental development. *Dev. Biol.* **236,** 124–135.
26. Basyuk, E., Cross, J. C., Corbin, J., et al. (1999) Murine Gcm1 gene is expressed in a subset of placental trophoblast cells. *Dev. Dyn.* **214,** 303–311.
27. Anson-Cartwright, L., Dawson, K., Holmyard, D., et al. (2000) The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. *Nat. Genet.* **25,** 311–314.
28. Yu, C., Shen, K., Lin, M., et al. (2002) GCMa regulates the syncytin-mediated trophoblastic fusion. *J. Biol. Chem.* **277,** 50,062–50,068.
29. Stecca, B., Nait-Oumesmar, B., Kelley, K. A., Voss, A. K., Thomas, T., and Lazzarini, R. A. (2002) Gcm1 expression defines three stages of chorio-allantoic interaction during placental development. *Mech. Dev.* **115,** 27–34.
30. Colosi, P., Swiergiel, J. J., Wilder, E. L., Oviedo, A., and Linzer, D. I. (1988) Characterization of proliferin-related protein. *Mol. Endocrinol.* **2,** 579–586.
31. Faria, T. N., Deb, S., Kwok, S. C., Talamantes, F., and Soares, M. J. (1990) Ontogeny of placental lactogen-I and placental lactogen-II expression in the developing rat placenta. *Dev. Biol.* **141,** 279–291.
32. Shida, M. M., Jackson-Grusby, L. L., Ross, S. R., and Linzer, D. I. (1992) Placental-specific expression from the mouse placental lactogen II gene promoter. *Proc. Natl. Acad. Sci. USA* **89,** 3864–3868.
33. Campbell, W. J., Deb, S., Kwok, S. C., Joslin, J. A., and Soares, M. J. (1989) Differential expression of placental lactogen-II and prolactin-like protein-A in the rat chorioallantoic placenta. *Endocrinology* **125,** 1565–1574.
34. Hamlin, G. P., Lu, X. J., Roby, K. F., and Soares, M. J. (1994) Recapitulation of the pathway for trophoblast giant cell differentiation in vitro: stage-specific expression of members of the prolactin gene family. *Endocrinology* **134,** 2390–2396.
35. Becker, S., Wang, Z. J., Massey, H., et al. (1997) A role for Indian hedgehog in extraembryonic endoderm differentiation in F9 cells and the early mouse embryo. *Dev. Biol.* **187,** 298–310.
36. Dziadek, M. and Adamson, E. (1978) Localization and synthesis of alphafoetoprotein in post-implantation mouse embryos. *J. Embryol. Exp. Morphol.* **43,** 289–313.