Video Article

Derivation of Mouse Trophoblast Stem Cells from Blastocysts

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Abstract

Specification of the trophectoderm is one of the earliest differentiation events of mammalian development. The trophoblast lineage derived from the trophectoderm mediates implantation and generates the fetal part of the placenta. As a result, the development of this lineage is essential for embryo survival. Derivation of trophoblast stem (TS) cells from mouse blastocysts was first described by Tanaka *et al.* 1998. The ability of TS cells to preserve the trophoblast specific property and their expression of stage- and cell type-specific markers after proper stimulation provides a valuable model system to investigate trophoblast lineage development whereby recapitulating early placentation events. Furthermore, trophoblast cells are one of the few somatic cell types undergoing natural genome amplification. Although the molecular pathways underlying trophoblast polyploidization have begun to unravel, the physiological role and advantage of trophoblast genome amplification remains largely elusive. The development of diploid stem cells into polyploid trophoblast cells in culture makes this *ex vivo* system an excellent tool for elucidating the regulatory mechanism of genome replication and instability in health and disease. Here we describe a protocol based on previous reports with modification published in Chiu *et al.* 2008.

Video Link

The video component of this article can be found at https://www.jove.com/video/1964/

Protocol

In this protocol, we describe the preparation of mouse blastocysts and the establishment of TS cell lines. General mouse manipulations prior to the collection of blastocysts, including the set up for natural mating and the induction of super ovulation, basically follow the standard protocol illustrated by Nagy *et al.* 2003 (pp146-150).

Derivation and maintenance of TS cells

1. Setup breeding cross between mice of interest.
2. Prepare mouse embryonic fibroblasts (MEFs) as feeder cells. Two days before the collection of blastocysts, MEFs (2 x 10^6 cells) are plated in 100 mm dishes. Next day, these cells are treated with 10 ml of TS medium containing mitomycin C (20 mg/ml) for a 2 hour-incubation at 37°C, 5% CO_2. This is followed by washing cells twice with PBS. The mitomycin C treated cells are then seeded in 12-well plates (1 x 10^5 cells/well).
3. On the day of blastocyst collection (3.5 dpc, referred to as day one), MEF culture medium is replaced by TS medium plus 1x FGF4/Heparin (0.5 ml/well).
4. Preparation for collection of the uterus: euthanize the mouse and locate the uterus (Figure 1A).
5. Remove the uterus by grasping it with forceps at the cervix (located behind the bladder) and cut across the junction of uterus and cervix (Figure 1B). Pull the uterus, trim the membrane and fat tissues away and cut the uterus below the junction with the oviduct (Figure 1C). Place the uteri (Figure 1D) in a small volume (0.2 ml) of TS medium in a 60 mm plate.
6. Flush each uterus horn with 1 ml of TS medium using a 1-ml syringe with a 26-gauge needle. Insert the needle into the base of uterus and flush slowly into a new 60 mm plate with 1 ml TS medium. The uterus should be inflated and extended fully during flushing.
7. Collect and transfer the blastocysts carefully into a clean 12 well plate containing TS medium using a mouth-controlled pipette. Wash blastocysts three times by serial transfer through TS medium in 12-well plates. Place one blastocyst per well (Figure 2A) in a 12-well plate containing the mitomycin C-treated MEF feeders and culture at 37°C, 5% CO_2.
8. The blastocysts will hatch from zona pellucida and attach to the wells in 24 to 36 hours. A small outgrowth will be readily observed on day 3. Feed the cultures with 500 µl fresh TS medium plus 1x FGF4/Heparin.
9. On day 4 or 5, the blastocyst outgrowth should be ready for disaggregation depending on its size. The ideal size for TS cell disaggregation is illustrated in Figure 3B. Wash the cultures once with 0.5 ml PBS. Add 100 ml 0.25% trypsin/EDTA and incubate for 5 minutes at 37°C, 5% CO_2. Break the outgrowth into small clumps by pipetting up and down vigorously with a P100 pipetteman. Stop the trypsinization by adding TS-70CM-1.5F4H (30% TS medium, 70% MEF-CM plus 1.5x FGF4/Heparin) into the well. Change medium 8 hours after disaggregation and continue to feed cells every 2 days.

10. Between days 6 and 10 (highly variable) TS cell colonies can be observed. They have flat, epithelial sheet like morphology with a clear colony boundary (Figure 2C). Continue to feed the cultures every 2 days
11. When TS cells reach 50% confluency (usually between days 15 and 20), wash once with PBS and add 100 ml 0.25% trypsin/EDTA. Pipette up and down during trypsinization to ensure a near single-cell suspension. Stop the trypsinization by adding TS-70CM-1.5F4H and transfer to new 6-well or 60mm plates containing the mitomycin C-treated MEF feeders (2.5 x 10^5 cells/6-well or 5 x10^5 cells/60mm plate).
12. Change medium 8 hours after passage and continue to feed the cultures every 2 days.
13. After one or two more passages (3-5 days between passages) on the MEF feeders, TS cells may be cultured without them. Because MEFs attach to the culture plate much faster than TS cells after trypsinization, such difference in the adherent rate can be utilized to separate TS cells from the MEF feeders. After passing the cultured cells, incubate them at 37°C, 5% CO_2 for 30 min. Most MEFs will attach to the plate and the TS population remains in suspension. Transfer the supernatant to a new plate. Repeat this process for several passages to obtain a pure TS population.
14. Maintain TS cells in TS-70CM-1.5F4H and pass them every 4 days (1:10 to 1:20 split) or when the culture reaches 70% confluency. Passing the cells with high density (higher than 1:5 split) or culturing them when overly confluent may cause their differentiation.
15. The identity of TS cells can be confirmed by immunostaining of a cell marker, Cdx2 (Figure 3).

Freezing TS cells

1. Prepare 2x freezing medium (50% FBS, 30% TS medium and 20% DMSO).
2. Collect TS cells by trypsinization, followed by centrifugation at 450g for 6 min. Resuspend them in TS medium with equal volume of 2x freezing medium.
3. Freeze the cells slowly using an isopropyl alcohol chamber at -70°C and transfer to liquid nitrogen after 24-48 hours.
4. A 60 mm plate (70% confluent) is usually kept in 2 vials.

Thawing TS cells

1. Quickly thaw the vial in 37°C, collect them in 10 ml TS media by centrifugation (450g, 6 min), and resuspend in 30% TS media, 70CM-1.5F4H.
2. One vial of cells is seeded in a 60 mm plate.

TS cell differentiation into TGC

Differentiation of TS cells into trophoblast giant cells (TGCs) can be induced by the withdrawal of MEF-CM, FGF4 and heparin. The TGC phenotype (large nuclei) can be detected four days after the induction (Tanaka et al., 1998 and Chiu *et al.* 2008). Immunostaining of p450scc detect the expression of this TGC marker in the differentiated culture (Figure 4A). Flow cytometric analysis of PI (propidium iodide) stained cells further shows the presence of differentiated cells containing higher DNA content (Figure 4B). These results suggest that TS cells undergo endoreduplication to become polyploid TGCs.

Representative Results

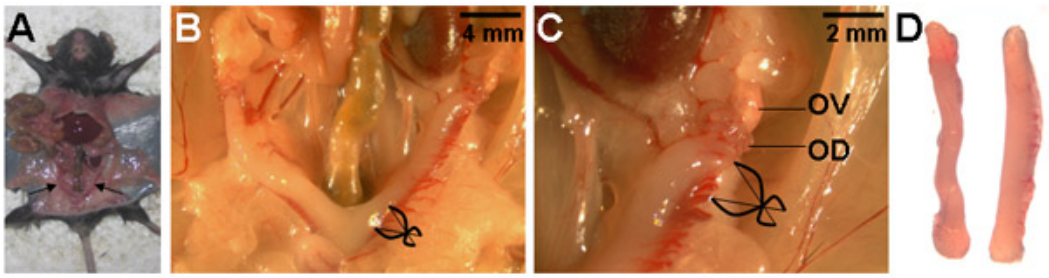

Figure 1. Dissection of mouse uteri. (A) Location of the uteri is indicated by arrows. (B) Cut across the junction of uterus and cervix as shown. (C) Cut the uterus just below the oviduct (OD) underneath the ovary (OV). (D) Image of the uteri.

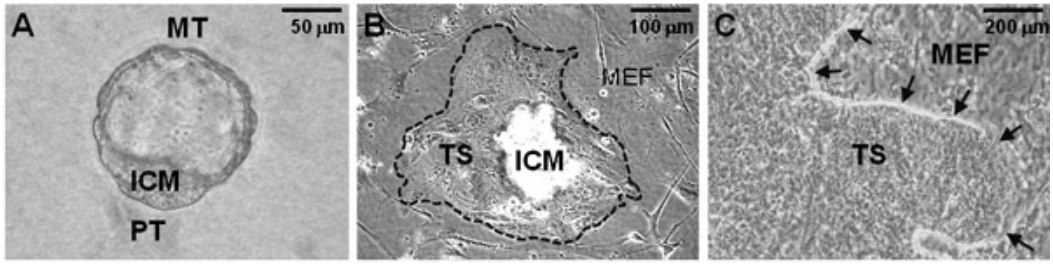

Figure 2. Representative pictures illustrate mouse blastocyst, blastocyst outgrowth and trophoblast colony. (A) A mouse blastocyst collected at E3.5 is shown. ICM, inner cell mass; MT, mural trophectoderm; PT, polar trophectoderm. (B) A trophoblast outgrowth on MEF feeders is shown. TS, trophoblast stem cell. (C) A trophoblast stem cell colony shows a clear edge on MEF feeders. Arrows indicate the cell boundary.

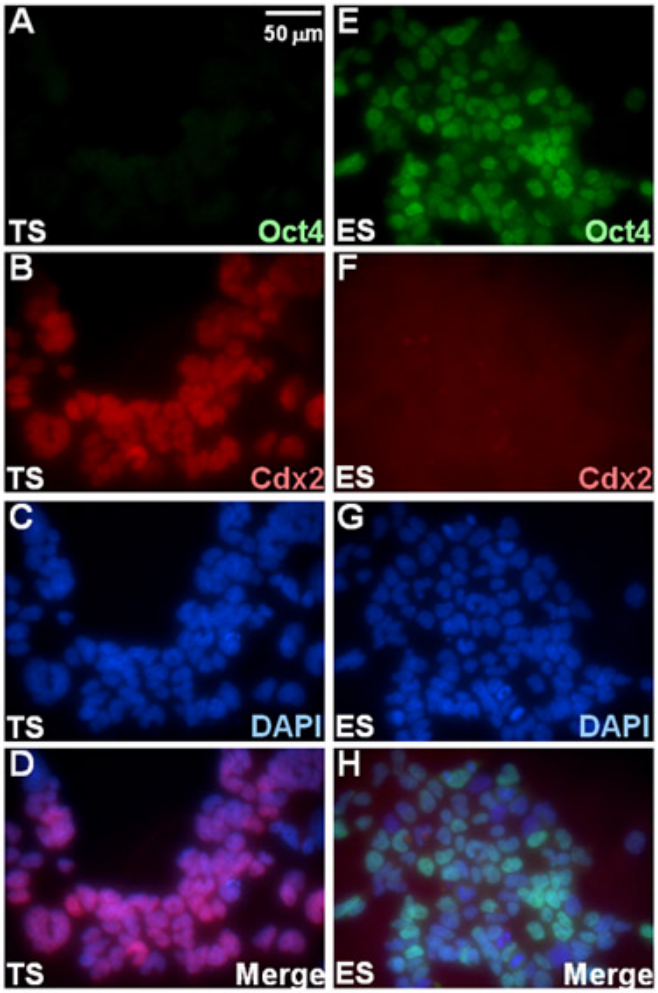

Figure 3. Identification of TS cells by immunostaining analysis. TS (A-D) and ES (control, E-H) cells are subject to marker identification. Cells are immunostained with antibodies to recognize Oct4 (A, E) or Cdx2 (B, F), and counterstained by DAPI (C, G) presented in merged images (D, H). Scale bar, 50 μm.

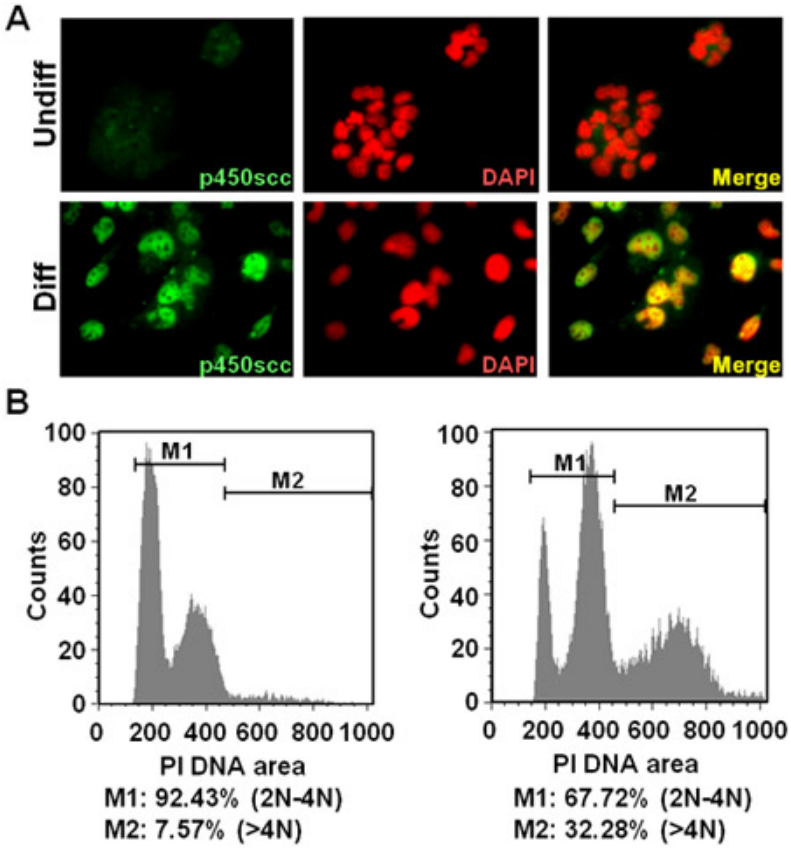

Figure 4. Differentiation of TS cells in vitro. (A) Undifferentiated (Undiff) or differentiated (Diff) cells in culture were examined for the expression of a TGC marker, p450scc (green), counterstained with DAPI (red). (B) Flow cytometric analysis of the differentiated cells, stained with PI, measures the DNA contents (M1, two to four copies; M2, more than four copies). The M2 population represents the polyploid trophoblast cells.

Discussion

In this video, we demonstrate the process to collect E3.5 blastocysts from uteri and experimental procedures to establish TS cell lines. We also describe the condition to maintain the stemness of TS cells and to induce their differentiation into differentiated cells. Two critical steps to obtain pure TS cell lines are the timing for outgrowth disaggregation (step 9) and processing the first passage (step 11). It has been reported that primitive endoderm-derived cells can arise in the culture after extensive blastocyst outgrow in step 9 or overgrowth of TS colonies (>50% confluency) in step 11 (Kunath et al., 2005). Although primitive endoderm-derived cells can be identified by their distinctive morphology with a rounded cell shape and reduced cell-cell contact compared to the TS cells, it is difficult to remove them from the culture because they grow well in the TS medium and do not seem to require MEF feeders or FGF4.

The differentiated TGCs may arise constantly in the TS culture even in the presence of MEF-CM and FGF4. If a pure TS population is desired, these differentiated cells may be separated on the basis of the differential adherent rates. Similar to MEFs, TGCs attach to culture plates faster than TS cells, providing a procedure possible to acquire a pure TS population as described in step 13. Alternatively, because TGCs are highly adherent and more resistant to trypsinization, a pure TS population also may be obtained by quick trypisinization (3 min), followed by collection of the suspended TS cells.

Disclosures

No conflicts of interest declared.

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