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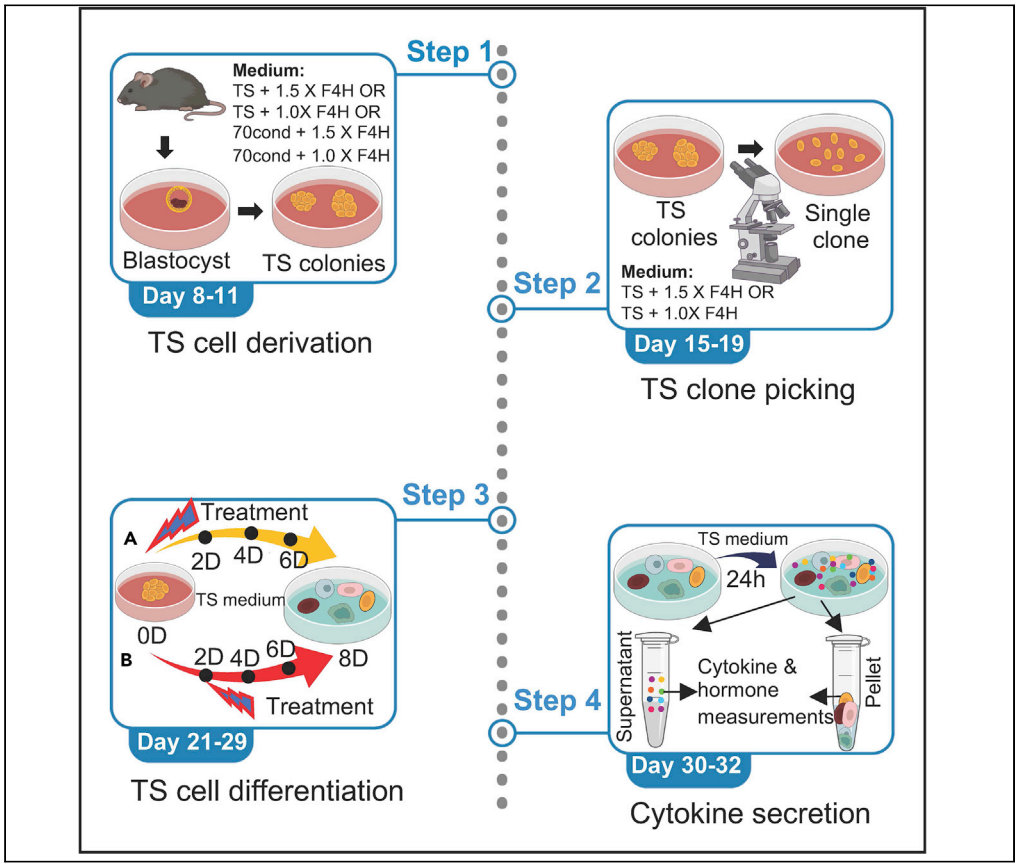
Protocol for mouse trophoblast stem cell isolation, differentiation, and cytokine detection



Trophoblast cells are the first differentiated cells formed from a fertilized egg during mammalian development, and they secrete several autocrine and paracrine factors essential for sustaining pregnancy. In pathological conditions, these cells secrete various proinflammatory cytokines affecting both maternal and fetal health. Here, we provide a detailed protocol for isolation, maintenance, differentiation, and detection of factors secreted from trophoblast stem (TS) cells. This protocol provides conditions for inducing genotoxic stress in differentiated TS cells and detecting the effects on cytokine production.

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HIGHLIGHTS

Describes mouse trophoblast stem cell isolation and differentiation protocol

Provides details for inducing genotoxic stress in differentiated TS cells

Provides methodology to detect factors secreted from differentiated TS cells

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Protocol for mouse trophoblast stem cell isolation, differentiation, and cytokine detection

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Summary

Trophoblast cells are the first differentiated cells formed from a fertilized egg during mammalian development, and they secrete several autocrine and paracrine factors essential for sustaining pregnancy. In pathological conditions, these cells secrete various proinflammatory cytokines affecting both maternal and fetal health. Here, we provide a detailed protocol for isolation, maintenance, differentiation, and detection of factors secreted from trophoblast stem (TS) cells. This protocol provides conditions for inducing genotoxic stress in differentiated TS cells and detecting the effects on cytokine production.

For complete details on the use and execution of this protocol, please refer to Singh et al. (2020).

Before you begin

General laboratory preparation

Timing: 4–6 h

1. All experiments were performed in a class II biosafety cabinet.
2. Set up humidified CO_2 incubator at 37°C.
3. Prepare all stock solutions.
4. Prepare all media.
5. Set water bath at 37°C or 56°C.

Mice preparation

Timing: 4–6 days

6. All mouse experimental protocols were performed as approved by the Institutional Animal Care and Use Committee (IACUC) of the Stowers Institute for Medical Research (Kansas City, MO).
7. C57BL/6J male and female mice of ~8–10 weeks of age were crossed and next morning plugs were checked, and the embryonic stage was considered 0.5 DPC. For blastocyst collection, female mice were culled at 3.5 DPC.

Mouse embryonic fibroblast (MEFs) preparation

Timing: 14–21 days

8. MEFs derived from 13.5 DPC stage embryos were used to make feeders (Millipore #pMEF-NL).
9. MEFs were mitotically inactivated with a 30 Gy dose of gamma irradiation using Gammacell 40 Exactor Irradiator.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti CDX2	Abcam	#ab76541
Goat anti-rabbit IgG Alexa Fluor 594	Thermo Fisher Scientific	#A11072
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde	Ted Pella	#18505
Tween 20	Sigma	#P9416
Goat serum	Vector Laboratories	#S-1000
DAPI	Vector Laboratories	#H-1200
BSA	Sigma	# A3311
Triton X-100	J.T. Baker	#X200-07
Crystal violet	Sigma	#V5265
ATM inhibitor KU55933	Sigma	#SML1109
ATR inhibitor VE821	Sigma	#SML1415
Caffeine	Sigma	#C0750
RPMI 1640	Fisher Scientific	#11875085
FBS	Hyclone	#SH3007103
Penicillin and streptomycin	Thermo Fisher Scientific	#15140122
Sodium pyruvate	Thermo Fisher Scientific	#11360070
β-Mercaptoethanol	Sigma	#M7522
GlutaMAX	Thermo Fisher Scientific	#35050061
FGF4	R&D System	#235-F4
Heparin	Sigma	#H3149
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	#25200114
DMEM/F12 medium	Invitrogen	#10565-018
TrypLE express enzyme	Thermo Fisher Scientific	# 12604021
β-Mercaptoethanol	Millipore	#ES-007-E
MEM non-essential amino acid solution	STEMCELL Technologies	#07600
TRIzol	Thermo Fisher Scientific	#15596026
Other		
Stripper tips for mouth pipets	ORIGIO	#MXL3-150
Standard tweezers straight	DUMONT	#0109-1-PO
Dissecting scissors, curved	VWR	#82027-584
30 mm dishes with cover slip	MatTek Corporation	#P35G-0-14-C
96-well clear TC-treated microplate	Corning	#3595
24-well clear TC-treated multiple well plates	Corning	#3526
6-well clear TC-treated multiple well plates	Corning	#3516
60 mm TC-treated culture dish	CELLSTAR	#628160
150 mm TC-treated culture dish	Corning	#430599
Cryogenic vials	Thermo Scientific	# 5000-0020

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Filter units, 0.45 μm PES, 150 mL	Thermo Fisher Scientific	#165-0045
Filter unit, 0.22 μm PES, 500 mL	Corning	# 431097
Cryogenic freezing container	Thermo Fisher Scientific	#5100-0001
RQ1 RNase-free DNase	Promega	#M6101
SuperScript III First-Strand Synthesis System	Thermo Fisher Scientific	#18080051
Phusion High-Fidelity PCR kit	New England BioLabs	#E0553S
Experimental models: cell lines		
Trophoblast stem cell (C57BL/6J genetic background)	This paper	N/A
Mouse embryonic fibroblast (MEFs)	Millipore	pMEF-NL
Oligonucleotides		
Pl1 (Prl3d1) -FP 5′ CGCTGCATTAA AAGGGACAT3′	This paper	N/A
Pl1 (Prl3d1) -RP 5′ AACTGAGGAGGG GAAAGCAT3′	This paper	N/A
Pl2 (Prl3b1) -FP 5′ AGGGTTTACCCA GGAGCTGT3′	This paper	N/A
Pl2 (Prl3b1) -RP5′GCACCGCCATAAG GTTCTAA3′	This paper	N/A
Plf (Prl2c2) -FP 5′ AGCCCCATGAGAT GCAATAC3′	This paper	N/A
Plf (Prl2c2) -RP 5′ CATCCAAAATCATGGCTCCT3′	This paper	N/A
18S-FP 5′GCTTAATTTGACTCAACACGGGA3′	(Singh et al., 2015)	N/A
18S-RP 5′AGCTATCAATCTGTCAATCCTGTC3′	(Singh et al., 2015)	N/A
Software and algorithms		
Fiji (ImageJ)	(Schneider et al., 2012)	https://imagej.net/Fiji
Code for quantification	This paper	https://github.com/jouyun
Deposited data		
Original data	This paper	http://www.stowers.org/research/publications/libpb-1581

Materials and equipment

TS medium		
Reagent	Final concentration	Amount
RPMI 1640 medium	n/a	500 mL
FBS	20%	130 mL
Penicillin and streptomycin (100×)	50 μg/mL	3.25 mL
Sodium pyruvate (100 mM)	1 mM	6.50 mL
β-Mercaptoethanol (20 mM)	0.1 mM	3.25 mL
GlutaMAX (200 mM)	2 mM	6.50 mL
ddH_2O	n/a	0.50 mL
Total	**n/a**	**650 mL**

⚠ CRITICAL: Filter sterilize with 0.22 μm filter. Store medium at 4°C and use within 1 month.

Note: Thaw FBS (500 mL) at 22°C–25°C (or 16–18 h at 4°C) and heat inactivate at 56°C for 30 min with constant slow shaking. Aliquot (40 mL) and store at −30°C.

β-Mercaptoethanol Stock		
Reagent	Final concentration	Amount
β-Mercaptoethanol	20 mM	14 μL
PBS (1×)	n/a	9986 μL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Filter sterilize with 0.22 μm filter. Store at 4°C.

BSA in PBS (0.1% W/V)		
Reagent	Final concentration	Amount
BSA	0.1%	10 mg
PBS (1×)	n/a	10 mL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Filter sterilize with 0.22 μm filter. Aliquot 1 mL and store at −80°C.

FGF4 stock solution (25 μg/mL)		
Reagent	Final concentration	Amount
FGF4	25 μg/mL	25 μg
BSA (0.1% in PBS)	n/a	1 mL
Total	**n/a**	**1 mL**

⚠ CRITICAL: Resuspend lyophilized FGF4 and store at −80°C in 100 μL aliquots.

Heparin Stock solution (1 mg/mL)		
Reagent	Final concentration	Amount
Heparin	1 mg/mL	10 mg
PBS (1×)	n/a	10 mL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Resuspend heparin in PBS and store at −80°C in 100 μL aliquots.

Feeder conditioned medium (feeder-CM)

- Seed 1 × 10^7 γ-irradiated MEFs in 20 mL TS medium for each 150 mm dish. The confluency should be around 90%.
- After 72 h collect the medium, spin at 200 × *g* using swing bucket rotor to remove dead cells and debris. Filter with a 0.45 μm filter and store at −20°C as feeder-CM.
- Use the same MEFs and add fresh 20 mL TS medium for each 150 mm dish. After 72 h collect the medium again to prepare more feeder-CM. Use these MEFs to collect conditioned medium for a maximum of 10 days.
- Thaw feeder-CM as needed; once thawed store at 4°C and use within 1 week.

70cond medium		
Reagent	Final concentration	Amount
TS medium	n/a	3 mL
Feeder-CM	n/a	7 mL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Store at 4°C and use within 1 week.

70cond + F4H medium		
Reagent	Final concentration	Amount
70cond medium	n/a	10 mL
FGF4 stock solution	25 ng/mL	10 μL
Heparin stock solution	1 μg/mL	10 μL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Filter with 0.45 μm filter. Store at 4°C and use within 1 week.

Note: To prepare 1.5× F4H use, 15 μL each of FGF4 and Heparin.

TS + F4H medium (1×)		
Reagent	Final concentration	Amount
TS Medium	n/a	10 mL
FGF4 stock solution	25 ng/mL	10 μL
Heparin stock solution	1 μg/mL	10 μL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Filter with 0.45 μm filter. Store at 4°C and use within 1 week.

Note: To prepare 1.5× F4H use 15 μL each of FGF4 and Heparin.

Freezing mix (1×)		
Reagent	Final concentration	Amount
FBS	90%	9 mL
DMSO	10%	1 mL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Filter with 0.22 mm filter and store at 4°C and use within 1 week.

PBSTB (1×)		
Reagent	Final concentration	Amount
PBS (10×)	1×	1 mL
Tween-20 (10%)	0.05%	50 μL
Goat serum	5%	500 μL
ddH_2O	n/a	8,450 μL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Store at 4°C and use within 1 week.

Permeabilization buffer		
Reagent	Final concentration	Amount
PBS (10×)	1×	1 mL
TritonX-100 (10%)	0.5%	500 μL
ddH_2O	n/a	8,500 μL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Store at 4°C and use within 1 month.

Fixative solution		
Reagent	Final concentration	Amount
PBS (10×)	1×	1 mL
Paraformaldehyde (16%)	4.0%	2.5 mL
ddH_2O	n/a	6.5 mL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Store at 4°C and use within 1 week.

0.01% crystal violet solution		
Reagent	Final concentration	Amount
Crystal violet (0.1%)	0.01%	1 mL
ddH_2O	n/a	9 mL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Store at 22°C–25°C and use within 1 week.

DNase treatment of RNA		
Reagent	Final concentration	Amount
Isolated RNA	100 ng/μL	1–8 μL
RNase -Free DNase	0.1 U/μL	1 μL
10× buffer	1×	1 μL
ddH_2O	n/a	0–7 μL
10× Stop solution	1×	1 μL
Total	**n/a**	**11 μL**

⚠ CRITICAL: 1× DNase buffer contains 10 mM $MgSO_4$. So, in cDNA synthesis mix additional Mg^{2+} is not required.

cDNA synthesis mix		
Reagent	Final concentration	Amount
DNase treated RNA	50 ng/μL	11 μL
Oligo (dT)	2.5 μM	1 μL
dNTPs	0.5 mM	1 μL
10X buffer	1×	2 μL
DTT	10 mM	2 μL
Reverse transcriptase	10 U/μL	1 μL
RNase inhibitor	2 U/μL	1 μL
ddH_2O	n/a	1 μL
RNase H	0.1 U/μL	1 μL
Total	**n/a**	**21 μL**

PCR mix		
Reagent	Final concentration	Amount
Phusion DNA polymerase	0.02 U/μL	0.25 μL
Phusion HF buffer	1×	5 μL
Primers (Forward & Reverse)	0.5 μM	1.25 μL
dNTPs	200 μM	1 μL
cDNA mix	n/a	1 μL
ddH_2O	n/a	16.50 μL
Total	**n/a**	**25 μL**

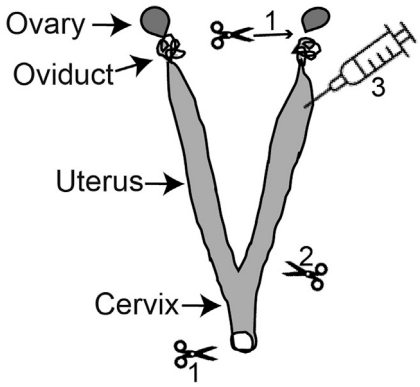

Figure 1. Flushing blastocysts from the uterus

(1) The uterus from a 3.5 DPC pregnant mouse is removed by cutting across the cervix and in between each ovary and oviduct. (2) The uterus is cut near the cervix to separate both horns. (3) The needle is inserted into the upper part of uterus to flush blastocysts toward the cervix and collect blastocysts in a 30 mm Petri dish.

Step-by-step method details

TS cell derivation from blastocyst

Timing: 8–11 days

TS cell derivation protocol was performed as described by Himeno et al. (2008) with some modifications.

1. Day before blastocyst collection, prepare 4- or 24-well plates with γ-irradiated MEFs by seeding 5×10^4 cells/ well in 0.5 mL TS medium.
2. On day 0 collect 3.5 DPC blastocysts from uterus by flushing under sterile conditions (Figure 1).
   a. Open abdominal cavity and take out uterus
      i. Cut across cervix using fine scissors.
      ii. Hold cervix with forceps and separate uterine horns by cutting mesometrium.
      iii. Cut between oviduct and ovaries. Keep uterotubal junction intact.
      iv. Keep uterus in small volume (0.2 mL) of TS medium in a 60 mm Petri dish.
   b. Insert a 26-gauge needle in upper part of uterus near oviduct and flush each horn with 1–2 mL of TS + F4H medium toward cervix.
   c. Collect medium with blastocysts in a 30 mm Petri dish.
3. Change medium of 4- or 24-well plates with feeder cells (from step 1) using 0.5 mL of TS + F4H medium.
4. Using a mouth pipet, transfer each blastocyst into a single well of a 4- or 24-well plate containing TS + F4H medium and incubate at 37°C and 5% CO_2.

   Note: Try to keep the blastocyst in the center of each well to monitor outgrowth properly.

5. On day 1 monitor the blastocyst for attachment and hatching. Do not change the medium.
6. On day 2 monitor the embryo for outgrowth and carefully remove medium and add fresh 0.5 mL TS + F4H medium.

   Note: If the outgrowth is loosely attached, remove only half of the medium and add 0.25 mL fresh TS + F4H medium.

7. On day 3 to 5 monitor outgrowth for size and once it is around 0.8 mm, disaggregate it in the same well.
   a. Wash the cells two times with 0.5 mL PBS.
   b. Aspirate the PBS and add 100 μL of 0.25% Trypsin.

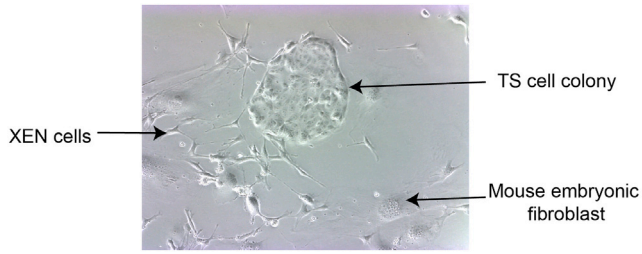

Figure 2. Colony morphology can be used to select TS cells in culture

A small TS cell colony growing with extraembryonic endoderm cells (XEN cells) and MEFs is shown. A TS cell colony grows with a well-defined border. To obtain a pure TS cell population, clones must be picked and separated from XEN cells.

c. Incubate for 5 min at 37°C with 5% CO_2.
d. Thoroughly disaggregate the outgrowth with P200 pipet by mixing up and down.

Note: Set pipet at 100 μL to avoid generation of air bubbles.

e. Stop trypsin action by immediate addition of 0.5 mL 70cond + 1.5× F4H medium.
f. Culture cells at 37°C and 5% CO_2 for 16 h and replace medium with fresh 70cond + F4H medium.

8. Hereafter change the 70cond + F4H medium every other day and monitor closely for colony formation.

⚠CRITICAL: Tight epithelial TS cell colonies will appear 3 - 7 days after step 8 is completed. Embryonic stem cells are very rare in this medium, but appearance of extraembryonic endoderm (XEN cells) is very common (Figure 2). After some passage it will be difficult to separate TS cells from XEN cells. So, we opted to pick colonies of TS cells to ensure pure TS cell lines.

TS cell colony picking

Timing: 4–8 days

TS cell colony picking helps to establish a pure TS cell population quickly.

9. Once TS cell colonies start appearing, prepare 4- or 24-well plates with γ-irradiated MEFs by seeding 5 × 10^4 cells/ well in 0.5 mL TS medium 1 day prior to colony picking.
10. On the day of TS cell colony picking, change the medium of feeders with 0.5 mL TS + 1.5× F4H medium and incubate at 37°C, 5% CO_2.
11. Move the dissecting microscope into a tissue culture hood.
12. Aspirate the medium from the plate containing TS cell colonies and wash with PBS two times. Add 0.5 mL PBS to the cells and do not remove this PBS.
13. Using the dissecting microscope pick TS cell colonies with the help of a P20 pipette (White tips and set pipette at 20 μL) and transfer each picked colony along with PBS separately in a single well of a 96 well plate.

Note: 20 μL white tips have a wide opening which facilitates picking colonies.

14. Once all colonies are picked, add 50 μL of 0.25% trypsin in each well and incubate at 37°C, 5% CO_2 for 5 min.

Note: Pick 5 to 10 colonies from each blastocyst.

⚠ CRITICAL: To avoid temperature shock, pick colonies within 10 min and if picking colonies from multiple plates do one at a time.

15. Add 150 μL of TS + 1.5× F4H medium to stop trypsin action and dissociate cells by pipetting up and down in each well.
16. Transfer each clone of the 96 well plate separately to 4- or 24-well plates prepared in step 9.
17. Incubate at 37°C with 5% CO_2 for 16 h and after incubation add fresh 0.5 mL TS + 1.5× F4H medium.
18. After 2 days, change medium every other day with 0.5 mL of fresh TS + 1× F4H medium until colonies reappear.

⚠ CRITICAL: TS cell colonies should appear in 3–7 days after picking.

Passaging and removing MEFs from TS cells

Timing: 15–20 days

TS cells can be maintained >50 passages in appropriate medium conditions without any chromosomal abnormalities and for many experiments we need TS cells to grow in feeder free condition.

19. Once TS cell culture appears 50%–60% confluent, passage it.
    a. Prepare 6-well plates with γ-irradiated MEFs by seeding 2 × 10^5 cells/ well in 2.0 mL TS medium 1 day prior to passaging TS cells and on day of passaging remove TS medium and add 1.5 mL TS + 1.5× F4H medium.
    b. Remove medium and wash the TS cells (from step 18) once with 0.5 mL PBS.
    c. Aspirate the PBS and add 100 μL of 0.25% Trypsin.
    d. Incubate for 5 min at 37°C with 5% CO_2.
    e. Stop trypsin action by immediate addition of 0.5 mL TS + 1.5× F4H medium and dissociate to single cells using 1.0 mL pipette (set pipette at 400 μL).
    f. Transfer all cells to 6- well plate containing γ-irradiated MEFs (step 19, a) and culture cells at 37°C, 5% CO_2 for 16 h.
    g. After 16 h add fresh 2.0 mL TS + 1.0× F4H medium to each well.
20. Keep changing the medium (TS + 1.0× F4H) every other day until TS cells become 60%–80% confluent.
21. Passage each clone 2 to 3 times (1:6 ratio) as mentioned in steps 19, 20 to obtain stable TS cell clones.

Note: Once stable clones are established on γ-irradiated MEFs, a portion of the TS cells can be frozen, and the rest can be maintained in feeder free condition.

22. For feeder free conditions, grow and passage TS cells in 70cond +F4H medium.
    a. Remove medium from 6-well plate and wash the TS cells (from step 21) once with 2.5 mL PBS.
    b. Aspirate the PBS and add 500 μL of 0.25% Trypsin.
    c. Incubate for 5 min at 37°C with 5% CO_2.
    d. Stop trypsin action by immediate addition of 2.5 mL TS medium and disaggregate cells by pipetting up and down.
    e. Transfer cell suspension to the 15 mL tube and centrifuge 5 min at 200 × *g*.
    f. Discard the supernatant and resuspend cells in 10 mL 70cond +F4H medium.
    g. Seed cells in 100 mm dish and incubate at 37°C with 5% CO_2 for 45 min to attach MEFs.

    Note: MEFs attach faster as compared to TS cells.
    h. Collect floating TS cells from 100 mm dish, count the cells and seed 1 × 10^5 cells in 2 mL 70cond +F4H medium in a single well of a tissue culture grade 6-well plate.

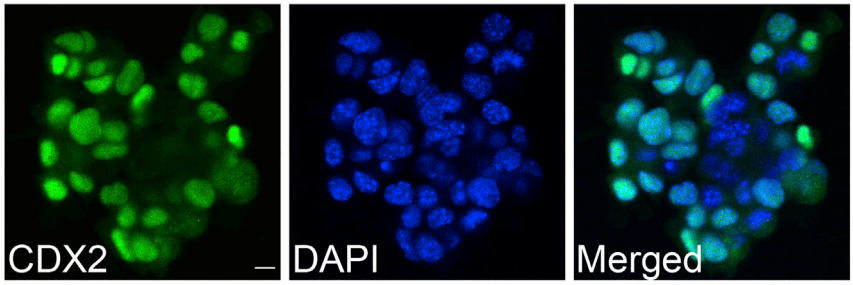

Figure 3. Immunofluorescence can be used to confirm TS cell identity

CDX2 is a marker for trophoblast stem (TS) cells which should be observed in at least 90% of cells. Isolated TS cell clones were grown without MEFs in a 30 mm dish (on glass cover slip) in 70cond + F4H medium for 48 h. Cells were fixed in 4% paraformaldehyde and immunofluorescence was performed using anti-CDX2 antibody. DAPI was used to stain DNA. Scale bar, 10 μm.

i. Passage cells 2 to 3 times to obtain a pure TS cell population.

Note: Once stable clones are established with typical TS cell colony morphology as indicated in Figure 2, in feeder free conditions, TS cells can be frozen. TS cell identity was validated with the TS cell specific marker CDX2 using immunofluorescence (Figure 3).

Freezing and thawing of TS cells

Timing: 1–4 days

Stable TS cell clones can be safely frozen and thawed for subsequent experiments.

23. For freezing cells:
    a. Prepare 1× freezing mix and keep on ice.
    b. Harvest TS cells from 60%–80% confluent culture (either from step 19 or 22) by trypsinization and pellet cells by centrifugation.
    c. Discard the supernatant and resuspend cells in 5 mL TS medium.
    d. Count the cells and pellet again by centrifugation.
    e. Discard the supernatant (leave 100 μL) and resuspend the pellet by tapping.
    f. Slowly add 1× freezing mix to the cells (2×10^5 cells per 1 mL freezing mix).
    g. Mix gently and aliquot 0.5 mL of the cell suspension per cryovial.
    h. Transfer tubes to cryogenic freezing container and place in −80°C freezer for 16–18 h.
    i. Transfer cryotubes to a liquid nitrogen container for long term storage.

Note: One vial is sufficient for a 30 mm dish (1×10^5 cells).

24. Thawing of cells:
    a. On the day before thawing TS cells, seed 2×10^5 γ-irradiated MEFs in a 30 mm dish with 2 mL TS medium.
    b. Quickly thaw a frozen vial of TS cells in a 37°C water bath.
    c. Transfer thawed TS cells to 10 mL of TS medium and centrifuge at 200 × *g* for 5 min.
    d. Discard the supernatant and resuspend the pellet in 2 mL TS + 1× F4H medium.
    e. Transfer cells on γ-irradiated MEFs prepared in step 24a and incubate at 37°C, 5% CO_2.
    f. Change the medium the next day and keep changing it every other day with TS + 1× F4H medium until confluency reaches 60%–80%.
    g. Make feeder free as mentioned in step 22 for subsequent experiments.

Note: A frozen stock of TS cells should be thawed onto MEF feeders to avoid differentiation. Alternatively, TS cells can be thawed in 70cond + 1.5 × F4H medium without feeders but expect some differentiation in the first passage after thawing.

Effect of genotoxic stress on TS cell differentiation

Timing: 3–10 days

The Fibroblast growth factor 4 (FGF4) signaling pathway is the main signaling pathway required for TS cell proliferation and self-renewal (Tanaka et al., 1998). In the presence of FGF4, heparin and MEFs, TS cells can be cultured in an undifferentiated state. After removal of FGF4 and heparin, TS cells can be differentiated to various placental types such as trophoblast giant cells (TGCs), spongiotrophoblasts (SpTs) and syncytiotrophoblasts (SyTs) (Natale et al., 2009; Ullah et al., 2008). This is a very useful tool to study trophoblast differentiation, genome maintenance, and factors secreted from these cells.

25. Harvest feeder free TS cells from 60%–80% confluent culture by trypsinization and count the cells.
26. Seed 1 × 10^5 cells in 10 mL of 70cond + F4H medium for 100 mm dish (1 × 10^4 cells for 30 mm dish).
27. After 24 h remove the medium and wash TS cells twice with 10 mL PBS.
28. Add 10 mL TS medium for differentiation.
29. Perform various treatments either just after addition of TS medium (Graphical abstract step 3-A) or after two days of differentiation (Graphical abstract step 3-B) and incubate cells at 37°C, 5% CO_2.
    a. DNA damage induction:
        i. For inducing DNA damage, seal the plates with parafilm and place the plate inside γ-irradiator and give various doses such as 0 Gy, 0.5 Gy, 10 Gy, or 30 Gy.
        ii. Change TS medium every other day.
    b. DNA damage response inhibition:
        i. For inhibiting the DNA damage response and downstream signaling pathway, add a DNA damage response inhibitor such as caffeine, ATM inhibitor KU55933, or ATR inhibitor VE821.
        ii. Change TS medium with inhibitors every other day.
    c. Keep one plate as a control without any treatment and keep changing TS medium every other day.
30. Collect cells every 2 days after treatment until the 8th day of differentiation, either by disaggregating with TrypLE for RNA/protein analysis or by fixing in 4% paraformaldehyde for immunofluorescence and colony formation assay.
31. For disaggregating differentiated TS cells, wash the cells with PBS and add 1 mL of TrypLE express enzyme (1×) in a 100 mm dish and incubate for 10–20 min at 37°C, followed by inactivation with 10 mL TS medium.
32. Disaggregate cells by pipetting up and down.
33. Pellet cells by centrifugation at 200 × *g* for 5 min and discard the supernatant.
34. Wash cells with PBS twice to remove any remaining medium. Store the pellet at −80°C for further analysis.

Note: Once TS cells are differentiated, it is difficult to disaggregate them with trypsin. Induction of DNA damage can be monitored by γH2A.X immunofluorescence and western blot analysis.

⚠ CRITICAL: Inducing DNA damage or inhibiting the DNA damage response just prior to TS cell differentiation causes cell death and affects the differentiation process (Figure 4). Inducing DNA damage or inhibiting the DNA damage response two days after initiating TS cell differentiation induces resistance to DNA damage (Figure 4).

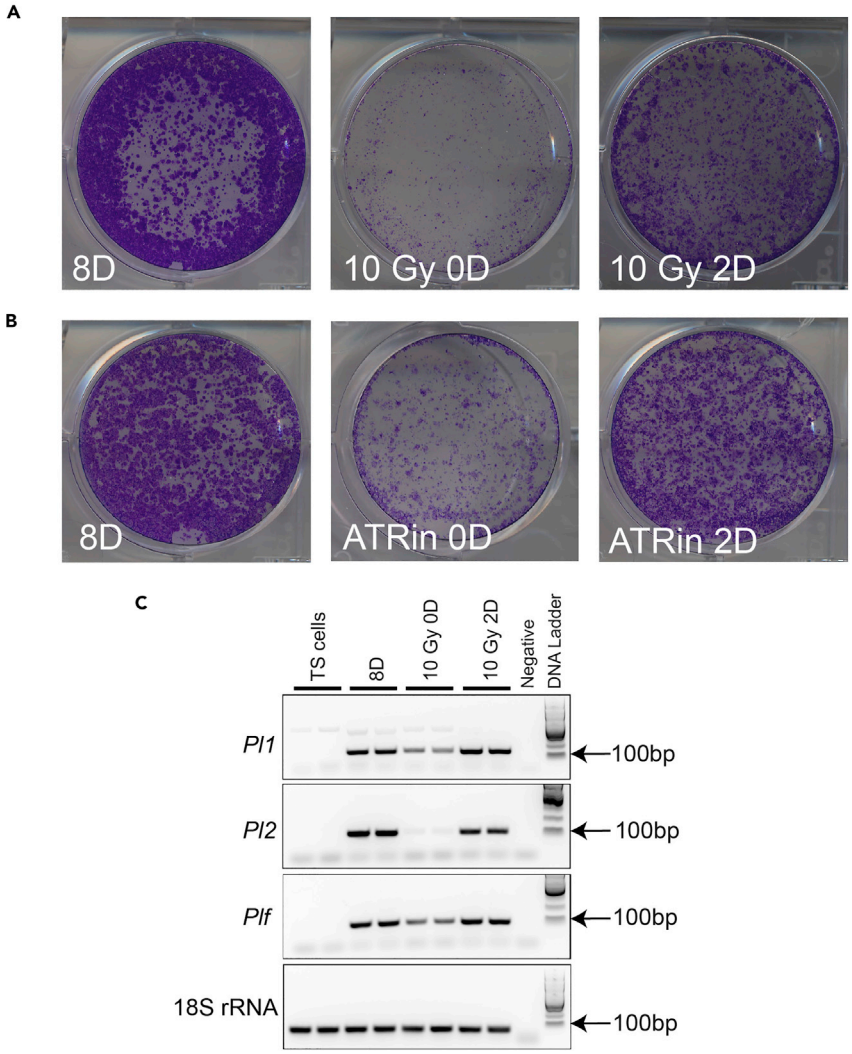

Figure 4. Various treatments can impact TS cell differentiation

To analyze the effect of DNA damage and DNA damage checkpoint inhibition on the differentiation of TS cells, colony formation assays along with cell type specific markers were analyzed.

(A and B) (A) DNA damage induction with 10 Gy γ-irradiation and (B) inhibition of the DNA damage response with an ATR inhibitor (ATRin) were performed either just after addition of differentiation TS medium (0D) or 2 days after differentiation (2D). After 8 days of total differentiation, cells were fixed in 4% paraformaldehyde for 10 min and stained with 0.01% crystal violet for 60 min, washed with water, dried and imaged (the same image is included in the main manuscript as Figure S3E, Singh et al., 2020). Treatment just after addition of differentiation TS medium (0D) resulted in reduced growth.

(C) Expression of TS cell differentiation markers such as *Pl1 (Prl3d1)*, *Pl2 (Prl3b1)*, and *Plf (Prl2c2)* was measured with 18S rRNA acting as a positive control. TS cells irradiated with 10 Gy either just after addition of differentiation TS medium (0D) or 2 days after differentiation (2D) were allowed to differentiate for a total of 8 days. Cells were lysed in TRizol to isolate RNA. RNA was DNase treated to digest any residual genomic DNA and reverse transcription was performed. PCR was performed using specific primers along with a negative "no template" control. PCR products were separated on a 2% agarose gel along with a 100 bp DNA ladder. Treatment just after addition of differentiation TS medium (0D) showed reduced levels of differentiation markers such as *Pl1*, *Pl2*, and *Plf*.

TS and differentiated TS cell marker analysis

Timing: 3–10 days

TS cell specific markers such as *Cdx2*, *Eomes*, and differentiation markers such *Pl1*, *Pl2*, *Tpbpa* are used to validate TS cell identity. Immunofluorescence is used to validate markers at the protein level and reverse transcription (RT) PCR is used to validate markers at the level of RNA expression.

35. For immunofluorescence, as mentioned in step 22 or 25 to 30, grow the cells in a 30 mm dish with bottom cover slip (#P35G-0-14-C) and fix for 10 min with 2 mL 4% paraformaldehyde at 22°C–25°C.
36. Wash cells three times with 2 mL PBS.
37. Add 2 mL permeabilization buffer to the dish for 10 min to permeabilize cells.
38. Wash cells three times with 2 mL PBS.
39. Add 2 mL 1× PBSTB blocking solution and incubate at 22°C–25°C for 1 h.
40. Dilute the primary antibody (such as CDX2, 1:500) in 1× PBSTB and add to the cells.
41. Incubate primary antibody 16–18 h at 4°C.
42. Next morning remove the primary antibody and wash the cells with 2 mL 1× PBSTB three times each for 10 min.
43. Dilute the secondary antibody (such as anti-rabbit 1:500) in 1× PBSTB and add to the cells.
44. Incubate the cells with the secondary antibody for 1 h at 22°C–25°C in the dark and wash the cells with 2 mL 1× PBSTB three times each for 10 min.
45. Mount the cells with a coverslip using DAPI.
46. Record images using a LSM780 confocal microscope (Zeiss) within 1 week and collect Z-stacks.
47. Process images using Fiji software (NIH; https://imagej.net/Fiji).
48. For RT-PCR, lyse the cells from step 34 using TRIzol and isolate RNA.
49. Use 1 μg of total RNA and digest with RQ1 RNase-free DNase (1 U) for 20 min at 37°C in 1× RQ1 RNase-free DNase reaction buffer.
50. Stop DNase action by adding RQ1 RNase-free DNase stop solution (Final concentration 1×) and incubate for 10 min at 65°C.
51. To the DNase digested RNA sample add 1 μL each of oligo (dT) and dNTPs and incubate for 5 min at 65°C.
52. Keep RNA sample on ice for 2 min and add cDNA synthesis mix which contains buffer, reverse transcriptase, RNase inhibitor, and DTT.
53. Incubate samples for 60 min at 50°C and inactivate enzymes for 5 min at 85°C.
54. Add 1 μL RNase H and incubate for 20 min at 37°C.
55. Take 1 μL of cDNA and perform PCR with TS cell marker primers such as *Pl1*, *Pl2*, *Plf*, and 18S rRNA.

Detection of cytokines secreted from differentiated TS cells

Timing: 3–10 days

Placental cells express various autocrine and paracrine factors. Differentiated TS cells are a good model to study the effect of genotoxic stress or genetic mutations on factors secreted by these cells.

56. To detect factors secreted by differentiated TS cells in different conditions, grow cells in a 30 mm dish as mentioned in steps 22 or 25 to 30.
57. Wash TS cells as well as differentiated TS cells with PBS and add fresh 1.2 mL TS medium.
58. Incubate the cells for 24 h at 37°C, 5% CO_2 and collect the supernatant.
59. Centrifuge the supernatant at 200 × *g* for 5 min.
60. Transfer the supernatant to a fresh tube and discard the pellet consisting of floating cells.
61. Store the supernatant at −80°C for further analysis such as measuring secreted cytokines, hormones, and other enzymes.
62. Add 500 μL of TrypLE express enzyme and incubate at 37°C for 10–20 min to disaggregate attached cells from the 30 mm dish (step 58).

63. Inactivate TrypLE by adding 2 mL of TS medium and pipette up and down to disaggregate all cells.
64. Transfer the cells to an Eppendorf tube and centrifuge at 200 × *g* for 5 min.
65. Wash the pellet two times with PBS and store the pellet at −80°C for further analysis such as measuring non-secreted cytokines and enzymes.

Note: Gamma irradiated mouse embryonic fibroblast cells also secrete several autocrine and paracrine factors. Therefore, we should not measure cytokines using feeder-CM or TS cells growing with MEF feeders. Hence it is possible that growing TS cells in TS medium for 24 h causes some degree of differentiation.

Expected outcomes

Isolation and derivation of TS cells from blastocysts in the presence of FGF4 was established in 1998 by Tanaka et. al. (Tanaka et al., 1998) and many further studies showed successful isolation of TS cells from embryonic stem cells by CDX2 overexpression (Niwa et al., 2000; Niwa et al., 2005). Here we established a differentiation protocol accompanied by various treatments such as inducing DNA damage or inhibiting the DNA damage response. We show that inducing DNA damage (Figure 4) or inhibiting the DNA damage response (Figure 4) immediately following TS cell differentiation causes cell death as evident from a colony formation assay. Once TS cells were allowed to differentiate for two days, inducing DNA damage or inhibiting the DNA damage response had a milder effect on cell death.

Limitations

In our cytokine detection protocol TS cell cannot be maintained without either γ-irradiated MEFs or feeder-CM, making it difficult to measure factors secreted by TS cells. As suggested by Erlebacher et al., TS cells can be maintained with TGFβ or activin in the absence of γ-irradiated MEFs or feeder-CM (Erlebacher et al., 2004). Alternatively, TS cells can be maintained with TGFβ or activin along with FGF4 for factors secreted by TS cells only.

In our protocol we allowed TS cells to differentiate for two days either before inducing DNA damage by γ-irradiation or before inhibiting the DNA damage response. It is possible that other kinds of treatments may differentially affect the growth of specific cell types and would require tracking of specific cell types throughout differentiation.

Troubleshooting

Problem 1

Spontaneous differentiation of TS cells.

Potential solution

Most blastocysts post-outgrowth and disaggregation form TS cell colonies. But after picking a colony, differentiation of TS cells is very common. Picking most of the colonies generated from a single blastocyst increases the efficiency of TS cell derivation.

Low efficiency in the derivation of TS cell lines and spontaneous differentiation during maintenance in feeder free condition is sometimes caused by low quality γ-irradiated MEFs and feeder-CM generated from these MEFs. Check each batch of γ-irradiated MEFs with established TS cell lines.

Thawing TS cells works best with γ-irradiated MEFs with TS + F4H medium to inhibit spontaneous differentiation. But if you are thawing in 70cond + 1.5× F4H medium, expect some differentiation of TS cells to TGCs in the first passage after thawing. TGCs are hard to disaggregate with 0.25% trypsin within 5 min, a property that can be used to enrich the TS cell population in subsequent passages.

Problem 2
Extraembryonic endoderm (XEN) cell contamination.

Potential solution
The appearance of XEN cells is very common during TS cell derivation. After disaggregation of the outgrowth, watch for the appearance of TS cell colonies and do not allow the culture to overgrow, otherwise it will be difficult to separate XEN cells from TS cells. Pick TS cell colonies carefully to avoid contamination with other cell types.

Problem 3
Disaggregation of differentiated TS cells without lysis.

Potential solution
Differentiated cells are hard to disaggregate with 0.25% trypsin in 5 min at 37°C and increased time causes lysis of some cells. TrypLE express enzyme can be used to disaggregate differentiated cells with minimum cell lysis.

Problem 4
Detection of less abundant cytokines.

Potential solution
Feeder-CM generated from γ-irradiated MEFs should not be used in the medium for assessing cytokine secretion because MEFs also secrete many cytokines. Some cytokines are secreted in very low amounts, so keep the volume of TS medium used for cell culturing as low as possible when cytokine detection is the goal. Alternatively, TS medium can be concentrated using a centricon device prior to downstream analysis such as ELISA, cytokine array etc. Furthermore, mass spectrometry can be used to identify secreted factors in an unbiased and high throughput manner (Napso et al., 2020).

Resource availability
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by lead contact Vijay Pratap Singh (vps@stowers.org).

Materials availability
Newly generated trophoblast cell lines are available but require an MTA for distribution.

Data and code availability
Original data underlying this manuscript can be accessed from the Stowers Original Data Repository at http://www.stowers.org/research/publications/libpb-1581. Codes used for quantification in this study are available at https://github.com/jouyun.

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Author contributions
V.P.S. and J.L.G. conceived the study, designed the experiments, and wrote the manuscript. V.P.S. performed the experiments.

Declaration of interests

The authors declare no competing interests.

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