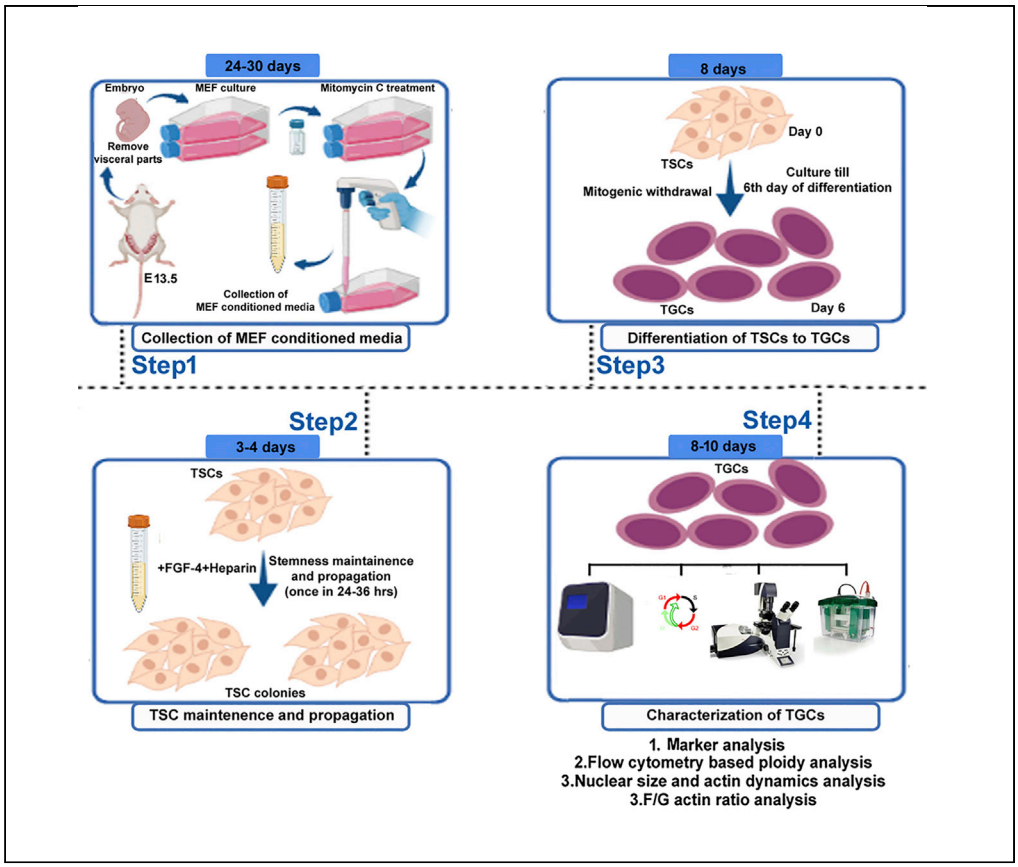
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for functional characterization of endoreduplicated murine trophoblast cells



Murine trophoblast stem cells (TSCs) have shaped placental research by providing resources for investigating trophoblast subtype specialization. Trophoblast giant cells (TGCs) are large polyploid cells, which undergo repetitive rounds of DNA replication without intervening mitosis by a process called endoreduplication. Endocrine and paracrine functions of TGCs aid in maternal adaptations to pregnancy. Here, we describe a protocol for *in vitro* differentiation of murine TSCs to TGCs together with the genotypic as well as phenotypic characterization of the endoreduplicated TGCs.

Trishita Basak, Madhurima Paul, Rupasri Ain

rupasri@iicb.res.in

Highlights

Protocol to harvest mouse embryonic fibroblasts and culture of TSCs

Protocol for differentiation of TSC to endoreduplicated TGCs and characterization

qRT-PCR for genetic marker and flow cytometry for ploidy analysis of TGCs

Hoechst/Phalloidin staining for nuclear size analysis and actin polymerization assay

Basak et al., STAR Protocols 3, 101573
September 16, 2022
https://doi.org/10.1016/j.xpro.2022.101573

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for functional characterization of endoreduplicated murine trophoblast cells

Trishita Basak,[1,2] Madhurima Paul,[1,2] and Rupasri Ain[1,3,*]

[1]Division of Cell Biology and Physiology, CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Kolkata 700032, India

[2]Technical contact

[3]Lead contact

*Correspondence: rupasri@iicb.res.in
https://doi.org/10.1016/j.xpro.2022.101573

SUMMARY

Murine trophoblast stem cells (TSCs) have shaped placental research by providing resources for investigating trophoblast subtype specialization. Trophoblast giant cells (TGCs) are large polyploid cells, which undergo repetitive rounds of DNA replication without intervening mitosis by a process called endoreduplication. Endocrine and paracrine functions of TGCs aid in maternal adaptations to pregnancy. Here, we describe a protocol for *in vitro* differentiation of murine TSCs to TGCs together with the genotypic as well as phenotypic characterization of the endoreduplicated TGCs.

For complete details on the use and execution of this protocol, please refer to Basak and Ain (2022).

BEFORE YOU BEGIN

Timing: 24–30 days

Institutional permissions

This protocol requires institutional permission for animal handling. For this protocol, mice handling and experimentation has been approved by IICB Animal Ethics and Care Committee as per guidelines set forward by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (http://cpcsea.nic.in).

Harvesting mouse embryonic fibroblast and mitotic inactivation

TSCs require certain secreted soluble factors in order to sustain indefinitely in an undifferentiated state. Preconditioned media from mitotically inactivated MEFs helps to maintain the stemness of TSCs by providing these soluble factors along with many other unknown components. The protocol for isolation and culture of mouse embryonic fibroblasts has been modified from a previous protocol (Quinn et al., 2006).

Set-up of mice mating for isolation of embryonic tissue

1. Cage sexually mature CD1 females overnight with fertile males.
2. Assign 0.5 day of pregnancy by the presence of a vaginal copulatory plug.
3. Euthanize the pregnant mice on embryonic day 13.5 (E13.5) (Figure 1A).

Derivation of mouse embryonic fibroblast from E13.5 embryos

4. Place the animal belly up in a sterile tissue culture hood on an absorbent paper towel.

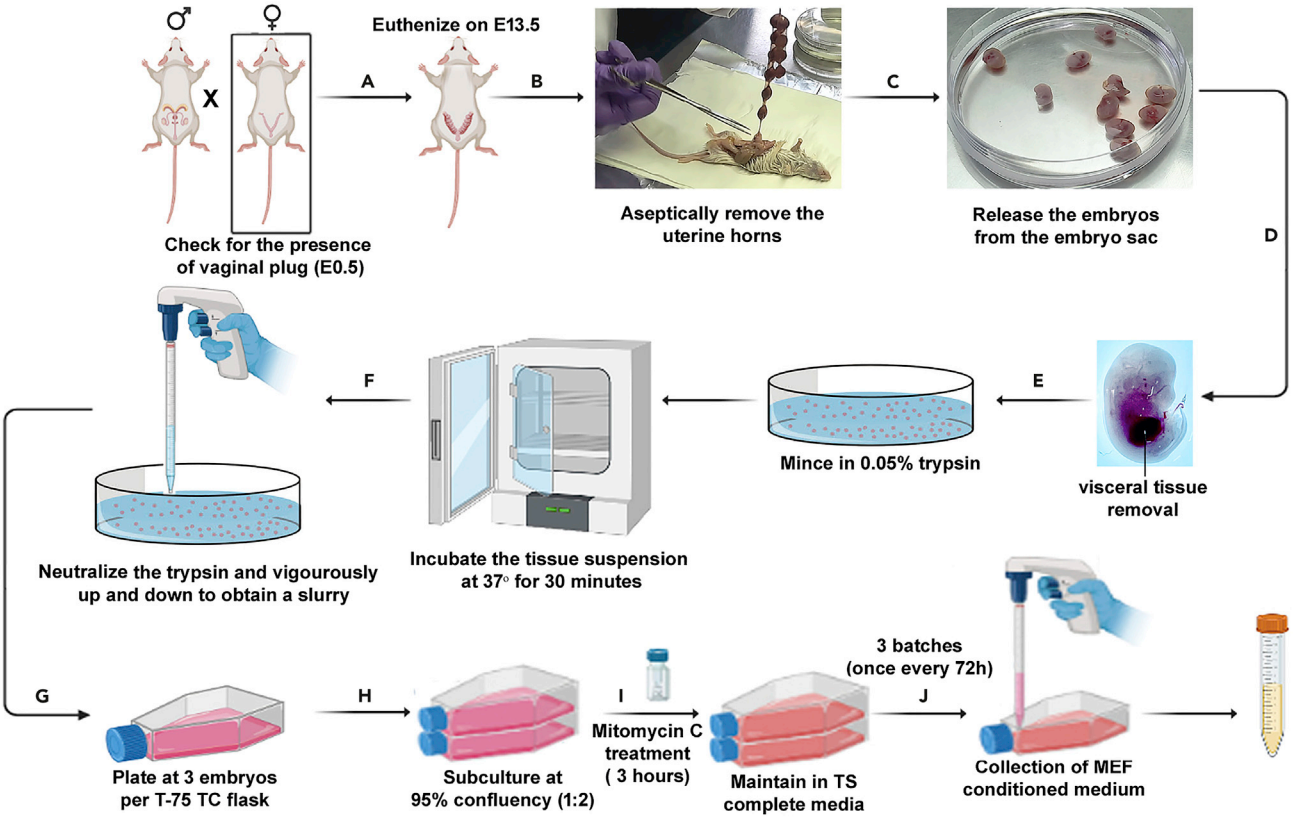


Figure 1. Detailed experimental procedure for isolation of MEF from pregnant mice at E13.5

Steps marked A–J have been detailed in the text.

5. Saturate the mouse abdomen with alcohol pad and using sterilized instruments cut back the skin to expose the peritoneum.
6. Re-sterilize the instruments using alcohol and glass bead sterilizer and cut the peritoneal walls to expose the uterine horns.
7. Remove the uterine horns and place them in a sterile disposable petri-plate. Wash the uterine horns three times with 15 mL sterile DPBS (Figure 1B).
8. Place the uterine horns in a fresh petri-plate and cut open the embryonic sacs to release the embryo using Dumont forceps and scissor (Figure 1C).
9. Place the embryos in a dish. Wash three times with 15 mL DPBS. Dissect out and remove the dark colored visceral tissues from the embryos. Remove excess PBS and place the embryos without visceral tissue in a fresh plate (Figure 1D).
10. Mince the embryo (except the visceral part) with dissecting scissors for 10 min into grain sized tissue. Add 2 mL 0.05% trypsin (vol/vol) and mince for few additional minutes.
11. Add 5 mL 0.05% trypsin (vol/vol) to obtain a suspension and place the tissue suspension in an incubator for 30 min at 37°C (Figure 1E).
12. Remove the minced tissue from the incubator and vigorously pipette the mixture up and down using a sterile 10 mL serological pipette until the mixture appears as a sludgy consistency.
13. Neutralize the action of trypsin by the addition of 20 mL MEF complete media (Figure 1F).
14. Plate the cells (3 embryos per flask) on a T-75 flask and culture them at 37°C in a humidified incubator at 5% CO_2 for 48 h (Figure 1G).
15. Observe for cell confluence and passage by trypsinizing with 0.05% Trypsin-EDTA at 95% confluence in 1:2 ratios in T75 flask pre-coated overnight with 0.1% gelatin (Figure 1H). Problem 1.

⚠ CRITICAL: Carry out all the dissection steps using sterilized surgical instruments in a sterile tissue culture hood under fully aseptic conditions. It is important that all tissues are digested. Make sure that there are no visible tissue clumps after tryptic digestion is complete.

Cryopreservation of isolated MEFs

16. At 100% confluence, trypsinize the MEFs in each T75 flask with 3 mL 0.05% Trypsin-EDTA. Incubate at 37°C incubator for 3 min. Neutralize the action of trypsin by addition of MEF complete media (5 times the volume of trypsin). Transfer the cell suspension to a 50 mL centrifuge tube. Centrifuge at 200 × *g* for 5 min and aspirate the trypsin containing media.
17. Dissolve the cell pellet obtained from each T75 flask in 1.5 mL MEF complete media.
18. Add 1.5 mL 2× MEF freezing media (90% MEF complete media, 10% DMSO) slowly to 1.5 mL cell suspension and mix by pipetting.
19. Freeze the cells at 1:3 ratios (3 vials from one flask) by placing 4×10^6 cells in 1 mL per freezing vial.
20. Place the tubes on an isopropanol slow freezer container and incubate overnight at −70°C for 24 h. Transfer vials to liquid nitrogen tank after 24 h (next day).

Thawing of MEFs

21. Thaw 10 vials of cryopreserved MEFs in 10 T75 flask pre-coated overnight with 0.1% gelatin. Allow the cells to attach and grow till 100% confluence.
22. Wash the cell monolayer with sterile DPBS. Remove the DPBS and trypsinize the MEFs in each T75 flask with 0.05% Trypsin-EDTA. Neutralize the action of trypsin by addition of MEF complete media (5 times the volume of trypsin). Transfer the cell suspension to a 50 mL centrifuge tube. Centrifuge at 200 × *g* for 5 min and aspirate the trypsin containing media.
23. Dissolve the cell pellet in 30 mL MEF complete media and distribute evenly in two T75 flask (1: 2 ratio). Thus, obtain a total of 20 T75 flasks. Do not use cells at more than passage 3.

Mitotic inactivation of MEFs and collection of MEF conditioned media

24. Wash the confluent cell monolayer gently with sterile DPBS (to remove traces of antibiotic in culture media) and mitotically inactivate the cells by incubating the cells with 10 mL antibiotic-free MEF culture media containing 10 μg/ mL Mitomycin-C for 3 h at 37°C in dark (Figure 1I).
25. After 3 h, remove the mitomycin containing media and wash the monolayer of cell division arrested MEFs three times with sterile DPBS (to remove traces of Mitomycin C) and maintain the cells in TS complete media for 72 h (Figure 1J).
26. Collect the MEF conditioned media in three consecutive batches, each after 72 h.
27. Spin down the collected media at 200 × *g* for 5 min and filter sterilize (0.45 μm) the supernatant and store in single use aliquots at −80°C until further use.

⚠ CRITICAL: Mitomycin C treatment should be carried out under complete darkness because it is readily decomposed in the presence of light. Generally, the first collected batch of conditioned media provides the best results, but the same feeder layer can be used to collect three consecutive batches of conditioned media.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal Anti-Cytokeratin	Sigma-Aldrich	C2931
Anti-mProliferin affinity purified goat IgG antibody	R&D Systems	AF1623

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pan-actin antibody	Cell Signaling Technology	4968
β-Actin (D6A8)	Cell Signaling Technology	8457
GAPDH antibody	Cell Signaling Technology	7118
rpL7 antibody	Bethyl Laboratories	A300-741A
Anti-rabbit HRP linked secondary antibody	Cell Signaling Technology	7074
Brilliant Violet™ 421-conjugated affinity pure anti mouse IgG (H+L) secondary antibody	Jackson ImmunoResearch	715-675-150
Anti-goat IgG APC conjugated antibody	R&D Systems	F0108
Chemicals, peptides, and recombinant proteins		
DPBS	Gibco	21600-010
Trypsin-EDTA (0.25%)	Gibco	25200-056
DMEM-high glucose	Gibco	12100-046
RPMI-1640	Gibco	31800-022
HI-FBS	Gibco	10082-147
Pen-strep	Gibco	15070-063
MEM NEAA	Gibco	11140-050
Glutamax	Gibco	35050-061
Sodium pyruvate	Sigma	P5280-254
Recombinant Human FGF-4	R&D Systems	235F-4
Heparin sodium salt from porcine intestinal mucosa	Sigma-Aldrich	H3149
β-mercaptoethanol	Sigma-Aldrich	M3148
Mitomycin C	Sigma-Aldrich	M4287
EmbryoMax 0.1% Gelatin solution	Merck Millipore	ES-006-B
DMSO	Sigma-Aldrich	D2650
TRIzol	Ambion	15596018
Chloroform	Merck	1070240521
Isopropanol	SRL	38445
Nuclease free water	Invitrogen	10977-015
DEPC	Sigma-Aldrich	D5758
5× FS buffer	Invitrogen	28025-013
$Oligo(dt)_{12–18}$	Invitrogen	18418012
10 mM dNTP mix	Thermo Scientific	R0192
0.1 M DTT	Invitrogen	28025-013
M-MLV Reverse transcriptase (200 U/μL)	Invitrogen	28025-013
RNaseOUT™ Recombinant Ribonuclease inhibitor	Invitrogen	10777019
RNase H	Invitrogen	18021071
Power SYBR® Green PCR Master Mix	Applied Biosystems	4367659
Paraformaldehyde	Sigma-Aldrich	P6148
DYLIGHT™ 544 Phalloidin	Cell Signaling Technology	13054
Hoechst 33258	Sigma-Aldrich	14533
Fluoroshield mounting media	Sigma-Aldrich	F6182
PIPES	Sigma-Aldrich	P6757
Cytochalasin D	Invitrogen	PHZ1063
ATP (100 mM)	Thermo Scientific	R0441
NaCl	HiMedia	GRM031
$MgCl_2$	Merck	105833
$MgSO_4$	Merck	105886
$CaCl_2$	Sigma-Aldrich	C5080
EGTA	Sigma-Aldrich	E8145
Glycerol	HiMedia	AS100-14
NP-40	HiMedia	RM2352
Triton-X	AMRESCO	0694
Tween-20	HiMedia	MB067
Protease inhibitor cocktail (PIC)	Cell Signaling Technology	5871

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell permeabilization buffer	Invitrogen	00-8333-56
Donkey serum	Jackson ImmunoResearch	017-000-001
30% Acrylamide/Bis solution (37.5:1)	Bio-Rad	1610158
SDS	AMRESCO	0227
Ammonium persulfate	Bio-Rad	1610700
TEMED	Bio-Rad	161-0800
Methanol	Finar	10930LL025
Ethanol	Merck	1009830511
Experimental models: Organisms/strains		
Mouse: CD1(8weeks old, female, wild type)		N/A
Experimental models: Cell lines		
Mouse trophoblast stem cells ($TSC_{3.5}$)	Janet Rossant's lab	N/A
Other		
Tissue culture dish (100 mm)	Corning	353003
Dumont forceps	Fine Science Tools	11252-00
Dissecting scissors	Fine Science Tools	14004-12, 15040-11, 14160-10
Sterilizer, dry heat with glass bead	Sigma-Aldrich	Z378569EU
Microscope glass slides	HiMedia	BG005
Cover glass	BLUE STAR	22*22 mm
Tissue culture plate,96 well flat bottom	Falcon	353072
T-25 tissue culture flask	Corning	430639
T-75 tissue culture flask	Corning	156499
15 mL centrifuge tubes	Corning	430791
1.5 mL microcentrifuge tubes	Tarsons	500010
Freezing cryovial	Thermo Scientific	368632
5 mL syringe	BD	309646
Hemocytometer	Thomas Scientific	5971R30
Milipore Express™ Plus (0.22 µm) filter	Merck	SCGPU05RE
28 mm diameter Syringe filters,0.2 µm pore PES membrane	Corning	431229
Amicon Ultra-15 centrifugal filter unit	Merck Millipore	UFC9010
10 mL serological pipet	Corning	4488
25 mL serological pipet	Corning	4489
5 mL serological pipet	Falcon	357543
FACS tubes	Falcon	352058
MicroAmp optical 8-cap strip	Applied Biosystems	4323032
MicroAmp optical 8-tube strip	Applied Biosystems	N8010580
SorvallTM WX + 90 Ultra series centrifuge (T890 rotor)	Thermo Scientific	75000090
Eppendorf Centrifuge 5427R	Eppendorf	EP022620701
Eppendorf Centrifuge 5702R	Eppendorf	EP022628012
Nano drop 2000 spectrophotometer	Thermo Scientific	R639
Nuve water bath (Model NB9)	Henderson Biomedical	02-2276
Forma Series II Water jacket CO_2 Incubator	Thermo Scientific	S111
Confocal microscope	Leica	TCS SP8
ProFlex PCR System	Applied Biosystems	4483636
7500 Real time PCR system	Applied Biosystems	4345241
UVP Biospectrum 810	UVP	97063001
Enspire Multi mode Plate reader	PerkinElmer	2300
Flow cytometer	BD	LSR Fortessa
Oligonucleotides		
mCdx2 f(5′-GAGCTGGCTGCACTTG-3′)	IDT	N/A
mCdx2 r(5′-CGGCTGTGGAGGCTGTTG -3′)	IDT	N/A
mPrl2c2 f(5′-CATCTCCAAAGCCACAGACATAAA-3′)	IDT	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
mPrl2c2 r(5′- TGAATGCGAGCATCTTCATTG-3′)	IDT	N/A
mRPL7 f(5′- AAGAAGCGGATTGCCTTGAC-3′)	IDT	N/A
mRPL7 r(5′-TAACTTGAAGGGCCACAGGAA-3′)	IDT	N/A

MATERIALS AND EQUIPMENT

- MEF culture.

100 mM Sodium pyruvate stock solution: Dissolve 0.011 g sodium pyruvate in 1 mL sterile DPBS to constitute a 100 mM stock in sterile DPBS. Filter sterilize and use freshly at a final concentration of 1 mM for TS complete media.

Reagent	Stock concentration	Volume of DPBS
Sodium pyruvate	100 mM	1 mL

MEF complete media: Prepare MEF complete media as per the following table.

Reagent	Final concentration	Amount
DMEM-high glucose	N/A	87 mL
HI-FBS	10%	10 mL
Pen-strep solution	1×	1 mL
NEAA	1×	1 mL
100 mM Na pyruvate solution	1 mM	1 mL
Total		100 mL
Store at 4°C after use up to 6 months.		

- Trophoblast stem cell culture.

FGF-4 stock solution (25 μg/mL; 1000×): Resuspend the lyophilized FGF-4 with 1 mL 0.1% (w/v) BSA in sterile DPBS. Mix very carefully with P200, make 50 μL aliquots.

Reagent	Stock concentration	Volume of 0.1% BSA
FGF-4	1000×	1 mL
Store all aliquots at −80°C after and until use.		

100 mM Sodium pyruvate stock solution: Resuspend the 0.011 g sodium pyruvate in 1 mL sterile DPBS to constitute a 100 mM stock in sterile DPBS. Filter sterilize and use freshly at a final concentration of 1 mM for TS complete media.

Heparin stock solution (10 mg/mL; 10,000×): Resuspend 10 mg heparin in 1 mL sterile DPBS. Filter sterilize, prepare aliquots.

Reagent	Stock concentration	Volume of DPBS
Heparin	10,000×	10 mg
Store all aliquots at −80°C until use. Do not refreeze after thawing.		

Reagent	Working stock concentration	Volume of DPBS
Sodium pyruvate	100 mM	1 mL

10 mM β-mercaptoethanol stock solution: Mix 1.4 μL β-mercaptoethanol solution in 2 mL sterile DPBS. Filter sterilize and use freshly at a final concentration of 100 μM.

Reagent	Stock concentration	Volume of DPBS
β-mercaptoethanol solution	10 mM	2 mL

TS complete media: Prepare TS complete media as per the following table.

Reagent	Final concentration	Amount
RPMI-1640	N/A	76 mL
HI-FBS	20%	20 mL
Pen-strep solution	1×	1 mL
Glutamax	1×	1 mL
100 mM sodium pyruvate	1 mM	1 mL
β-mercaptoethanol	100 μM	1 mL
Total		100 mL
Store at 4°C after use up to 6 months.		

TSC maintenance media: Prepare 100 mL TSC maintenance media as described below freshly before use as per requirement.

Reagent	Final concentration	Amount
TS complete media	30%	30 mL
MEF conditioned media	70%	70 mL
FGF4	25 ng/mL	100 μL
Heparin	1 μg/mL	10 μL
Total		100 mL
Prepare freshly during each use.		

2× TSC freezing media: Prepare 2× TSC freezing media as described below freshly and cool to 4°C before use.

Reagent	Final concentration	Amount
TS complete media	30%	0.6 mL
HI-FBS	50%	1 mL
DMSO	20%	0.4 mL
Total		2 mL
Prepare freshly during each use.		

- **RNA isolation and cDNA preparation.**

Reverse transcription RNA mix: Prepare the following mix in a nuclease-free 100 μL tube. Calculations are provided for individual samples.

Reagent	Final concentration	Amount
RNA	–	5 μg
Nuclease free water	70%	(11-RNA) μL
500 μg/mL Oligo $dT_{(12–18)}$	25 μg/mL	1 μL
10 mM dNTP mix	10 nM	1 μL
Total		12 μL

Reverse transcription enzyme mix: Prepare the following mix in a nuclease-free 100 μL tube. Calculations are provided for individual samples.

Reagent	Final concentration	Amount
5× First strand buffer	1×	4 μL
0.1 M DTT	5 mM	2 μL
RNase OUT (40 U/μL)	2 U	1 μL
Total		7 μL

Low TE buffer: Prepare low TE buffer as described below. Store at 4°C after use.

Reagent	Final concentration	Amount
1 M Tris HCl (pH 8.0)	0.01 M	1 mL
0.5 M EDTA	0.1 mM	20 μL
Nuclease-free water		98.98 mL
Total		100 mL
Store at 4°C after use up to 1 year.		

- **Ploidy and Nuclear size analysis.**

Hoechst 33258 stock solution (2 mg/mL, 1000×): Measure 2 mg Hoechst 33258 and add 1 mL distilled water. Sonicate with 3 pulses (30 s each at 8 KHz). Centrifuge at 12,000 × *g* for 5 min to allow the undissolved particles to settle down.

Reagent	Stock concentration	Volume of distilled water
Hoechst 33258	1000×	1 mL
Store at 4°C after use up to 1 year.		

4% Paraformaldehyde solution: Weigh 2 gm paraformaldehyde and dissolve it in 50 mL DPBS by repeated heating, cooling and stirring until all particles have dissolved completely.

Reagent	Stock concentration	Volume of DPBS
Paraformaldehyde	4%	50 mL
Prepare freshly during each use.		

- **Actin polymerization assay.**

F-actin stabilization buffer: Prepare the following as mentioned below:

Reagent	Final concentration	Amount
1 M PIPES	50 mM	50 μL
5 M NaCl	50 mM	10 μL
2.5 M $MgCl_2$	5 mM	2 μL
0.5 M EGTA	5 mM	10 μL
Glycerol	5%	50 μL
NP-40	0.1%	1 μL
Triton-X	0.1%	1 μL
Tween-20	0.1%	1 μL
β-mercaptoethanol	0.1%	1 μL
100 mM ATP	1 mM	10 μL
100 × PIC	1×	10 μL
Nuclease free water		854 μL
Total		1 mL
Prepare freshly during each use.		

F-actin de-polymerization buffer: Prepare the following as mentioned below:

Reagent	Final concentration	Amount
1 M PIPES	100 mM	100 μL
1 M $MgSO_4$	100 mM	100 μL
1 M $CaCl_2$	10 mM	10 μL
500× Cytochalasin D	5 ×	10 μL
100 × PIC	1×	10 μL
Nuclease free water		770 μL
Total		1 mL
Prepare freshly during each use.		

STEP-BY-STEP METHOD DETAILS

Derivation of endoreduplicated trophoblast giant cells (TGCs) from trophoblast stem cells (TSCs)

TSCs were a kind gift from Professor Janet Rossant, The Hospital for Sick Children (SickKids), Toronto, Canada were derived as described before (Tanaka et al., 1998). These cells are not commercially available.

Murine TSCs have also been derived by Ohinata and Tsukiyama (2014). These cells are also not available commercially.

TSC maintenance

Timing: 3–4 days

This section describes the thawing of cryopreserved TSCs, maintenance and the propagation steps.

Thawing.

1. Thaw a frozen vial of murine TSC in a 37°C water bath.
2. Transfer the contents of the vial gently onto a 15 mL sterile centrifuge tube containing 5 mL TS complete media (Figure 2).
3. Centrifuge at 200 × *g* for 5 min.

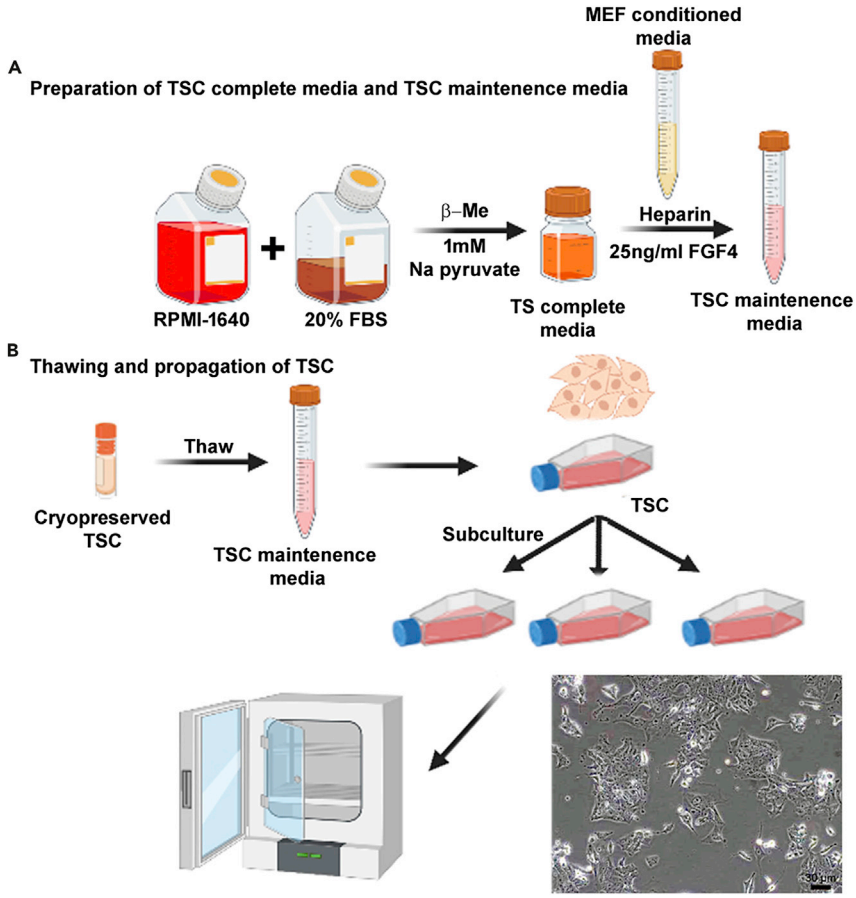


Figure 2. Trophoblast stem cell (TSC) culture

(A) Preparation of TSC complete media and TSC maintenance media.

(B) Schematic illustration of thawing and sub-culturing of TSCs. Scale: 30 μm.

4. Aspirate the media to remove the DMSO containing freezing media completely.
5. Resuspend the cell pellet in 5 mL TSC maintenance media (Figure 2) and maintain the cells at 37°C in a humidified incubator supplied with 5% CO_2 for 48 h.

⚠ CRITICAL: To ensure maximum viability of TSCs, perform rapid cell thawing. Make sure to remove all DMSO present in the freezing media during the thawing procedure. Traces of residual DMSO might affect the viability of TSCs.

Subculturing.

6. Passage the cells at 60% confluence with 0.05% Trypsin-EDTA (Figure 2). For sub-culturing, wash the cell monolayer with sterile DPBS to remove traces of FBS, incubate TSCs with sufficient amount of 0.05% Trypsin-EDTA to cover the entire surface for 30 s at 37°C in an incubator and neutralize with double amount of TS complete media.
7. Mix the cell suspension very gently and transfer to a 15 mL centrifuge tube. Centrifuge at 200 × *g* for 5 min. Aspirate the supernatant, resuspend the pellet in TSC maintenance media and subculture at 1:3 ratios.

⚠ CRITICAL: During preparation of TSC maintenance media, mix properly after the addition of mitogens such that FGF4 and heparin are distributed homogenously in the media. Do

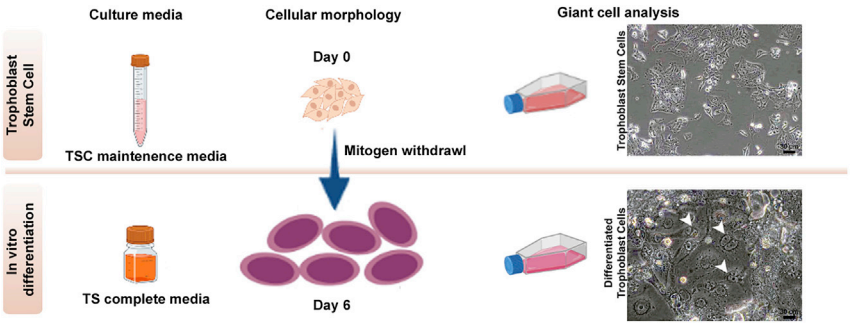


Figure 3. Comparative experimental strategy for propagation of TSCs and in vitro differentiation of TSC to TGC
Scale: 30 μm.

not allow the cells to grow at more than 65% confluence as this might result in spontaneous differentiation of the stem cells. Do not use till high passages for experimental purposes as this might result in a gradual loss of the stem cell population. Recommended, till passage 22. Do not perform trypsinization for more than five minutes as this might result in the loss of surface receptors.

a. At this point, cells can be cryopreserved as mentioned previously in MEF harvesting point 16.

Differentiation of TSCs into TGCs

Timing: 8 days (TSCs cultured for 2 days and differentiation for 6 days).

TSCs are induced to differentiate by withdrawal of mitogens.

8. Obtain a pellet of TSCs as mentioned in b.
9. Wash the cell pellet with 5 mL sterile DPBS to remove all the traces of mitogens present.
10. Resuspend the cells in TS complete media (Figure 3) and plate at a density of 60,000 cells per T25 flask and maintain for 6 consecutive days.
11. Replenish the media once in the first two days and daily from the third day till the fifth day. Harvest on the 6^{th} day of differentiation, when the TSCs are fully differentiated to TGCs as per the experimental requirement mentioned in the next section (Figure 3).

Functional characterization of endoreduplicated trophoblast giant cells (TGCs)

Timing: 8–10 days

TGCs can be genotypically and phenotypically distinguished from TSCs using various indigenous methodologies as described.

Detailed data example for functional characterization of endoreduplicated TGCs have been illustrated in Figure 4. TSCs have been used as the control sample in the experimental procedures detailed hereafter.

Marker analysis by qPCR

Trophoblast giant cells formed by differentiation of TSCs can be characterized by analysis of genetic markers specifying endoreduplicated TGCs like *Prl2c2*. TSC marker *Cdx2* is used as control (Figure 4).

RNA isolation and cDNA preparation.

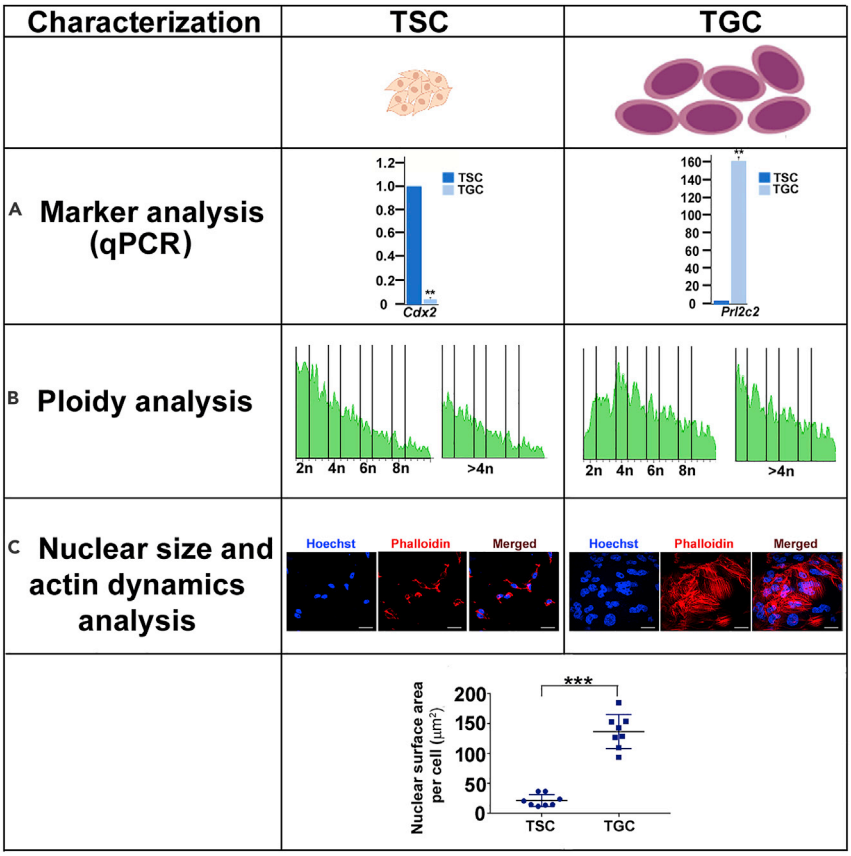


Figure 4. Phenotypic characterization of TGCs and example of data

(A) qPCR-based analysis of genetic marker specifying TSC (*Cdx2*) and TGC (*Prl2c2*). Mouse RPL7 has been used as endogenous control for normalization.

(B) Left panel shows flow cytometry-based analysis of the ploidy state of trophoblast cells stained with Hoechst. Cells with DNA content >4n (TGCs) are represented on the right panel.

(C) Hoechst and Phalloidin staining of TSCs and TGCs for quantification of the nuclear size. The nuclei have been stained using Hoechst (blue) and counterstaining of the cytosolic actin filaments has been done using DyLight™ 554 Phalloidin (red). Scale: 20 μm. Quantification of the nuclear size using ImageJ software has been shown below.

12. For isolating RNA from TSCs, aspirate the media and wash the cell monolayer with sterile DPBS.
13. Add 1 mL 0.05% Trypsin-EDTA per T25 flask and incubate at 37°C in an incubator. Neutralize the action of trypsin with double the volume of TS complete media and transfer the cell suspension to a 15 mL centrifuge tube.
14. Centrifuge at 200 × *g* for 5 min and aspirate the trypsin containing media. Wash the cells with 2 mL sterile DPBS at 200 × *g* for 5 min. Discard the supernatant and dissolve the pellet in 1 mL TRIzol. Incubate at room temperature for 5 min.

⚠ CRITICAL: RNA isolation from TSCs must be carried out within 36 hours of seeding in stemness condition (doubling time of TSCs is 36 h) by gentle trypsinization and not cell scraping in order to avoid any chances of possible differentiated cell contamination which might happen if cells are scraped off. Although differentiation does not occur that fast, yet there remain chances of spontaneous cell differentiation. As a result, gentle trypsinization helps to obtain a pure stem cell population.

15. For isolating RNA from TGCs (differentiated till day 6), aspirate the media and wash the cell monolayer with sterile DPBS.

16. Add 1 mL TRIzol and incubate at room temperature for 5 min. Directly scrape off the cell monolayer with a cell scraper.

⚠ CRITICAL: RNA isolation from TGCs must be carried out after 6 days of seeding in differentiated condition.

17. Transfer 1 mL lysed suspension into a RNase free (DEPC treated) 1.5 mL Eppendorf tube and incubate for 5 min to allow complete dissociation of the nucleoprotein complexes.
18. Add 200 μL chloroform to the tube containing the lysate and mix very gently by inverting the tube in "figure-of-eight" pattern and then incubate it for 3 min.
19. Centrifuge at 12,000 × *g* for 15 min at 4°C. Collect the aqueous phase containing the RNA in a fresh RNase free tube and add 500 μL isopropanol to the tube. Incubate for 10 min at 4°C.
20. Centrifuge at 12,000 × *g* for 10 min at 4°C. Discard the supernatant and wash the RNA pellet with 1 mL of 75% ethanol at 7,500 × *g* for 5 min at 4°C.
21. Discard the supernatant completely and depending on the pellet size, dissolve it in nuclease free water (volume of water depends on the size of the pellet) by gentle pipetting.

Note: Other commercially used available kits for total RNA isolation like RNeasy kits can also be used.

22. Heat the RNA sample at 65°C for 5 min. Quick chill on ice for 2 min and measure the quality and total amount of RNA spectrophotometrically by determining the 260/280 ratio and concentration respectively. Use 5 μg RNA to reverse transcribe first-strand cDNA using oligo dT primers.
23. Prepare individual reverse transcription RNA mix as described in materials and equipment. Heat the mix to 65°C for 5 min followed by a quick chilling in ice for 2 min.
24. Meanwhile prepare the reverse transcription enzyme mix as described in materials and equipment. Add 7 μL of the mix to each tube and mix gently.
25. Mix the contents and incubate at 37°C for 2 min. Add 1 μL (200 U) M-MLV RT and mix gently by pipetting up and down. The total contents of each tube are tabulated below.

Reagent	Amount
Reverse transcription RNA mix	12 μL
Reverse transcription enzyme mix	7 μL
M-MLV RT (200 U)	1 μL
Total	20 μL

26. Incubate for 50 min at 37°C.
27. Heat inactivate the enzyme by incubating at 70°C.
28. Remove the RNA present as hybrid with cDNA by adding 0.5 μL (1 U) of *E.coli* RNaseH by incubating at 37°C for 20 min.

Quantitative real-time PCR.

Primers used in the analysis have been adopted from Basak et al. (2021).

29. Prepare a 10 μM sub-stock of individual 100 μM forward and reverse primers in low TE buffer. Prepare a 1 μM primer mix containing both forward and reverse primers in low TE buffer.
30. Prepare a 1:10 dilution of cDNA by adding 1 μL cDNA to 9 μL nuclease-free water.
31. Prepare a qPCR mix containing 1 μL of 1:10 diluted cDNA, 5 μL FG-Power SYBR Green, 1 μL of 1 μM primer mix and 3 μL water. Problem 2. The total contents of each tube are tabulated below.

Reagent	Amount
Water	3 μL
1 μM primer mix	1 μL
FG-Power SYBR Green	5 μL
cDNA (1:10 dilution)	1 μL
Total	10 μL

32. Run the qPCR as per the cycle conditions mentioned below.

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	
Denaturation	95°C	15 s	40
Annealing	60°C	1 min	
Dissociation	95°C	15 s	
Extension	60°C	1 min	
Final denaturation	95°C	30 s	

33. Analyze the C_t values of the target gene and the endogenous control (*Rpl7*) and calculate the fold change using $2^{-\Delta\Delta Ct}$ method. Problem 2.

Marker analysis by flow cytometry

Harvesting TSCs.

34. Grow TSCs at 60% confluency in T25 flask. At the time of harvesting cells, aspirate the media and wash the cell monolayer with sterile DPBS (pH 7.4).
35. Add 1 mL 0.05% Trypsin-EDTA per T25 flask and incubate at 37°C in an incubator for 30 s.
36. Neutralize the action of trypsin with double the volume of TS complete media and transfer the cell suspension to a 15 mL centrifuge tube.
37. Centrifuge at 200 × *g* for 5 min. Wash the cells once in 1× sterile DPBS (pH 7.4) at 200 × *g* for 5 min.

Harvesting TGCs.

38. To harvest TGCs on day6 of differentiation, aspirate the media and wash the cell monolayer with sterile DPBS (pH 7.4).
39. Add 1 mL 0.25% Trypsin-EDTA per T25 flask and incubate at 37°C in an incubator for 4 min for complete trypsinization of the giant cells.
40. Neutralize the action of trypsin with five times the volume of TS complete media and transfer the cell suspension to a 15 mL centrifuge tube.
41. Centrifuge at 200 × *g* for 5 min. Wash the cells once in 1× sterile DPBS (pH 7.4) at 200 × *g* for 5 min.

Sample preparation and staining.

42. Aspirate the supernatant and re-suspend the cell pellet in 1 mL DPBS (pH 7.4). Problem 4.
43. Count the cells in a hemocytometer and take 5 × 10^5 cells in a 1.5 mL microcentrifuge tube per analysis.
44. Centrifuge at 200 × *g* for 5 min, aspirate the supernatant and resuspend the pellet in 100 μL of blocking buffer containing 0.5% donkey serum dissolved in 1× cell permeabilization buffer and incubate for 30 min at 4°C.
45. Centrifuge cells at 200 × *g* for 5 min.

Table1. List of Trophoblast markers

Cell type	Markers
Trophoblast cells	Cytokeratin
Trophoblast stem cells (TSC)	*Cdx2, Eomes, Essrb*
Trophoblast giant cells (TGC)	*Prl2c2, Prl3d1, Prl3b1*

46. Aspirate the supernatant completely and incubate in 100 μL 1× cell permeabilization buffer containing primary antibodies specific to the trophoblast marker Cytokeratin and the TGC marker Prl2c2 at 1:100 dilutions for approximately 1 h 30 min at 4°C. Control unstained cells were resuspended in permeabilization buffer without the antibodies. Other antibodies that can be used for trophoblast subtype characterization have been listed in Table 1.
47. Following primary antibody incubation, centrifuge cells at 200 × *g* for 5 min at 4°C and aspirate the supernatant. Then wash three times in 500 μL DPBS (pH 7.4) by centrifuging at same speed for same time interval. Problem 4.
48. Incubate cell pellets in 100 μL 1× cell permeabilization buffer containing fluorochrome conjugated anti-mouse and anti-goat secondary antibodies for Cytokeratin and Prl2c2 respectively at 1:200 dilutions for 1 h 30 min at 4°C.
49. Following incubation centrifuge cells at 200 × *g* for 5 min, aspirate supernatant and wash cell pellet with 500 μL 1× DPBS (pH 7.4) twice. Problem 4.
50. Resuspend cell pellet in 500 μL 1× DPBS (pH 7.4) before analyzing in a flow-cytometer using appropriate filters for respective fluorochromes.
51. To compensate for any background signal from fluorochrome conjugated secondary antibodies, incubate cells only with fluorochrome conjugated IgGs, under conditions identical to the stained samples (Isotype control).
52. Do primary gating based on forward scatter and side scatter to exclude debris and dead cells. Problem 5.
53. In case of simultaneous detection of multiple markers, use fluorochromes with minimal spectral overlap for individual markers detected.
54. Set fluorescence gates for the markers, based on the unstained samples, isotype and single stained controls to gate compensations for the spectral overlap.
55. Draw quadrants to quantitate individual cell populations based on their marker specificity.

Flow cytometric analysis of ploidy state of endoreduplicated TGCs

The protocol described here has been adapted from Chakraborty and Ain (2018) and Basak and Ain (2022).

Harvesting TSCs.

56. Stained TSCs are used for gating in flow cytometry. To harvest TSCs for flow cytometry, aspirate the media and wash the cell monolayer with sterile DPBS (pH 7.4).
57. Add 1 mL 0.05% Trypsin-EDTA per T25 flask and incubate at 37°C in an incubator for 30 s.
58. Neutralize the action of trypsin with double the volume of TS complete media and transfer the cell suspension to a 15 mL centrifuge tube.

Harvesting TGCs.

59. To harvest TGCs for flow cytometry, aspirate the media and wash the cell monolayer with sterile DPBS. Add 1 mL 0.25% Trypsin-EDTA per T25 flask and incubate at 37°C in an incubator for 4 min to completely dislodge the giant cells. Neutralize the action of trypsin with five times the volume of TS complete media and transfer the cell suspension to a 15 mL centrifuge tube.

Sample preparation and staining.

60. Centrifuge at 200 × *g* for 5 min and aspirate the trypsin containing media. Wash the cell pellet once with sterile DPBS at 200 × *g* for 5 min.
61. Resuspend the cell pellet in 500 μL sterile DPBS and count the number of cells.
62. Prepare a 250 μL cell suspension of having a concentration of 10^6cells/mL.
63. Stain the nuclei with Hoechst33258 at a final concentration of 2 μg/mL. For staining the nuclei, add 0.25 μL of 2 mg/mL Hoechst 33258 stock solution to 250 μL cell suspension. Mix gently by pipetting up down and incubate for 30 min at 37°C in an incubator.
64. Set the flow cytometer for ultraviolet excitation (340–380 nm) and measure the cell fluorescence using pulse width vs pulse area signal to discriminate between G2 population and doublets.
65. Use the stained TSCs to perform gating and analyze the percentage of polyploids in TGCs (>4n and ≤10n) using DNA content frequency histogram (Figure 4B). 1000 TGCs are analyzed per run.

Hoechst and Phalloidin staining for analysis of nuclear size and actin dynamics

TGCs are characterized by endoreduplicated nuclei where the size of the nuclei increases significantly as compared to stem cells Thus, the size of the nuclei provides a direct correlation of the TGC state. Staining of the nucleus using Hoechst can provide a potential method to characterize the endoreduplicated TGC state. Also, distinct filaments of F-actin support the cytoskeleton in TGCs in contrast to the weak F-actin filaments which are observed in TSCs. *The protocol described here has been adapted from* Basak and Ain (2022).

66. Plate 2 × 10^5 TSCs and 20,000 TGCs onto a coverslip in a 35 mm dish. TSCs should be maintained for no longer than 36 h whereas for TGCs, harvest the cells on the 6^{th} day of differentiation.

Sample preparation and staining.

67. Remove the media and fix the cells with freshly prepared 4% paraformaldehyde for 15 min at room temperature. Wash the cells three times (5 min each) with 1 mL DPBS by gentle rotation.
68. Stain the F-actin filaments with DyLight™ 554 Phalloidin at a dilution of 1:200 in DPBS for 20 min at room temperature under complete darkness.
69. Wash the cells three times (5 min each) with 1 mL DPBS by gentle rotation.
70. Counterstain the nuclei with Hoechst 33258 at a final concentration of 2 μg/mL for 20 min at room temperature under complete darkness.
71. Wash the cells five times (5 min each) with 1 mL DPBS by gentle rotation.
72. Mount the coverslips onto clean slides with Fluoroshield. Allow it to dry and then seal the edges. The samples are now ready for image acquisition.

Image Acquisition.

73. Set up the confocal microscope for 340 nm and 550 nm excitation to capture the fluorescence image of Hoechst 33258 and Phalloidin respectively.

Measurement of nuclear size.

74. Open the Hoechst-stained image of the nuclei (containing the scale bar in μm) using ImageJ software.
75. Use the line selection tool to draw a line over the scale bar and use the software to measure it in pixels. Initialize the software to establish a relationship of the provided distance of the scale bar (in μm) with equivalent distance in pixels.

Step 76 is mandatory if the nuclei appear very crowded and touching each other otherwise there are chances that two or more nuclei will be considered as a single nucleus. However, because only the size of the nuclei is measured, there is no requirement of background subtraction.

76. Go to Process/Smooth for smoothing the image.
77. Set the threshold of the image to an optimum value such that all the nuclei appear as an evenly highlighted region.

Based on the threshold set, some areas might appear over-saturated and some areas under-saturated. Either remove or enhance the pixels from saturation, respectively.

78. Outline the periphery/ boundary of individual nuclei by selecting the "overlay outlines". Use the boundary markings and optimize the pixel size discrimination to such a value that all artifacts (which aren't nuclei) are eliminated.
79. Analyze and calculate the area of the nuclei by Analyze/Analyze particles.
80. The results tab provides the area of each nuclei selected.
81. Export this data to GraphPad Prism software to statistically analyze the changes in nuclear size (Figure 4C). Bigger nucleus generally indicates increased DNA content, and hence increased ploidy and better TGC formation.

Actin polymerization assay

The protocol described here has been adapted from Chakraborty and Ain (2018).

Harvesting TSCs.

82. To harvest TSCs for actin polymerization assay, aspirate the media and wash the cell monolayer with sterile DPBS.
83. Add 1 mL 0.05% Trypsin-EDTA per T25 flask and incubate at 37°C in an incubator for 30 s.
84. Neutralize the action of trypsin with double the volume of TS complete media and transfer the cell suspension to a 15 mL centrifuge tube. Centrifuge at 200 × *g* for 5 min.

Harvesting TGCs.

85. To harvest TGCs for flow cytometry, aspirate the media and wash the cell monolayer with sterile DPBS.
86. Add 1 mL 0.25% Trypsin-EDTA per T25 flask and incubate at 37°C in an incubator for 4 min to completely dislodge the giant cells.
87. Neutralize the action of trypsin with five times the volume of TS complete media and transfer the cell suspension to a 15 mL centrifuge tube. Centrifuge at 200 × *g* for 5 min.

Sample preparation.

88. Aspirate the supernatant and re-suspend the cell pellet in 1 mL F-actin stabilization buffer (pH 6.9).
89. Homogenize the cells by passing the cell suspension repeatedly through a 25-gauge needle attached to a 5 mL syringe.
90. Incubate the homogenate at 37°C for 10 min. Centrifuge and pellet down the cell debris at 326 × *g* for 5 min.
91. Collect the supernatant.
92. Subject the supernatant to immunoblot analysis and measure the total amount of endogenous controls [rPL7 (1:5000), GAPDH (1:1000) and total β-actin (1:1000)].
93. Ultracentrifuge the supernatant at 100,000 × *g* for 1 h to separate the G-actin and F-actin fraction using Sorvall WX Ultra Series centrifuge, T890 rotor.
94. Collect the supernatant as the G-actin fraction and concentrate it with a 10 kD cut-off centrifugal-filter unit by centrifuging it at 4,000 × *g* for 10 min.
95. Re-suspend the cell pellet (F-actin fraction) in equal volume of F-actin de-polymerization buffer (pH 6.9) and incubate at 4°C for 1 h.

96. Resolve equal volumes of individual fractions in TSCs and TGCs in immunoblot assay and probe the blots with Pan-actin antibody (1:1000).

EXPECTED OUTCOMES

This protocol is described for a reliable and reproducible culture of TSCs, differentiation of TSCs to TGCs and characterization of the differentiated TGCs using multiple routine in-house experimental approaches such as qPCR, flow-cytometry and fluorescence-based staining. TGCs derived using this protocol by culturing for 6 days shows ~150-fold enrichment of TGC marker *Prl2c2*, a greater number of cells with ploidy >4n and giant-sized nuclei. The protocols described here may be used to test efficacy of any protein, non-coding RNA or any other small molecule to affect self-renewal or differentiation of trophoblast cells.

LIMITATION

TSCs tend to lose their self-renewal potential with successive generation thereby hindering with the results. It is therefore recommended to culture TSCs at low passages ($\leq$20).

TROUBLESHOOTING

Problem 1

Presence of yeast in the MEF culture flask (step 15 in before you begin).

Potential solution

Perform the dissection steps under fully aseptic condition using a pair of clean gloves and apron.

If minute colonies of yeast are microscopically visible, add Amphotericin B (Sigma #A2942) at a concentration of 0.25 μg/mL.

Problem 2

Detectable Ct in the non-template control of quantitative real-time PCR (steps 31 and 33 in step-by-step method details).

Potential solution

Prepare the real-time mix inside a laminar flow to prevent false-positive artifacts.

Use fresh aliquots of nuclease-free water and low TE buffer for every reaction.

Problem 3

Presence of differentiation markers in TSC population:

Potential solution

Routinely check for TSC markers in every passage to prevent the drift in stemness.

Do not allow trypsinization of TSCs for more than 30–45 s.

Do not allow TSCs to grow more than 70% confluence. Maintenance of cell density is very critical as too much cells might result in a mixed population of stem cells and differentiated cells.

Use low passage number cells to prevent spontaneous differentiation.

Problem 4

Lower yield of cells (steps 42, 47 and 49 in step-by-step method details).

Potential solution

Minimize the number of washing steps.

Remove the supernatant very carefully post centrifugation to minimize cell loss.

Increase the antibody concentration (marker analysis by flow cytometry).

Problem 5

Presence of doublets during gating in flow cytometry (step 52 step-by-step method details).

Potential solution

Optimize the time of trypsinization such that all TGCs are lifted up from the surface. Mix the cell suspension very gently and evenly such that there are no cell clumps present.

RESOURCE AVAILABILITY

Lead contact

Any further information and requests should be directed to and will be fulfilled by the lead contact.

Materials availability

This study did not generate any new reagents.

Data and code availability

No new codes have been generated. All data related to this manuscript are available within the manuscript. Raw data are available with R.A.

ACKNOWLEDGMENTS

The authors thank Prof. Janet Rossant, Hospital for Sick Children, Toronto, Canada, for providing the TS cells. This work was supported by seed grant from CSIR-Indian Institute of Chemical Biology. Financial support from DBT-RA program in Biotechnology and Life Sciences to Dr. Madhurima Paul is acknowledged.

AUTHOR CONTRIBUTIONS

T.B. wrote the first draft of most part of the manuscript except flow cytometry. M.P. wrote the first draft of the flow cytometry part of the manuscript and provided the relevant data and figure associated with this. T.B. made the figures. R.A. supervised and edited the manuscript and provided funding. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

REFERENCES

Basak, T., Dey, A.K., Banerjee, R., Paul, S., Maiti, T.K., and Ain, R. (2021). Sequestration of eIF4A by Angiomotin: a novel mechanism to restrict global protein synthesis in trophoblast cells. Stem Cells *39*, 210–226. https://doi.org/10.1002/stem.3305.

Basak, T., and Ain, R. (2022). Molecular regulation of trophoblast stem-cell self-renewal and giant cell differentiation by the Hippo components YAP and LATS1. Stem Cell Res. Ther. *13*, 1–17. https://doi.org/10.1186/s13287-022-02844-w.

Chakraborty, S., and Ain, R. (2018). NOSTRIN: a novel modulator of trophoblast giant cell differentiation. Stem Cell Res. *31*, 135–146. https://doi.org/10.1016/j.scr.2018.07.023.

Ohinata, Y., and Tsukiyama, T. (2014). Establishment of trophoblast stem cells under defined culture conditions in mice. PLoS One *9*, e107308. https://doi.org/10.1371/journal.pone.0107308.

Quinn, J., Kunath, T., and Rossant, J. (2006). Mouse Trophoblast Stem Cells. In Placenta and Trophoblast. Methods in Molecular MedicineTM, *121*, M. Soares, J and J. Hunt, S, eds (Humana Press). https://doi.org/10.1385/1-59259-983-4:123.

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. https://doi.org/10.1126/science.282.5396.2072.