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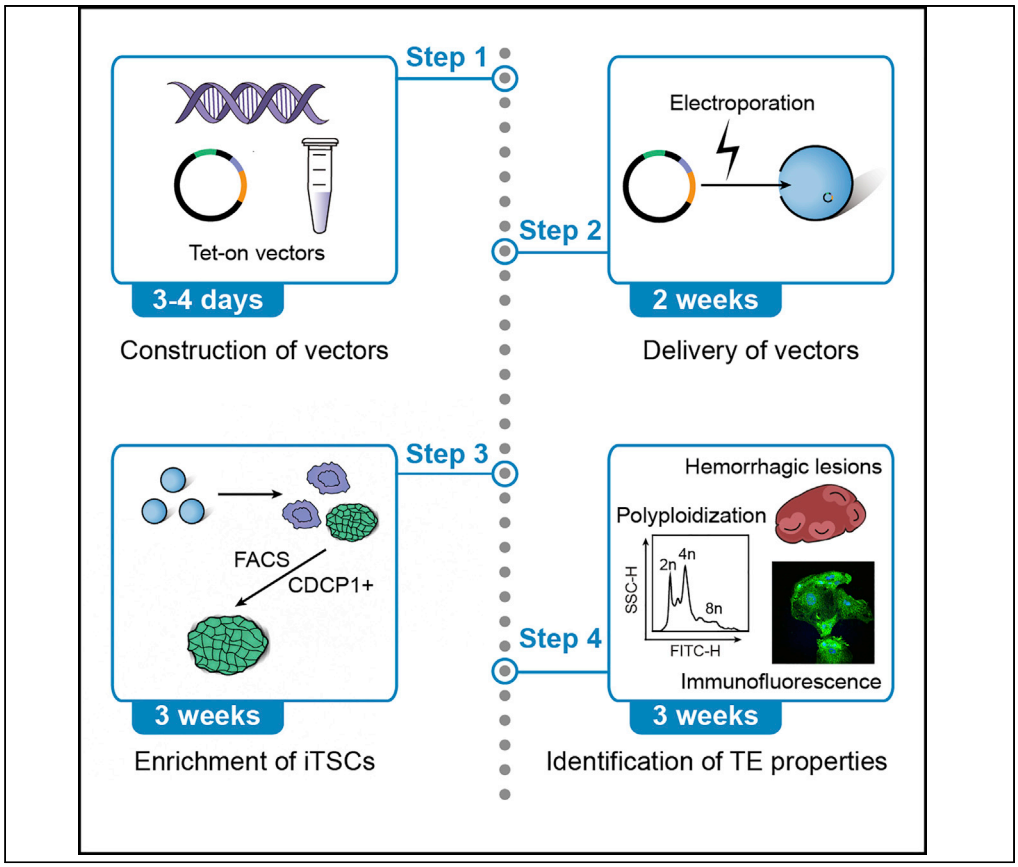
Protocol to generate induced trophoblast stem cells from embryonic stem cells in mice



Conversion of trophectoderm (TE)-derived trophoblast stem cells (TSCs) from inner-cell-mass-derived embryonic stem cells (ESCs) in mice is difficult to achieve naturally. Here, we introduce a reliable and repeatable protocol to generate induced TSCs (iTSCs) from ESCs via a Tet-on system *in vitro*. The iTSCs show typical TSC properties and have the potential to differentiate into syncytiotrophoblast cells (STCs) and trophoblast giant cells (TGCs). This cell fate transition provides a general platform to robustly investigate the mechanisms underlying TE specification.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for inducing a *Hmgn3*-OE Tet-on system into ESCs

Generation of induced TSCs from ESCs with the assistance of *Hmgn3*-OE

TE identification in induced TSCs by immunofluorescence and random differentiation

Zhao et al., STAR Protocols 4, 102092
March 17, 2023
https://doi.org/10.1016/j.xpro.2023.102092

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Protocol to generate induced trophoblast stem cells from embryonic stem cells in mice

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https://doi.org/10.1016/j.xpro.2023.102092

SUMMARY

Conversion of trophectoderm (TE)-derived trophoblast stem cells (TSCs) from inner-cell-mass-derived embryonic stem cells (ESCs) in mice is difficult to achieve naturally. Here, we introduce a reliable and repeatable protocol to generate induced TSCs (iTSCs) from ESCs via a Tet-on system *in vitro*. The iTSCs show typical TSC properties and have the potential to differentiate into syncytiotrophoblast cells (STCs) and trophoblast giant cells (TGCs). This cell fate transition provides a general platform to robustly investigate the mechanisms underlying TE specification.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2022).[1]

BEFORE YOU BEGIN

Construction of *Hmgn3*-overexpression inducible vectors

Timing: 3–5 days

The first cell fate decision yields the first two different lineages, trophectoderm (TE) and inner cell mass (ICM). It is of interest to determine the key barriers between the TE and ICM; thus, TE-derived trophoblast stem cells (TSCs) and ICM-derived embryonic stem cells (ESCs) are ideal platforms to study this issue *in vitro*. Previous research showed that the overexpression (OE) of *Cdx2* or knockdown of *Oct4* induced a TE fate in mouse ESCs. However, stable maintenance of TSCs cannot be achieved in this way, mainly due to the failure of demethylation in TSC-specific genes.[2] Whether there are other critical genes regulating the complete ESC-to-TSC transition in mice is of interest. *Hmgn3* (full name is high mobility group nucleosomal binding domain 3)[3] is highly expressed in many organs, including eyes, and has been reported to be an important gene that promotes the ESC-to-TSC transition.[1] Here, we constructed inducible *Hmgn3* overexpression vectors based on a doxycycline (DOX)-inducible system. Briefly, the coding sequence (CDS) of *Hmgn3* was inserted downstream of TRE-miniCMV to induce exogenous expression of *Hmgn3* in response to DOX (Figure 1A).

1. PBase (SBI, PB210PA-1) was purchased without modification. PB-rtTA-Hyg^r (PB1) is a modified vector in our lab.[4]
2. For constructing the Tet-on *Hmgn3*-OE vector (PB2), design primers for the *Hmgn3*-CDS using SnapGene or other suitable software before vector construction.

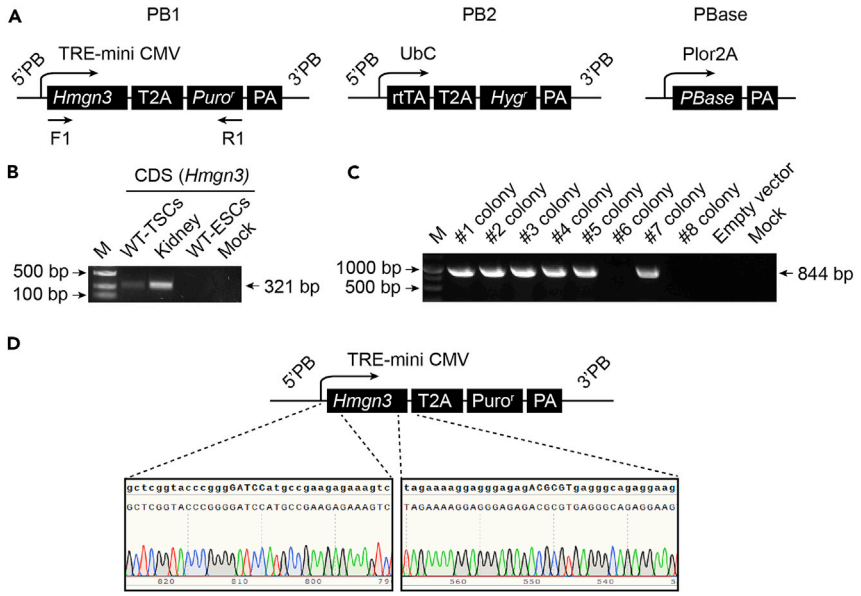

Figure 1. Construction of the Tet-on *Hmgn3*-OE vectors

(A) Schematic overview of the vectors PB1 and PB2. Primers F1/R1 were used for plasmid construction verification.

(B) The fragment (*Hmgn3*-CDS) required for Tet-on *Hmgn3*-OE vector construction. *Hmgn3*-CDS (321 bp) is cloned from TSCs and kidney cDNA. ESC cDNA was used as a negative control.

(C) Colony-PCR for the constructed Tet-on *Hmgn3*-OE vectors amplified by primers F1/R1. Seven colonies were verified to be correct (844 bp). Empty vector was used as a negative control.

(D) Alignment of the successfully ligated *Hmgn3*-CDS fragment in a Tet-on *Hmgn3*-OE plasmid as designed.

⚠ CRITICAL: For primer design, Tm should be lower than 60°C, and the primers are should be reviewed in Primer-Blast of NCBI. The oligo sequence containing 6–8 bases was added to the restriction sites of primers to improve the efficiency of digestion. If you need 2 restriction sites at the same position, the two sites must be separated from another base.

3. Amplify *Hmgn3*-CDS from the cDNA of wild-type (WT) TSCs. Perform PCR using a Phanta® Max Super-Fidelity DNA Polymerase kit and mix the following components in a sterile 200 μL PCR tube. Primer pairs are shown in Table S1.

PCR mix

Reagent	Amount
ddH_2O	Up to 50 μL
2× Phanta Max Buffer	25 μL
dNTP Mix (10 mM)	1 μL
Template (WT-TSCs cDNA)	5–10 ng
Forward primer (*Hmgn3*-CDS-F, 10 μM)	2 μL
Reverse primer (*Hmgn3*-CDS-R, 10 μM)	2 μL
Phanta Max Super-Fidelity DNA Polymerase	1 μL

PCR conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	15 s	35 cycles
Annealing	55°C–60°C	15 s	
Extension	72°C	30 s/kb	

(*Continued on next page*)

Continued			
Steps	Temperature	Time	Cycles
Final extension	72°C	5 min	1
Hold	4°C	forever	–

⚠ CRITICAL: It is important to use a high-fidelity polymerase to minimize error in the amplification of cDNA.

4. Load PCR products onto a 1% (wt/vol) agarose gel in 1× TAE for electrophoresis (Figure 1B) and purify them using a SanPrep Column DNA Gel Extraction Kit.
5. Digest the purified *Hmgn3*-CDS fragment and PB-TRE-Puro^r vector with BamHI and MluI at 37°C for 30 min. The products are purified with a SanPrep Column PCR Product Purification Kit.

Digest reaction mix	
Reagent	Amount
DNA	1–2 μg
10× Thermo Scientific FastDigest Buffer	2 μL
FastDigest Restriction Enzyme I (BamHI)	1 μL
FastDigest Restriction Enzyme II (MluI)	1 μL
ddH_2O	Up to 10 μL

6. Ligate the two products above using T4 ligase according to the manufacturer's instructions.
7. Transform the ligated products into the Trans1-T1 Phage Resistant Chemically Competent Cell following the manufacturer's instructions.
8. Randomly pick 8 colonies to perform individual bacterial colony PCR to identify the inserted DNA (Figure 1C). Primer pairs are shown in Table S1.

⚠ CRITICAL: To avoid false-positive results, the forward and reverse primers must be designed across the inserted fragment and backbone, respectively.

PCR mix	
Reagent	Amount
ddH_2O	6 μL
2× Taq Master Mix	10 μL
Forward Primer (F1, 10 μM)	1 μL
Reverse Primer (R1, 10 μM)	1 μL
Bacteria-water suspension	2 μL

PCR conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	15 s	35 cycles
Annealing	55°C–60°C	15 s	
Extension	72°C	60 s/kb	
Final extension	72°C	5 min	1
Hold	4°C	forever	–

9. Visualize PCR (F1/R1) products on a 1% (wt/vol) agarose gel and culture the correct colonies with an 844 bp band (Figure 1C) for further Sanger sequencing by a local company (Genewiz). Align the sequences with the designed sequence by SnapGene (Figure 1D).

10. Perform endotoxin-free plasmid extraction with a GoldHi Endofree Plasmid Maxiprep Kit. Mix the plasmid DNA using ethanol (2.5-fold of the plasmid volume) and 3 mol/L sodium acetate (pH 5.2, 0.1-fold of the plasmid volume) at −20°C for 2-3 h. Centrifuge the mixture for 30 min at 12,000 × *g* at 4°C. Wash the plasmid pellet using 75% (vol/vol) ethanol and solute it with ddH_2O.

⚠ CRITICAL: Guarantee the elimination of endotoxin to avoid affecting the transfection efficiency.

⚠ CRITICAL: Ensure no ethanol is left in the plasmid solution.

ESC daily culture and passaging

Timing: 4–5 days

WT-ESCs with a 129Sv/Jae background used in this protocol were derived from a zygote blastocyst in our lab with T2i/L medium (see materials and equipment as follows)[5] on tissue culture (TC)-treated dishes with feeder cells (mitomycin-C deactivated murine embryonic fibroblasts (MEFs)) in T2i/L medium. Usually, 1 × 10^5 feeder cells per 35 mm dish (or one well of a six-well plate) would meet the requirements.

11. Thaw a tube of frozen ESCs at 37°C as quickly as possible and resuspend them with 2 mL of MEF medium (see materials and equipment as follows).
12. Centrifuge the cells for 3 min at 200 × *g*, discard the supernatant and resuspend the cell pellet with prewarmed T2i/L medium.
13. Change the MEF medium with the prewarmed T2i/L medium in the dish and seed the single cells of ESCs. Typically, 5.0 × 10^5 ESCs are plated in a 35 mm dish or one well of a six-well plate.
14. Culture the cells at 37°C and 5% CO_2 in an incubator and change the culture medium every day.

⚠ CRITICAL: The cell lines used in this research should be regularly detected as mycoplasma free.

15. Passage the ESCs when the cells reach 80% confluence. To passage ESCs into single cells, incubate cells with 0.05% trypsin-EDTA for 3 min at 37°C. Add two volumes of MEF medium to stop trypsinizing in a 15 mL centrifuge tube. Centrifuge for 3 min at 200 × *g*, discard the supernatant and resuspend the cell pellet with T2i/L medium. Plate these cells in a feeder-coated dish and culture them with T2i/L medium.

TSC daily culture and passaging

Timing: 4–5 days

We cultured WT-TSCs on TC-treated dishes with feeder cells in TSC medium (see materials and equipment as follows).[6] Usually, 1 × 10^5 feeders per 35 mm dish would meet the requirements.

16. Thaw a tube of frozen TSCs at 37°C as quickly as possible and resuspend them with 2 mL of MEF medium.
17. Centrifuge the cells for 3 min at 200 × *g*, discard the supernatant and resuspend the cell pellet with TSC medium.
18. Exchange the MEF medium with the prewarmed TSC medium in the dish and seed the single cells of TSCs. Typically, 5.0 × 10^5 TSCs are plated in a 35 mm dish.
19. Culture the cells at 37°C and 5% CO_2 in an incubator, and change the culture medium every day.

⚠ CRITICAL: The cell lines used in this research should be regularly detected as mycoplasma free.

20. Refer to step 15 above to routinely passage the TSCs.

Transfection of the *Hmgn3*-overexpression vectors into ESCs

Timing: 2 weeks

21. Prepare the plasmid-R buffer (one component of the Neon™ Transfection System kit) mixture containing 3 μg Tet-on *Hmgn3*-OE plasmid, 3 μg PB-rtTA-Hyg^r plasmid and 1 μg PBase plasmids in 100 μL of R buffer.
22. Trypsinize the cells and wash ESCs with PBS with 0.05% trypsin-EDTA for 3 min at 37°C. Stop the trypsinizing with MEF medium and centrifuge the cells at 200 × *g* for 3 min. Discard the supernatant and resuspend the cell pellet with 1 mL of PBS.
23. Count live cells using a cell counter according to the manufacturer's instructions.
24. Centrifuge the cells at 200 × *g* for 3 min and discard the supernatant. Resuspend the cell pellet with plasmid-R buffer mixture (approximately 1 × 10^6 ESCs in per 100 μL of plasmid-R buffer mixture).
25. Electroporate the mixture in 4 mL of E2 buffer (another component of the Neon™ Transfection System kit) using the Neon™ Transfection System at 1,400 V for 10 ms for 3 pulses.
26. Plate the transfected cells on fresh fibronectin (16.7 μg/mL)-recoated dishes with T2i/L medium and culture them at 37°C and 5% CO_2.
27. Add DOX (final concentration: 1 μg/mL) into the medium 2–3 days after electroporation to induce overexpression of *Hmgn3* and puromycin resistant gene (*Puro*^{r}). Change the culture medium with DOX (1 μg/mL) and puromycin (1 μg/mL) every day. After puromycin selection for two days, surviving colonies are potential inducible *Hmgn3*-OE ESCs for further detection (Figure 2A).

⚠ CRITICAL: 1 μg/mL is a suggested concentration of DOX according to our previous protocol.[4] Electroporated ESCs (without DOX) must be prepared and treated with puromycin as a negative control. Puromycin should be withdrawn once all the control cells are dead.

28. Passage the surviving cells according to step 15 above and seed the cells on feeder cells with T2i/L medium.
29. Detect the expression of *Hmgn3*, pluripotent genes and TE marker genes by quantitative PCR (qPCR) in the surviving cells treated with DOX (Figures 2B and 2C). Primer pairs are shown in Table S1.
    a. Precoat the 35 mm dish with 1 mL 0.2% (wt/vol) gelatin at 37°C overnight.
    b. Dissociate the ESCs when the cells reach 80% confluence according to step 15 above. Resuspend the cell pellet with culture medium and plate them on gelatin-coated dish. Incubate them for 30 min in an incubator to separate ESCs from feeder cells.
    c. Collect the cell suspensions and centrifuge at 200 × *g* for 3 min. The pellet of ESCs is prepared for qPCR.

⚠ CRITICAL: The feeder cells more easily adhere to gelatin than ESCs. The attachment of feeder cells to the bottom can be observed under a microscope to eliminate feeder cells.

    d. Extract total RNA using TRIzol reagent according to the manufactures' instructions. Measure the RNA concentration with a NanoDrop™ Spectrophotometer (A260/A280 ratio).
    e. Eliminate genomic DNA and perform reverse-transcription reaction using a Hifair Strand cDNA Synthesis SuperMix for qPCR kit according to the manufacturer's instructions. Detect the expression of marker genes in ESCs by qPCR using qPCR SYBR Green Master Mix (No ROX) according to the manufacturer's instructions on a real-time PCR detection system.

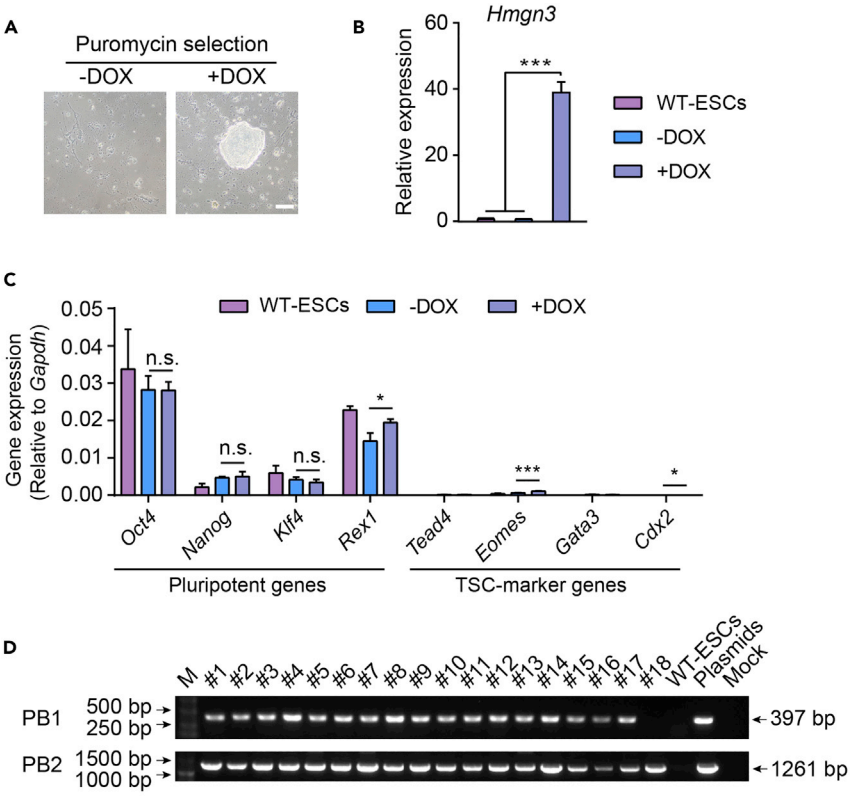

Figure 2. Establishment of the inducible *Hmgn3*-OE system

(A) Bright field image of surviving ESCs with *Hmgn3*-OE vectors with DOX (right) and puromycin selection. The transfected ESCs without DOX (-DOX) were used as a negative control. Scale bar, 100 μm.

(B) The expression levels of *Hmgn3* in *Hmgn3*-OE ESCs treated with and without DOX, with WT-ESCs as a control. t test, ***p < 0.001. Data are represented as the mean G SEM.

(C) The relative expression levels of pluripotent genes (*Oct4*, *Nanog*, *Klf4* and *Rex1*) and TSC marker genes (*Tead4*, *Eomes*, *Gata3* and *Cdx2*) in *Hmgn3*-OE ESCs treated with and without DOX, with WT-ESCs as a control. t test, n.s. not significant, ***p < 0.001.

(D) Genotype of subclones from surviving cells using the primers PB1-F/R and PB2-F/R. WT-ESCs are a negative control, and plasmids are a positive control.

30. Randomly pick 18 subclones from the surviving cells, and culture each subclone in one well of a 24-well plate for expansion. Extract genome DNA and genotype the inserted vectors (PB1 and PB2) using primers PB1-F/R and PB2-F/R, respectively, with WT-ESCs and vectors as controls (Figure 2D).
31. Choose correct subclones for further experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-EOMES (1:500)	Abcam	Cat#ab23345; RRID: AB_778267
Anti-CDX2 (1:500)	Abcam	Cat#ab76541; RRID: AB_1523334
Anti-TPBPA (1 μg/mL)	Abcam	Cat#ab104401; RRID: AB_10901888
Anti-TFAP2C (1:100)	Santa Cruz	Cat#sc-12762; RRID: AB_667770
Anti-CDCP1 (1:100)	R&D Systems	Cat#AF4515; RRID: AB_2078800s
Anti-GCM1 (1:50)	Santa Cruz	Cat# sc-101173; RRID: AB_2108121

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-SDC1 (1:50)	Abclonal	Cat#A4174; RRID: AB_2863202
Anti-CD31 (1:100)	Santa Cruz	Cat#sc-376764; RRID: AB_2801330
FITC Goat Anti-Mouse IgG (H+L) (1:200)	Abclonal	Cat#AS001; RRID: AB_2769475
FITC Goat Anti-Rabbit IgG (H+L) (1:200)	Abclonal	Cat#AS011; RRID: AB_2769476
Alexa Fluor 488 AffiniPure Donkey Anti-Sheep IgG(H+L) Alexa Fluor 488 (1:50)	Yeason	Cat#34906ES60; RRID: AB_2340745
Chemicals, peptides, and recombinant proteins		
DMEM/F-12	Thermo Fisher Scientific	11320033
DMEM	Thermo Fisher Scientific	12800017
RPMI 1640 Medium, GlutaMAX™ Supplement	Thermo Fisher Scientific	61870036
GlutaMAX™ Supplement	Thermo Fisher Scientific	35050061
MEM Non-Essential Amino Acids Solution (100×) (NEAA)	Thermo Fisher Scientific	11140050
Penicillin–Streptomycin	Thermo Fisher Scientific	15140122
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	25300062
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	25300072
Hoechst 33342	Thermo Fisher Scientific	H3570
2-Mercaptoethanol	Thermo Fisher Scientific	21985023
Neon™ Transfection System kit	Thermo Fisher Scientific	MPK10096
Phosphate buffered saline (PBS)	Sigma-Aldrich	D8537
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
Bovine serum albumin[7]	Sigma-Aldrich	A1933
Sodium pyruvate	Sigma-Aldrich	P4562
Triton X-100	Sigma-Aldrich	T8787
Gelatin	Sigma-Aldrich	V900863
Paraformaldehyde (PFA)	Sigma-Aldrich	158127
Fetal bovine serum (FBS)	Biological Industries	04-002-1A
PD0325901	MCE	HY-10254
CHIR-99021	MCE	HY-10182
Doxycycline	MCE	HY-N0565B
Y27632	MCE	HY-10071
Mitomycin-C	MCE	HY-13316
LIF Protein, Mouse, Recombinant	Sino Biological	50756-MNAH
Recombinant Human FGF-4	Peprotech	100-31
Agarose gel	Biowest	111860
TAE Buffer,50×	Solarbio	T1060
TRIzol reagent	Thermo Fisher	15596018
Hifair strand cDNA synthesis SuperMix for PCR	Yeasen	11141ES60
qPCR SYBR Green master mix (no ROX)	Yeasen	H0001451
Fibronectin	Millipore	ECM001
Puromycin	Thermo Fisher	A1113803
Hygromycin	Millipore	400050
Phanta® Max Super-Fidelity DNA Polymerase kit	Vazyme	P505-d1
SanPrep Column DNA Gel Extraction Kit	Sangon Biotech	B518131
SanPrep Column PCR Product Purification Kit	Sangon Biotech	B518141
Trans1-T1 Phage Resistant Chemically Competent Cell	Transgen	CD501-02
GoldHi EndoFree Plasmid Maxi Kit	CWBIO	CW2104
Heparin	Millipore	375095
Experimental models: Cell lines		
WT-ESCs	Our lab	N/A
WT-TSCs	Our lab	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
SCID mice	Vital River Laboratories	N/A
Recombinant DNA		
PB-rtTA-Hygr vector	Xu et al.[4]	N/A
PBase vector	Xu et al.[4]	SBI Plasmid # PB210PA-1
Software and algorithms		
FlowJo™ v10 Software	This paper	https://www.bdbiosciences.com/en-us/products/software/flowjo-v10-software/
ImageJ v1.53c	This paper	https://imagej.nih.gov/ij/
SnapGene	This paper	https://www.snapgene.cn/
GraphPad	This paper	https://www.graphpad.com/
Other		
Research cell sorter	Beckman Coulter	MoFlo Astrios EQ
Research cell sorter analyzer	BD	BD LSRFortessa™ X-20
Confocal microscope	Leica	TCS SP8
Cell incubator	Thermo Fisher	Forma 4111
Neon™ Transfection System	Thermo Fisher	MPK5000
Real-time PCR detection system	Bio-Rad	CFX96
NanoDrop eight	Thermo Fisher	M020
LUNA-II™ Automated Cell Counter	LUNA	LUNA-II
Corning tissue-culture treated culture dishes	Corning	CLS430165
Costar tissue culture plates, 6-well	Corning	3516
Costar tissue culture plates, 24-well	Corning	3524
100 mm tissue culture dish	Corning	430167
0.22-μm sterile syringe filter	Millipore	SLGP033RK
40-μm cell strainer	BD	352340
1.5-mL Eppendorf (EP) tube	Axygen	MCT-150-C
Round Coverslips	Electron Microscopy Sciences	72196-12
200 μL PCR tube	NEST	401001
Sterile 15-mL tube	Corning	430790
Sterile 50-mL tube	Corning	430828
5-mL BD tube	BD	352063

MATERIALS AND EQUIPMENT

"2i" solution stock

PD0325901 (100 mM)	
Reagent	Amount
PD0325901	10 mg
DMSO	207 μL
Prepare aliquots in sterile 1.5-mL EP tubes and store at −80°C for up to 6 months.	

CHIR99021 (50 mM)	
Reagent	Amount
CHIR99021	5 mg
DMSO	2,150 μL
Prepare aliquots in sterile 1.5-mL EP tubes and store at −80°C for up to 6 months.	

Lif solution stock (10^7 U/mL)	
Reagent	Amount
mouse Lif	100 μg
1 × PBS	100 μL
Prepare aliquots in sterile 1.5-mL EP tubes and store at −80°C for up to 6 months.	

⚠ CRITICAL: Do not refreeze after thawing.

Modified T2i/L medium		
Reagent	Final concentration (mM or μM)	Amount
DMEM/F-12	81% (vol/vol)	405 mL
FBS	15% (vol/vol)	75 mL
GlutaMAX	1×	5 mL
NEAA	1×	5 mL
Penicillin–Streptomycin	1×	5 mL
2-Mercaptoethanol	0.1 mM	500 μL
LIF	1,000 U/mL	50 μL
PD0325901	0.2 μM	1 μL
CHIR99021	3 μM	30 μL
Total	**N/A**	**500 mL**
Store at 4°C and use within 2 weeks.		

⚠ CRITICAL: 2-mercaptoethanol is toxic. Use personal protective equipment and discard waste appropriately.

FGF-4 solution stock (0.1 mg/mL)	
Reagent	Amount
FGF-4	100 μg
0.1% (wt/vol) BSA	1 mL
Prepare aliquots in sterile 1.5-mL EP tubes and store at −80°C for up to 8 weeks.	

Heparin solution stock (20 mg/mL)	
Reagent	Amount
Heparin	100 mg
1 × PBS	5 mL
Prepare aliquots in sterile 1.5-mL EP tubes and store at −80°C for up to 6 months.	

TSC basic medium		
Reagent	Final concentration (mM or μM)	Amount
RPMI 1640	N/A	77 mL
FBS	20% (vol/vol)	20 mL
GlutaMAX	1×	1 mL
2-Mercaptoethanol	0.1 mM	100 μL
Sodium pyruvate	1 mM	1 mL
Penicillin–Streptomycin	1×	1 mL
Total	**N/A**	**100 mL**
Store at 4°C and use within 2 weeks.		

TSC medium (70CM+F4H)		
Reagent	Final concentration (mM or μM)	Amount
TSC basic medium	30% (vol/vol)	30 mL
Conditioned medium	70% (vol/vol)	70 mL
Human FGF-4	25 ng/mL	25 μL
Heparin	1 μg/mL	5 μL
Total	**N/A**	**100 mL**
Store at 4°C and use within 1 week.		

MEF medium: Prepare MEF medium composed of the following reagents		
Reagent	Final concentration (mM or μM)	Amount
DMEM	N/A	89 mL
FBS	10%	10 mL
Penicillin–streptomycin	1×	1 mL
Total	**N/A**	**100 mL**
Store at 4°C and use within 1 month.		

TSC differentiation medium		
Reagent	Final concentration (mM or μM)	Amount
DMEM	N/A	82 mL
FBS	15% (vol/vol)	15 mL
2-Mercaptoethanol	0.1 mM	100 μL
NEAA	1×	1 mL
Sodium pyruvate	1 mM	1 mL
Penicillin–Streptomycin	1×	1 mL
Total	**N/A**	**100 mL**
Store at 4°C and use within 1 week.		

Mouse CDCP1 antibody solution. Reconstitute stock solution (0.2 mg/mL) in sterile PBS according to the manufacturer's instructions. Prepare aliquots in sterile 1.5-mL EP tubes and store them at −20°C for up to 6 months. Do not refreeze after thawing.

4% (wt/vol) PFA solution. Dilute 2 g PFA powder in 50 mL of PBS at 65°C to prepare 4% (wt/vol) PFA. Adjust the pH to 7.0–7.2 and store it at −20°C.

STEP-BY-STEP METHOD DETAILS

Induction of iTSCs from *Hmgn3*-OE ESCs

Timing: 3 weeks

1. Precoat the 35 mm TC dish with 1 mL 0.2% (wt/vol) gelatin at 37°C overnight.
2. Dissociate the *Hmgn3*-OE ESCs with 0.05% trypsin-EDTA at 37°C for 3 min and stop trypsinization with MEF medium in 15-mL tubes. Centrifuge at 200 × *g* for 3 min and discard the supernatant.
3. Resuspend the cells with fresh TSC basic medium and plate 5.0×10^4 cells on each gelatin pre-coated dish in TSC basic medium with 1 μg/mL DOX.
4. Culture the cells at 5% CO_2 and 37°C in an incubator, with the medium changed every day. Observe the cell transition every day (Figure 3A).

⚠ CRITICAL: A low confluence of cells in the beginning is necessary to avoid frequent passaging during ESC-to-TSC transition process.

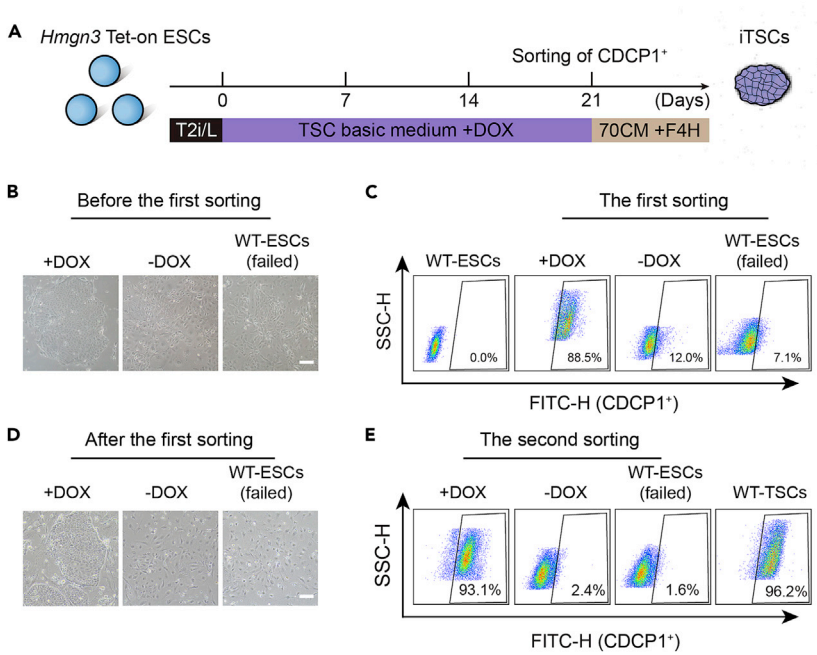

Figure 3. Induction of iTSCs from *Hmgn3*-OE ESCs

(A) Schematic overview of the transition process from *Hmgn3*-OE ESCs to iTSCs.

(B) Morphological images of cell cultures in bright field during the ESC-to-TSC transition. *Hmgn3*-OE (+DOX, before sorting), *Hmgn3*-non-OE (-DOX, before sorting) and WT-ESCs (failed) are shown. Scale bar, 100 μm.

(C) The proportion of CDCP1-positive cells in WT-ESCs, *Hmgn3*-OE iTSCs (+DOX), *Hmgn3*-non-OE (-DOX) and WT-ESCs (failed) at the 1st sorting.

(D) Morphological images of cell cultures in bright field of *Hmgn3*-OE, with *Hmgn3*-non-OE (-DOX) group and WT-ESCs (failed) as controls. Scale bar, 100 μm.

(E) The proportion of CDCP1-positive cells in *Hmgn3*-OE iTSCs (+DOX), *Hmgn3*-non-OE (-DOX), WT-ESCs (failed) and WT-TSCs at the 2nd sorting.

5. After culture for 21 days, approximately 50% typical TSC-like colonies emerged in the cell cultures of the *Hmgn3*-OE group (Figure 3B). CDCP1 is a specific marker of mouse TSCs, the antibody of which is extensively used for TSC enrichment,[8] so the CDCP1-positive cells were sorted for further culture in TSC medium (70CM+F4H).

Sorting for CDCP1-positive cells

Timing: 2 h

6. Dissociate the cells into single cells using 0.05% trypsin-EDTA.
7. Resuspend cells with CDCP1 antibody solution and incubate them at 4°C for 30 min. Usually, 40 μL of anti-CDCP1 solution is suggested per million cells.
8. Centrifuge at 200 × *g* for 3 min, and discard the supernatant. Resuspend cells with 3 mL of PBS to wash the primary antibody by centrifuging and discarding the supernatant.
9. Resuspend cells with 1 mL of diluted Alexa Fluor 488 secondary antibody and incubate them at 4°C for 30 min.
10. Centrifuge at 200 × *g* for 3 min and discard the supernatant. Resuspend cells with 3 mL of PBS to wash the secondary antibody by centrifuging and discarding the supernatant. Resuspend the cells in 0.5 mL of TSC medium.
11. Filter the cells of each sample with a 40-μm cell strainer into a new 5-mL BD tube to remove cell clumps and prepare a control sample (GFP negative) in parallel (Figure 3C).

12. Prepare a harvesting tube with 1 mL of fresh TSC medium. Sort the samples on a research cell sorter according to the following steps.
    a. Find the main population for live cells on the SSC-H versus FSC-H plot.
    b. Find main GFP positive cells by setting the gate according to the GFP negative control.
    c. Sort the cell population of the GFP-positive gate into harvesting tubes.
    d. Transfer the sorted cells from the harvesting tubes into 15-mL tubes, centrifuge at 200 × *g* for 3 min and resuspend in TSC medium.
    e. Plate the cells in a fresh feeder-coated well of a 24-well plate with TSC medium. Usually, 2 × 10^5 cells are seeded in each well of a 24-well plate.

⚠ CRITICAL: To increase cell viability, 10 μM Y27632 could be added to the TSC medium on the first day after sorting.

13. Observe the colony morphology after sorting. Only the *Hmgn3*-OE group can achieve iTSCs with typical colonies, whereas the group without DOX (non-OE group) cannot achieve this (Figure 3D).
14. Confirm the established *Hmgn3*-OE iTSCs by second sorting for CDCP1-positive cells, with the WT-TSCs and non-OE group as controls (Figure 3E).

Characterization of *Hmgn3*-OE iTSCs

Timing: 2 days

15. Detect the TSC markers CDX2, EOMES and TFAP2C in *Hmgn3*-OE iTSCs *via* immunofluorescence (IF) (Figure 4A).
    a. Prepare *Hmgn3*-OE iTSCs and WT-TSCs on 0.2% (wt/vol) gelatin-coated coverslips for IF.
    b. Fix *Hmgn3*-OE and WT-TSCs in 4% (wt/vol) PFA for 15 min at 25°C.
    c. Permeabilize and block the cells with 2% (wt/vol) BSA (supplemented with 0.1% Triton X-100 in PBS) for 2 h at 25°C.
    d. Incubate cells with primary antibodies overnight at 4°C.
    e. Wash coverslips in PBS 3 times (5–10 min each).
    f. Incubate cells with secondary antibodies for 1 h at 25°C. Wash coverslips in PBS 3 times (10–20 min each).
    g. Incubate cells with Hoechst 33342 for 15 min at 25°C. Wash coverslips in PBS 3 times (5 min each).
    h. Capture images on a SP8 confocal microscope.

Differentiation of *Hmgn3*-OE iTSCs

Timing: 3 weeks

16. Differentiate *Hmgn3*-OE iTSCs and WT-TSCs *in vitro* (Figures 4B and 4C).
    a. Place round coverslips into the wells of 24-well plates coated with 0.2% (wt/vol) gelatin overnight.
    b. Plate the *Hmgn3*-OE iTSCs and WT-TSCs onto gelatin-coated round coverslips and culture them with TSC differentiation medium at 37°C and 5% CO_2.
    c. Culture them for 6 days to induce random differentiation.
    d. Fix cells and identify the trophoblast lineage-specific markers TPBPA, SDC1 and GCM1 by IF.
17. Perform DNA content analysis by PI staining (Figure 4D).
    a. Precool prepared 75% ethanol solution at 4°C.
    b. Centrifuge the differentiated cells from *Hmgn3*-OE iTSCs and WT-TSCs (on Days 0, 2, 4, and 6) at 200 × *g* for 5 min and discard the supernatant.

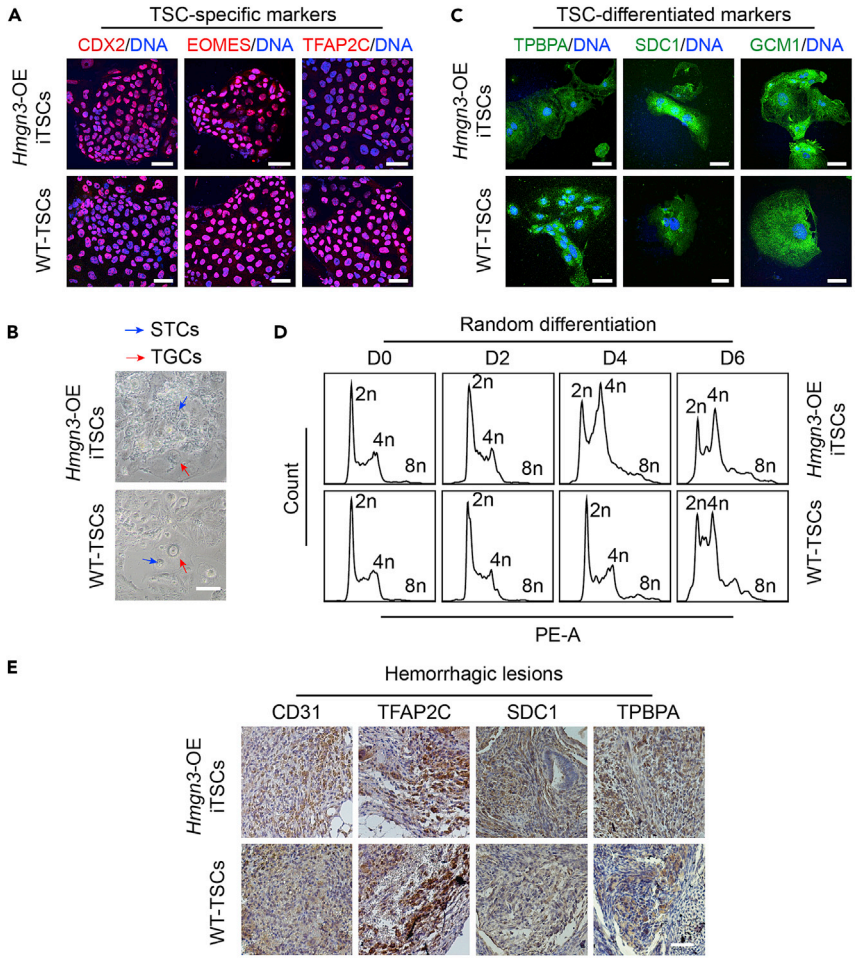

Figure 4. Characteristics of *Hmgn3*-OE iTSCs

(A) IF of TSC markers (CDX2, EOMES and TFAP2C) in *Hmgn3*-OE iTSCs and WT TSCs. DNA was stained with Hoechst 33342 (5 μg/mL Hoechst 33342 staining solution (diluted in PBS)). Scale bar, 50 μm.

(B) Morphological images of cell cultures in bright field of differentiated cells from *Hmgn3*-OE iTSCs. The differentiated cells from WT-TSCs are the control. The blue arrow indicates syncytiotrophoblast cells (STCs), and the red arrow indicates trophoblast giant cells (TGCs). Scale bar, 100 μm.

(C) IF of STC marker (TPBPA), STB marker (SDC1) and TGC marker (GCM1) in the randomly differentiated derivatives from iTSCs. DNA was stained with Hoechst 33342. Scale bar, 50 μm.

(D) DNA content analysis of *Hmgn3*-OE iTSCs during random differentiation on Days 0, 2, 4 and 6. The diploid (2n), tetraploid (4n), and octaploid (8n) DNA contents are indicated. The differentiation of WT-TSCs was used as a control.

(E) IHC analysis of hemorrhagic lesions derived from *Hmgn3*-OE iTSCs and WT-TSCs *in vivo* for the endothelial marker CD31 and the trophoblast markers TFAP2C, SDC1 and TPBPA. Scale bar, 20 μm.

c. Resuspend the cells with 200 μL of PBS, add 4 mL of precooled 75% ethanol solution slowly and mix gently. Place the tube at 4°C for at least one night.

⚠ CRITICAL: The 75% ethanol solution must be freshly prepared. Centrifuge the cells at 200 × *g* for 5 min and discard the supernatant.

d. Resuspend cells with 50 μg/mL PI staining solution (diluted in PBS) and incubate them in a water bath for 20 min at 37°C in the dark.

e. Centrifuge the cells at 200 × *g* for 5 min, discard the supernatant, and resuspend the cells in PBS.

f. Perform DNA content analysis on a research cell sorter analyzer.

18. Differentiation of *Hmgn3*-OE iTSCs *in vivo*.
    a. Prepare 1 × 10^7 *Hmgn3*-OE iTSCs and WT-TSCs independently. Dissociate the cells as in step 15 in the before you begin. Resuspend cell pellet with 150 μL of PBS in a 1.5 mL EP tube and plate them on ice.
    b. Inject cell suspensions of *Hmgn3*-OE iTSCs and WT-TSCs into the limbs of an 8-week-old male SCID mouse independently.
    c. Dissect the fully-grown hemorrhagic lesions on Day 21 and fix them with 4% PFA overnight at 4°C.
19. Perform paraffin section according to the manufacturer's instruction. Use primary antibodies against the endothelial marker CD31 and the trophoblast markers TFAP2C, SDC1 and TPBPA to perform immunohistochemistry (IHC) analysis. Capture images on a microscope (Figure 4E).

EXPECTED OUTCOMES

In this protocol, we show our design and the construction steps of the DOX-inducible *Hmgn3*-OE vectors in detail (Figure 1A). For PB1 vector construction, we inserted the *Hmgn3*-CDS fragment (amplified from TSC cDNA) (Figure 1B) into the PB basic backbone with the TRE mini-CMV cassette and *Puro*^{r} gene (Figure 1A). The completed vector sequence was confirmed by Sanger sequencing (Figure 1D). After electroporation, induction of *Hmgn3*-OE and enrichment of *Hmgn3*-OE cells (Figure 2A), we derived inducible *Hmgn3*-OE ESCs (Figure 2D). The inducible *Hmgn3*-OE ESCs could maintain their ESC morphology and differentiation potential during the continuous overexpression of *Hmgn3* in T2i/L medium for long-term culture, as we have reported.[1]

The protocol for iTSCs induced from ESCs is detailed, which guarantees the successful generation of highly purified iTSCs with differentiation potential, and the iTSCs could maintain TSC properties for at least 30 passages without karyotypic changes. We also provide a comprehensive platform to validate the effect of candidate genes related to the transition from ESCs to TSCs. According to this protocol, iTSCs could be induced from *Hmgn3*-OE ESCs in TSC basic medium and enriched by CDCP1-positive cell sorting during transition (Figure 3). The detailed introduction of IF, PI staining and IHC provides the methodology for TSC property identification (Figure 4).

LIMITATIONS

The derivation of iTSCs requires regular cell sorting. Physical and chemical injuries during sorting limit the efficiency of deriving iTSCs. Another concern is the heterogeneity. After the conversion of TSCs from ESCs, it is necessary to sort the CDCP1-positive cells periodically to purify iTSCs (usually 2–3 weeks). The mechanism behind this transition needs further study, and it would be helpful to optimize the transition.

TROUBLESHOOTING

Problem 1

It is difficult to generate pure typical TSC-like colonies during the transition (see steps 1–5).

Potential solution

During the transition, cell cultures expand quickly in the beginning. Therefore, ESCs with low starting confluence are necessary to avoid frequent passaging during transition and improve the survival efficiency. In addition, some EpiSC-like cells (relying on FGF signaling for self-renewal) are generated during the transition and are partially CDCP1-positive. To reduce the EpiSC-like cells, FGF4 should not be added to the medium during the transition before the first sorting for CDCP1 antibody.

Problem 2

There are some false-positive cells during CDCP1 antibody sorting when preparing samples (see steps 6–14).

Potential solution

During CDCP1 antibody staining, washing the secondary antibody with PBS carefully is necessary; otherwise, the nonspecific staining of the secondary antibody results in false-positive results. The proportion of CDCP1-positive cells in the first sorting was approximately 80%. If the proportion is extremely high, it is probably caused by the nonspecific staining of secondary antibody.

Problem 3

The efficiency of the ESC-to-TSC transition is very low (see steps 6–14).

Potential solution

The process of the ESC-to-TSC transition relies on high-qualified FBS; if the FBS is low grade, it is difficult to generate iTSCs. You should choose ESC-standard qualified FBS in this procedure. To exclude the influence of FBS, a serum-free system named TX medium could also be an option.[9]

Problem 4

Cell viability is low after electroporation (see steps 21–26 in the before you begin) or sorting (see steps 6–14).

Potential solution

For cell damage caused by electroporation, the addition of 10 μM Y27632 into the culture medium for 24 h after plating is helpful. During cell sorting, an excessively fast sample current speed would impact the purity and viability of the sorted cells. Reducing the sample current speed (at most 10,000 cells per second) optimizes the cell viability. The addition of 10 μM Y27632 into the culture medium for 24 h after plating is also suitable for the sorted cells.

Problem 5

There are not enough cell numbers during the differentiation by DNA content analysis (see steps 16 and 17).

Potential solution

The differentiation of iTSCs is always accompanied by cell death. Abundant starting cell numbers for differentiation are necessary, especially for the Day 6 group.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and would be fulfilled by the lead contact, Ling Shuai, PhD, lshuai@nankai.edu.cn.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102092.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (2019YFA0109901 to L.S.), the National Natural Science Foundation of China (32022025 and 31872841 to L.S., 82201840 to Q.G.), the Strategic Collaborative Research Program of the Ferring Institute of Reproductive Medicine (FIRMD181102 to L.S.), and funding from the National Clinical

Research Center for Obstetrics and Gynecology (Peking University Third Hospital) (BY-SYSZKF2021007 to L.S.).

AUTHOR CONTRIBUTIONS

Y.Z., Q.W., W.Z., and Q.J. performed the experiments. Y.Z., W.Z., and L.S. wrote the manuscript draft. L.S. and Q.G. provided funding, supervised this project, and reviewed the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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