Fast In Vitro Procedure to Identify Extraembryonic Differentiation Defect of Mouse Embryonic Stem Cells

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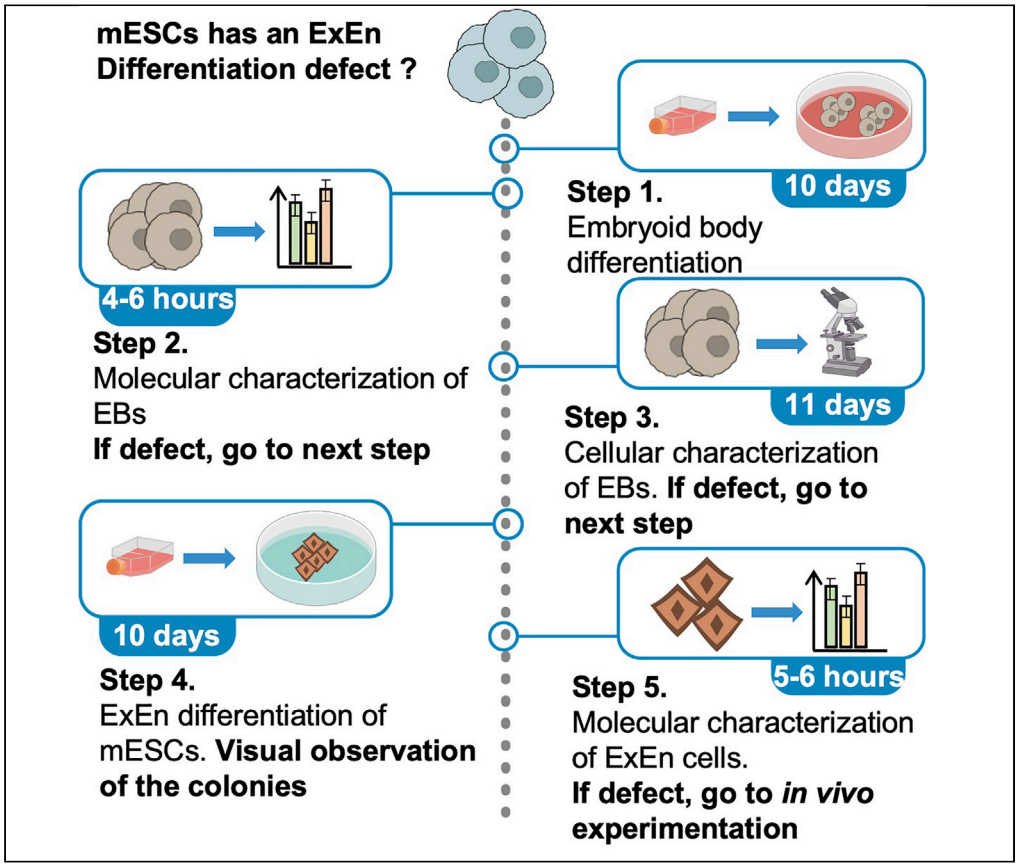

Mouse embryonic stem cells (mESCs) are a powerful model to study early mouse development. These blastocyst-derived cells can maintain pluripotency and differentiate into the three embryonic germ layers and an extraembryonic layer, the extraembryonic endoderm (ExEn), which shares similar molecular markers to the definitive endoderm. Here, we present a fast procedure to identify a differentiation defect of mESCs toward ExEn *in vitro* through the molecular and cellular characterization of embryoid bodies, followed by direct differentiation of mESCs into ExEn.

Richard Patryk Ngondo, Michel Cohen-Tannoudji, Constance Ciaudo

patryk.ngondo@ibmp-cnrs.unistra.fr (R.P.N.)
cciaudo@ethz.ch (C.C.)

HIGHLIGHTS

In vitro protocols to identify extraembryonic endoderm differentiation defect of mESCs

Molecular and cellular characterization of embryoid bodies

Molecular characterization of extraembryonic endoderm cells

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Richard Patryk Ngondo,[3,4,5,*] Michel Cohen-Tannoudji,[2] and Constance Ciaudo[1,6,*]

[1]Swiss Federal Institute of Technology Zurich, IMHS, Chair of RNAi and Genome Integrity, 8093 Zurich, Switzerland

[2]Early Mammalian Development and Stem Cell Biology, Institut Pasteur, CNRS UMR 3738, 25 rue du docteur Roux, 75015 Paris, France

[3]University of Strasbourg, Strasbourg, France

[4]Institut de Biologie Moléculaire des Plantes UPR-CNRS 2357, 12 rue du Général Zimmer, 67084 Strasbourg, France

[5]Technical Contact

[6]Lead Contact

*Correspondence: patryk.ngondo@ibmp-cnrs.unistra.fr (R.P.N.), cciaudo@ethz.ch (C.C.)
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SUMMARY

Mouse embryonic stem cells (mESCs) are a powerful model to study early mouse development. These blastocyst-derived cells can maintain pluripotency and differentiate into the three embryonic germ layers and an extraembryonic layer, the extraembryonic endoderm (ExEn), which shares similar molecular markers to the definitive endoderm. Here, we present a fast procedure to identify a differentiation defect of mESCs toward ExEn *in vitro* through the molecular and cellular characterization of embryoid bodies, followed by direct differentiation of mESCs into ExEn.

For complete details on the use and execution of this protocol, please refer to Ngondo et al. (2018).

BEFORE YOU BEGIN

This protocol should be used to identify ExEn differentiation defects of a mutant mESC line compared to correctly differentiating control wild-type mESCs.

⚠ CRITICAL: When proceeding with this protocol always perform in parallel a control experiment using a control mESC wild-type line.

We use for this protocol mESCs adapted to grow on gelatin-coated plates. If your cells grow on feeders, you would need to first adapt them to gelatin-coated plates.

Preparing Reagents

Timing: 0.5–1 h

1. Prepare culture media and preheat all culture reagents at 37°C.
2. Before thawing cell, prepare gelatin-coated culture flasks. Pour 1–2 mL of 0.2% gelatin on the culture vessel (25 cm^2 = T25) and let it rest for at least 15 min at 20°C–25°C or leave it 16–24 h in the cell culture incubator.

⚠ CRITICAL: The gelatin must cover the entire culture surface.

Thawing mESCs

Timing: 0.5–1 h

Note: We routinely freeze cells in serum supplemented with 10% DMSO. Each CryoTube vial contains about 2–3 million cells in 1 mL of freezing medium.

3. Take out a stock vial from the liquid nitrogen storage tank.
4. Put cells in 37°C water-bath until thawed and no more than 5 min.
5. Pipet the whole volume of cells into a 15 mL tube containing 3 mL of preheated MF culture medium.
6. Centrifuge 5 min at 182 × *g* to pellet the cells and discard the supernatant.
7. Remove the gelatin from the flask (25 cm^2 = T25).
8. Resuspend pellet in 4 mL preheated ESC medium and transfer to the flask.
9. Let the cells grow in a humidified incubator at 37°C and 8% CO_2.

⚠ CRITICAL: Change culture media the next morning. To change the medium, pour out the ESC medium and replace it with 4 mL of fresh ESC medium.

Maintaining and Passaging mESCs

Timing: 0.5 h

Cells should be passaged at least once before starting any experiment.

Passage the cells every 2–3 days by diluting them 1:10.

10. Prepare a gelatin-coated T25 flask.
11. Harvest mESCs by first pouring out the ESC medium. Then, gently wash the cells 1 time with 3 mL PBS 1× by slowly tilting several times the T25 flask.
12. Discard the PBS 1× and add 1 mL of 0.05% Trypsin-EDTA. Then, incubate 5 min at 37°C.
13. Add 3 mL MF culture medium to the flask and dissociate into individual cells by pipetting up and down with a 5 mL pipette.
14. Add 400 µL of the cellular suspension to a gelatin-coated T25 flask containing 4 mL of ESC medium.
15. Let the cells grow in a humidified incubator at 37°C and 8% CO_2.

⚠ CRITICAL: The cells should not reach 100% confluency. The ideal confluency should be under 80% but the most important is that the individual colonies should not be in contact with each other (Figure 1).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat Monoclonal Anti-Nanog (eBioMLC-51)	eBioscience	14-5761-80
Rabbit monoclonal Anti-FOXA2 (EPR4466)	Abcam	ab108422
Goat polyclonal Anti-GATA6	R&D Systems	AF1700
Goat polyclonal Anti-SOX17	R&D Systems	AF1924

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Goat polyclonal Anti-GATA4 (C-20)	Santa Cruz	sc-1237
Mouse monoclonal Anti-DAB2 (52/p96)	BD bioscience	610464
Alexa Fluor-conjugated secondary antibodies	Invitrogen	A32723 A32731 A32849 A11006
Chemicals, Peptides, and Recombinant Proteins		
Trizol	Life Technologies	15596018
ESGRO recombinant mouse LIF protein	Millipore	ESG1107
DMEM Medium	Sigma-Aldrich	D6429-500ML
Penicillin/Streptomycin	Sigma-Aldrich	P0781-100ML
0.05% Trypsin-EDTA	Life Technologies	25300054
PBS 1×	Life Technologies	10010015
2-β-mercaptoethanol	Life Technologies	31350010
FBS	Life Technologies	10270-106
RPMI 1640 Glutamax	Life Technologies	100305
Retinoic acid	Sigma-Aldrich	R2625
Gelatin powder	Sigma-Aldrich	G-1890
DMSO	Sigma-Aldrich	D8418-100ML
BSA	Sigma-Aldrich	05470
Paraformaldehyde (PFA)	Sigma-Aldrich	158127
Hoechst	Sigma-Aldrich	B2261
Sucrose	Sigma-Aldrich	S7903
OCT Compound	Sakura	4583
Triton X-100	Sigma-Aldrich	X100
Vectashield	Vector lab	H-1000
Fetal calf serum	Fisher scientific	11563397
Normal donkey serum	Dutscher	S2170-100
Goat serum	Thermofischer	16210064
Critical Commercial Assays		
KAPA SYBR FAST for Roche LightCycler 480	Sigma-Aldrich	KK4611
GoScript KIT	Promega	A2801
Experimental Models: Cell Lines		
WT Mouse ESCs (E14Tg2a)	ATCC	CRL-1821
Ago2_KO mESCs	Ngondo et al., 2018	n/a
Oligonucleotides		
qPCR primers	This paper	Tables 1, 2, and 4
Other		
Nunc® CryoTube® vials	Sigma-Aldrich	V7884-450EA
6-Well plates for mESCs and ExEn	TPP	TPP92006
Petri dishes 94/16 MM for EB	Greiner	633180
T75: 75 cm^2 Filter Flask	TPP	TPP90076
T25: 25 cm^2 Filter Flask	TPP	TPP90026
Cryomold	Fisher scientific	NC9511236

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SuperFrost Plus histological slides	Fisher scientific	10149870
Transport box for 5 slides	Dutscher	049723
50 mL conical bottom falcons	Bioswisstec	53050
15 mL conical bottom falcons	Greiner bio-one	188271
Safe-Lock Tubes 1.5 mL tubes	Eppendorf	0030 120.086
Dakopen	Agilent	S200230-2
Light Cycler® 480 Instrument	ROCHE	n/a
Zeiss Axiovert 200M with a Zeiss apotome system	ZEISS	n/a
Cryostat CM 3050S	Leica	n/a

MATERIALS AND EQUIPMENT

Culture Conditions

mESCs and mExEn are maintained on 0.2% gelatin-coated TPP cell culture dishes at 8% CO_2, 37°C.

Gelatin 0.2% Solution

1. Weight 1 g of gelatin and put it in the 500 mL empty autoclaved bottle.
2. Add 500 mL of autoclaved water.
3. Mix vigorously.
4. Put the bottle in the microwave until the powder is dissolved (between 2–5 min).

⚠ CRITICAL: Do not close the bottle lid completely. If too many bubbles appear, stop, mix, and restart the microwave.

5. Make sure that gelatin is completely diluted and the solution is homogeneous.
6. Autoclave the bottle.
7. Make aliquots, under the hood: 45 mL in 50 mL Falcon and store them at 4°C.

ESC Medium (Serum Culture)

Reagent	Stock Concentration	Final Concentration	Amount
DMEM	n/a	n/a	42 mL
FBS (ES tested)	n/a	15%	7.5 mL
LIF	10^7 U/mL	1,000 U/mL	5 µL
2-β-mercaptoethanol	50 mM	0.1 mM	100 µL
Penicillin/Streptomycin	10,000 U/ mL	100 U/ mL	500 µL
Total			**50 mL**

MF Medium (Differentiation Medium)

Reagent	Stock Concentration	Final Concentration	Amount
DMEM	n/a	n/a	44.5 mL
FBS	n/a	10%	5 mL
2-β-mercaptoethanol	50 mM	0.1 mM	100 µL
Penicillin/Streptomycin	10,000 U/ mL	100 U/ mL	500 µL
Total			**50 mL**

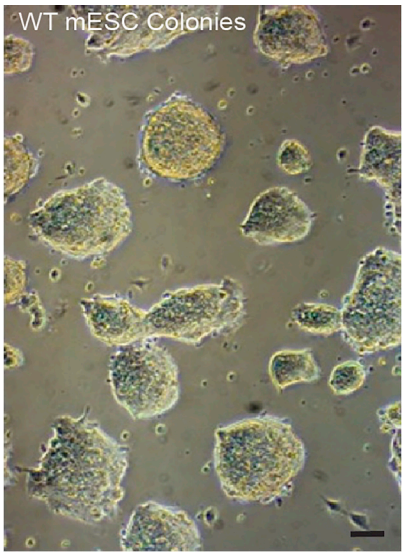

Figure 1. Representative Picture of WT mESC Growing at 70%–80% Confluency on Gelatin-Coated Plates
The scale bar is 50 μm.

ExEn Medium

Reagent	Stock Concentration	Final Concentration	Amount
RPMI 1640 Glutamax	n/a	n/a	42 mL
FBS	n/a	15%	7.5 mL
2-β-mercaptoethanol	50 mM	0.1 mM	100 μL
Activin A[a]	50 mg/mL	10 ng/mL	10 μL
Retinoic acid[a]	1 mM (in DMSO)	0.1 μM	5 μL
Penicillin/Streptomycin	10,000 U/mL	100 U/mL	500 μL
Total			**50 mL**

[a]Add Activin A and Retinoic acid just before use in required volume.

Note: Reconstitute Activin A in water supplemented with 1% BSA.

⚠ CRITICAL: Retinoic acid is toxic for reproduction. Please read the MSDS before working with this chemical. Gloves and safety glasses should be worn.

⚠ CRITICAL: Protect retinoic acid from light.

STEP-BY-STEP METHOD DETAILS

This protocol includes three major steps:

1. Perform an ESC differentiation into embryoid bodies.
2. Analyze the differentiation outcome to identify an extraembryonic differentiation defect using molecular and cellular approaches.
3. Characterize the identified defect using a directed differentiation protocol of mESCs into extra-embryonic endoderm. We recommend doing this series of *in vitro* experiments before attempting *in vivo* approaches.

Embryoid Body Differentiation

Timing: 10 days

The embryoid body differentiation protocol is classically used to spontaneously differentiate mESCs into the three embryonic germ layers (Robertson, 1987; Keller, 1995), but is rarely used to assess extraembryonic endoderm defects. The first steps of the procedure are similar to the previously described protocol (Bodak and Ciaudo, 2016), with minor changes consisting on non-plating back the cells on adherent gelatin-coated plates, allowing the cellular analysis described further.

4. Start the EB differentiation with 80% confluent mESC T25 flask (Figure 1).
5. Prepare a T75 gelatin-coated flask– add 2 mL of 0.2% gelatin to a T75 flask and incubate at 37°C minimum 15 min before adding cells.

Note: Make sure to distribute the gelatin on the whole surface of the flask by tapping and tipping the flask.

6. Harvest mESCs by first pouring out the ESC medium. Then, wash gently the cells 1 time with 3 mL PBS 1× by tilting the T25 flask
7. Discard the PBS 1× and add 1 mL of 0.05% Trypsin-EDTA before to incubate 5 min at 37°C.
8. Add 3 mL MF culture medium to the flask and dissociate into individual cells by pipetting up and down with a 5 mL pipette.
9. Transfer 2 mL of cell suspension into 2 × 15 mL Falcons (Falcons A and B) and centrifuge 5 min at 182 × *g*.
10. Falcon A is used to amplify undifferentiated mESCs to collect dry pellets (sample day0):
    a. Remove the gelatin from the T75 flask.
    b. Carefully remove the supernatant from falcon A and resuspend the pellet in 12 mL of mESC medium.
    c. Dissociate into individual cells by pipetting up and down with a 10 mL pipette.
    d. Transfer the cells in the T75 flask and Incubate at 37°C, 8% CO_2.
    e. Change the culture media the next morning, by pouring out the ESC medium and replace it with 12 mL of fresh ESC medium.
    f. When the T75 flask is 80%–90% confluent wash 1 time with 4 mL PBS 1×.
    g. Add 2 mL of 0.05% Trypsin-EDTA and incubate 5 min at 37°C.
    h. Add 4 mL MF culture medium to flask and dissociate into individual cells by pipetting up and down with a 5 mL pipette.
    i. Transfer cells to 15 mL Falcon and centrifuge 5 min at 182 × *g*.
    j. Aspirate the supernatant.
    k. Gently resuspend the cells in 3 mL PBS 1×.
    l. Transfer to labeled 3 × 1.5 mL Eppendorf tube (mESCs day0).
    m. Centrifuge 182 × *g* for 5 min.
    n. Aspirate supernatant.
    o. Freeze dry pellet at −80°C.

Pause Point: The collected pellets can be stored at −80°C for several months.

11. Annotate under the hood 2× Petri dishes (10 cm): EB day6 and EB day10.

⚠ CRITICAL: The petri dishes are not cell culture treated (we use bacterial petri dishes). The cells here should not attach to the dish in order to form aggregates.

Troubleshooting 1.

12. Falcon B is used to start the EB differentiation and obtain the samples day6 and day10:
    a. Carefully remove the supernatant from falcon B and resuspend the pellet in 10 mL of MF medium.
    b. Dissociate into individual cells by pipetting up and down with a 10 mL pipette.
    c. Transfer the cells in the two petri dishes (5 mL each) and add to each dish 5 mL of MF medium for a total of 10 mL of medium.
    d. Incubate at 37°C, 8% CO_2.

⚠ CRITICAL: Do not manipulate the petri dishes during the 3 first days of the differentiation to let the cells aggregate properly.

    e. At day 3 of differentiation change the medium of the 2 petri dishes.
    f. With a 10 mL pipette, transfer gently the medium and bodies into a 15 mL falcon.
    g. Add 5 mL of fresh MF medium into the 2 petri dishes.
    h. Wait 10 min that the EB sediment at the bottom of the falcon tube.
    i. Carefully remove the supernatant.
    j. Add 5 mL of fresh MF medium into the falcon tubes and transfer back the EBs in their original petri dishes.

Note: Repeat the medium replacement every 2–3 days depending on the acidification of the medium. Keep checking the cells every day and change the medium if you observe a color change toward light orange-yellow.

13. At days 6 and 10, collect EBs for molecular characterization:
    a. With a 10 mL pipette, transfer the medium and bodies into a 15 mL falcon.
    b. Centrifuge at 182 × *g* for 5 min.
    c. Gently resuspend the cells in 2–3 mL PBS 1×.
    d. Transfer to labeled 2–3 × 1.5 mL Eppendorf tube (mESCs day6 or day10).
    e. Centrifuge 182 × *g* for 5 min.
    f. Aspirate supernatant.
    g. Freeze dry pellet at −80°C.

Pause Point: The collected pellets can be stored at −80°C for several months.

Molecular and Cellular Characterization of EBs

The molecular characterization of the EBs will allow the identification of differentiation defects from the three embryonic germ layers. If a defect is observed for endoderm markers, a cellular characterization of the EBs is necessary to distinguish between extraembryonic endoderm and definitive endoderm defects as both endoderm layers share the same molecular markers.

Molecular Characterization of EBs

Timing: 5–6 h

The expression of three germ layers markers is analyzed by RT-qPCR.

14. Previously collected frozen pellets are directly used for RNA extraction.

Note: The methods of RNA extraction and reverse transcription are not specific. Step-by-step protocols used in the laboratory has been already described in (Bodak & Ciaudo 2016). We usually perform the reverse transcription reaction using 1–2 µg of RNA.

15. Perform quantitative PCR (qPCR) with the following markers (Table 1) to assess ectoderm, endoderm, and mesoderm formation.
    a. Example of qPCR reaction 1×

Table 1. Markers for EB Differentiation

Primers for mRNAs qPCR		Purpose
Oct4_Fw	CAACTCCCGAGGAGTCCCA	Pluripotency marker
Oct4_Rev	CTGGGTGTACCCCAAGGTGA	
Nanog_Fw	CAGAAAAACCAGTGGTTGAAGA	Pluripotency marker
Nanog_Rev	GCAATGGATGCTGGGATACTC	
Sox2_Fw	CACAGATGCAACCGATGCA	Pluripotency marker
Sox2_Rev	GGTGCCCTGCTGCGAGTA	
Nestin_Fw	CTCTTCCCCCTTGCCTAATACC	Ectoderm marker
Nestin_Rev	TTTAGGATAGGGAGCCTCAGACAT	
Fgf5_Fw	GCCTGTCCTTGCTCTTCCTCAT	Ectoderm marker
Fgf5_Rev	GGAGAAGCTGCGACTGGTGA	
Pax6_Fw	TAACGGAGAAGACTCGGATGAAGC	Ectoderm marker
Pax6_Rev	CGGGCAAACACATCTGGATAATGG	
Brachyury_Fw	TCTCTGGTCTGTGAGCAATGGT	Mesoderm marker
Brachyury_Rev	TGCGTCAGTGGTGTGTAATGTG	
Fgf8_Fw	CGCTTTAGTTGAGGAACTCGAAGCG	Mesoderm marker
Fgf8_Rev	ACATGGCCTTTACCCGCAAG	
Actc1_Fw	CCAAAGCTGTGCCAGGATGT	Mesoderm marker
Actc1_Rev	GCCATTGTCACACACCAAAGC	
Gata6_Fw	GAAGCGCGTGCCTTCATC	General endoderm marker
Gata6_Rev	GTAGTGGTTGTGGTGTGACAGTTG	
Gata4_Fw	CTGGAAGACACCCCAATCTC	General endoderm marker
Gata4_Rev	GTAGTGTCCCGTCCCATCTC	
Dab2_Fw	TTGATGATGTGCCTGATGCT	General endoderm marker
Dab2_Rev	TTTGCTTGTGTTGTCCCTGA	
Rrm2_Fw	CCGAGCTGGAAAGTAAAGCG	Normalizer gene
Rrm2_Rev	ATGGGAAAGACAACGAAGCG	

Reagent	Amount
KAPA SYBR® FAST qPCR Master Mix (2×)	5 μL
cDNA (Reverse Transcription reaction product diluted 5×)	2 μL
Fw primer (5 μM)	1 μL
Rev primer (5 μM)	1 μL
H_2O	1 μL
Total	**10 μL**

b. Example of settings for qPCR

PCR Cycling conditions				
Steps	Temperature	Acquisition	Time	Cycles
Initial Denaturation	95°C	None	3 min	1
Denaturation	95°C	None	10 sec	

(*Continued on next page*)

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PCR Cycling conditions				
Annealing	60°C	None	20 sec	40
Extension	72°C	Single	1 sec	
	95°C	None	5 sec	
Melting curve	65°C	None	1 min	1
	97°C	Continuous	5–10 acq/°C	
Cooling	40°C	None	10 sec	1

Note: We performed qPCR on a LightCycler® 480 instrument (Roche, Penzberg). To analyze the qPCR, we calculate the differences between samples and controls using the $2^{-\Delta CT}$ method and we use Rrm2 transcript as a normalizer. The expression level of each marker in differentiated cells is calculated relative to its respective non-differentiated control sample.

16. Analyze your qPCR results as presented in "Quantification and Statistical Analysis" section (Table 5).

Troubleshooting 2.

Anticipated results for the molecular characterization of EBs are presented in the "Expected Outcomes" section.

Cellular Characterization of EBs

Timing: 1–2 days

The cellular characterization of the EBs will allow the identification of a specific extraembryonic differentiation defect.

17. Prepare the solutions.
    a. PFA 4% solution. You can purchase concentrated liquid PFA to dissolve in PBS 1× or start from powder. To prepare 100 mL of 4% PFA solution in PBS 1× from powder, dissolve 4 g of PFA in 80 mL of PBS 1× under the hood on a stir plate at 60°C. Add 1 N NaOH to rise the pH dropwise until the solution clears. Then adjust the pH to 6.9 with HCL. Fill with PBS 1× to 100 mL.

⚠ CRITICAL: Formaldehyde is toxic. Please read the MSDS before working with this chemical. Gloves and safety glasses should be worn and solutions made inside a fume hood.

    b. PBS/0.25% Triton X-100: add 500 µL of Triton 10% in 20 mL of PBS 1×.
    c. PBT: PBS/0.1% Triton X-100.
    d. Blocking solution: 10% fetal calf serum, 3% normal donkey serum (if secondary antibodies were made in donkey, otherwise use goat serum, …), 1% (w/v) BSA in PBT.
18. Perform an EB differentiation until day 10 (see embryoid body differentiation protocol above).
19. On day 10, fix the bodies 16–24 h in 4% PFA in PBS 1×, at 4°C:
    a. With a 10 mL pipette, transfer the medium and bodies in a falcon 15 mL conical tube.
    b. Centrifuge 182 × *g* for 5 min.
    c. Gently resuspend the cells in 10 mL of 4% PFA and place them at 4°C 16–24 h.
20. Cryoprotect the EBs with PBS containing 30% (w/v) sucrose:

Note: The addition of cryoprotectants such as sucrose prevent the formation of ice crystal that would damage tissue morphology. A direct transfer of the sample into 30% sucrose will also alter tissue morphology because of too great difference in osmalorality. Therefore, concentration of sucrose must be increased progressively.

a. Let EBs decant for 5 min, gently remove the supernatant and add 10 mL of PBS 1×.
b. Place on a tilting shaker for 10 min.
c. Repeat steps a and b 3 times.

⚠ CRITICAL: Perform all manipulations inside a fume hood until the moment when EBs are in the second PBS 1× wash solution. PFA and first PBS 1× wash solution containing traces of PFA should be disposed of in accordance with local regulations.

d. Increase progressively sucrose concentration by removing about 5 mL of PBS 1× and replacing it with the same volume of 30% sucrose solution.
e. Place on a tilting shaker for 10–15 min until equilibration (bodies should float before equilibration and easily decant after).
f. Repeat steps d and e 3–4 times.

21. Embed in OCT compound and section using a cryostat:
    a. Place a Pyrex beaker in a Styrofoam container and fill it with isopentane up to about 10 cm.
    b. Slowly add some dry ice pellets in the beaker and wait a few minutes until the pellets stop bubbling vigorously.

⚠ CRITICAL: Isopentane is highly flammable and all manipulations should be performed inside a fume hood. Wear gloves and protectives glasses when manipulating dry ice.

    c. Transfer EBs into in a small cryomold using a P1000 and a cut tip and remove sucrose solution as much as possible.
    d. Fill the mold with OCT and gather EBs with a tip.
    e. Carefully plunge the mold in cold isopentane and hold until the OCT is frozen.
    f. Remove sample from mold and place into tin foil in the cryostat chamber for 1 h to allow the temperature of the sample to equilibrate with that of the cryostat.
    g. Section the EBs at 12 μm using the cryostat.
    h. Mount sections onto SuperFrost Plus histological slides.

Pause Point: The embedded EBs and the sections can be stored at −80°C for several months.

22. Immunostaining of frozen EBs sections:

Note: Most primary and secondary antibodies (Table 2) are used between 0.5 and 5 μg/μL respectively. Choose carefully the fluorophore coupled secondary antibody depending on specie of the primary antibody or of the combination of primary antibodies you wish to use. In case of multiplexing verify the compatibility of fluorophores.

    a. Dry sections 10 min at 20°C–25°C.
    b. Draw incubation chambers with a Dakopen.
    c. Place the slide 5 min in a container filled with PBS 1× to rehydrate the sections. We use a 5 slides transport box filled with 10 mL PBS 1×.
    d. Permeabilize with PBS/0.25% Triton X-100 10 min at 20°C–25°C.
    e. Wash 3 × 5 min in PBT and incubate with blocking solution for 1 h at 20°C–25°C.

Table 2. Antibodies Used for Immunofluorescence

Marker	
NANOG	Pluripotency marker
FOXA2	Endoderm marker
GATA6	Endoderm marker
SOX17	Endoderm marker
GATA4	Endoderm marker
DAB2	Endoderm marker

f. Add 40–100 μL of primary antibody diluted in blocking solution in each incubation chamber and gently cover with parafilm avoiding air bubbles.
g. Incubate 1.5 h at 20°C–25°C (can be extended 16–24 h at 4°C) in the dark in a humidified chamber.
h. Wash 3 × 5 min with PBT and incubate with the appropriate secondary antibody diluted in blocking solution 1 h at 20°C–25°C in a humidified chamber.
i. Wash 2 × 5 min with PBT, 1 × 5 min with PBS containing Hoescht (10 μg/mL) and 2 × 5 min with PBS 1×.
j. Quickly rinse in distilled water, add a drop of Vectashield on sections and careful place a coverslip on top avoiding air bubbles.
k. Absorb residual liquid on slide and coverslip and seal with nail polish.
l. Observe under a fluorescent microscope.

Pause Point: The sealed slides can be stored in the dark at 4°C for several weeks.

Anticipated results for the cellular characterization of EBs are presented in the "Expected Outcomes" section.

Extraembryonic Endoderm Differentiation and Molecular Characterization

The protocol for ExEn differentiation presented here is based on a previously published protocol paper (Niakan et al. 2013). The differentiation conditions (medium containing activin A and low doses of Retinoic Acid) are the same as previously published. Our contribution consisted on implementing a differentiation time course using different amounts of cells (Ngondo et al 2018).

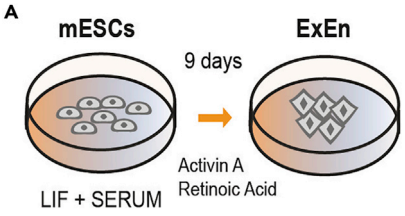
ExEn Differentiation

Timing: 10 days

mESCs are converted to ExEn cells by treating them with Activin A and low concentrations of retinoic acid.

Note: The Extraembryonic endoderm (ExEn) differentiation is performed in 6 well plate but can be scale up if needed.

23. Prepare a 0.2% gelatin-coated 6 well plate.
24. Start with an 80% confluent mESC T25 flask (Figure 1).
25. Harvest mESCs by first pouring out the old ESC medium and wash the cells by tilting gently with 3 mL of PBS 1×.
26. Discard the PBS 1× and add 1 mL of 0.05% Trypsin-EDTA before to incubate 5 min at 37°C.
27. Add 3 mL MF culture medium to the flask and dissociate into individual cells by pipetting up and down with a 5 mL pipette.
28. Count the cells.



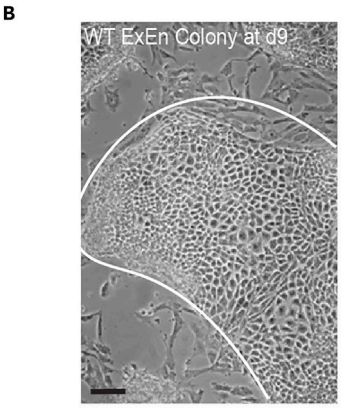


Figure 2. ExEn Differentiation Outcome at Day 9

(A) Schematic representation of ExEn differentiation protocol.

(B) Photography of wild-type cells after 9 days of ExEn differentiation. The scale bar is 50 μm.

29. For a time-course differentiation, we recommend to start with different amounts of cells for the various time points.

Note: If the cells are too confluent before reaching the collection day, try to seed less cells for these time points. Do not passage the cells during the differentiation time course to avoid introducing a selection bias.

Troubleshooting 3.

30. Aliquot the cells in 15 mL falcon tubes. The cells from each tube will be transferred into a 6-well.
31. Centrifuge 182 × *g*, 5 min.
32. Aspirate supernatant.
33. Resuspend the cells in 2 mL of ExEn medium.
34. Remove the gelatin from the 6-well plate.
35. Transfer the cells.
36. Incubate at 37°C, 8% CO_2.
37. Change the medium every two days by pouring out the medium using an aspiration pipet on the edge of the well and replace it with 2 mL of freshly prepared ExEn medium.

Note: A lot of cell death is normally observed within the first 3 days. To eliminate the maximum of dead cells, tilt gently the plate to resuspend the floating cells before pouring out the old medium.

Note: Visual observation of ExEn cells can give you an indication of the success or the failure of the differentiation. As previously control wild-type cells should be used as a reference. After 4–5 days of differentiation, ExEn colonies should start appearing. They are easily distinguishable from the other cells because of their epithelial morphology. A photograph of an ExEn colony after 9 days of differentiation is presented Figure 2.

Molecular Characterization of ExEn

Timing: 5–6 h

Table 3. Quantity of mESCs Required Per Well to Start the ExEn Differentiation in Order to Collect Different Time Points

Days	Number of Cells per 6-well
1	800,000
2	600,000
3	500,000
4	300,000
5	200,000
6	150,000
7	100,000*
8	100,000*

The molecular characterization of ExEn will allow the identification of a specific extraembryonic differentiation defect.

38. Harvest the cells and prepare dry pellets at chosen time points:
    a. Wash the cells 1 time with 2 mL PBS 1×.
    b. Add 0.5 mL of 0.05% Trypsin-EDTA and incubate 5 min at 37°C.
    c. Add 2 mL MF culture medium to the well and dissociate into individual cells by pipetting up and down.
    d. Transfer cells to 15 mL Falcon and centrifuge 5 min, 182 × *g*.
    e. Aspirate the supernatant.
    f. Gently resuspend the cells in 1 mL PBS 1×.
    g. Transfer to labeled 1.5 mL Eppendorf tube.
    h. Centrifuge 182 × *g* for 5 min.
    i. Aspirate supernatant.
    j. Freeze dry pellet at −80°C.

Pause Point: The collected pellets can be stored at −80°C for several months.

39. Perform RNA extraction and Reverse transcription as previously for EB (see step 17). Use the following markers (Table 4) to assess the success of the differentiation by qPCR (Table 5).

EXPECTED OUTCOMES

Molecular Characterization of EBs

The EB differentiation experiment (Figure 3A) should be repeated at least three times in order to statistically validate the observed differences. Please refer to the "Quantification and Statistical Analysis."

If a decrease in endoderm markers is observed in the EB samples from mutant ESC line compare to WT cells (Figure 3B), proceed to the cellular characterization of the EBs to determine if the endoderm defect comes from the extraembryonic endoderm.

Cellular Characterization of EBs

Extraembryonic endoderm specification occurs *in vitro* in the outer layer of mESC aggregates during EB differentiation (Hamazaki et al. 2004). The immunofluorescence imaging of wild-type EB sections should reveal a strong expression of endodermal markers (such as GATA6, SOX17, GATA4, or DAB2) in cells from the outer layer of the EB (as shown on the scheme in red, Figure 4A). The absence of signal or strongly reduced signal in the outer layer strongly suggests an extraembryonic differentiation defect. An example of expected results is presented on Figure 4B.

Table 4. Markers for ExEn Differentiation

Primers for mRNAs qPCR		Purpose
Gata6_Fw	GAAGCGCGTGCCTTCATC	Endoderm marker
Gata6_Rev	GTAGTGGTTGTGGTGTGACAGTTG	
Gata4_Fw	CTGGAAGACACCCCAATCTC	Endoderm marker
Gata4_Rev	GTAGTGTCCCGTCCCATCTC	
Dab2_Fw	TTGATGATGTGCCTGATGCT	Endoderm marker
Dab2_Rev	TTTGCTTGTGTTGTCCCTGA	
Foxa2_Fw	AGCACCATTACGCCTTCAAC	Endoderm marker
Foxa2_Rev	CCTTGAGGTCCATTTTGTGG	
Rrm2_Fw	CCGAGCTGGAAAGTAAAGCG	Normalizer gene
Rrm2_Rev	ATGGGAAAGACAACGAAGCG	

If such a defect is observed, it can be confirmed by attempting a directed differentiation of mESCs toward ExEn.

Molecular Characterization of ExEn

The expression of ExEn markers is induced already after 4–5 days of differentiation. This timing correlates to the appearance of ExEn colonies on the plate. After 6–7 days we usually reach a plateau of expression for *Gata6*, *Gata4*, *Dab2*, and *Foxa2* markers. An induction of more than 100 times fold compare to non-differentiated cells is expected for the wild-type cells. The data can be represented as a grouped bar plot for all the markers. The global induction of ExEn markers in the "mutant" cells should be compared to wild-type cells. Expected results are presented in Figure 5. A delayed or weak induction of all the markers is strongly suggesting a differentiation failure. Once the defect has been revealed *in vitro*, you can proceed with confidence to *in vivo* experimentation.

Table 5. Example of a Relative Expression Calculation Method for RT-qPCR Data

		(a) Mean CT			(b) Expression normalized to Rrm2 ($2^{-\Delta CT}$)		(c) Relative expression ($2^{-\Delta\Delta CT}$)	
		Rrm2	Marke 1	Marker 2	Marker 1	Marker 2	Marker 1	Marker 2
Wild-type cells	day 0 #	20.1	30	28	$2^{-(30-20.1)} = 0.00105$	0.004	$\frac{0.00105}{0.00105} = 1$	1
	day 1	19.5	29	26	$2^{-(29-19.5)} = 0.00138$	0.011	$\frac{0.00138}{0.00105} = 1.314$	2.639
	…	…	…	…	…	…	…	…
	day 9	20	18	15	$2^{-(18-20)} = 4$	32.000	$\frac{4}{0.00105} = 3809.524$	7643.406
Your cells of interest	day 0 #	20.1	30	29	$2^{-(30-20.1)} = 0.00105$	0.002	$\frac{0.00105}{0.00105} = 1$	0.5
	day 1	19.8	29	28	$2^{-(29-19.8.1)} = 0.00170$	0.003	$\frac{0.00170}{0.00105} = 1.619$	0.812
	…	…	…	…	…	…	…	…
	day 9	20	29	28.5	$2^{-(29-20.)} = 0.00195$	0.003	$\frac{0.00195}{0.00105} = 1.857$	0.660

A calculation spreadsheet describing the ΔΔCT method used measure the relative expression of two markers (Marker 1 and Marker 2) in "your cells of interest" compared to "wild-type cells." The normalized gene expression (b) of the markers is calculated relative to Rrm2 housekeeping gene levels and is obtained by applying the following formula $2^{-\Delta CT}$ using the difference between mean CT values (a). The relative expression (c) is obtained by dividing normalized expression (b) of each point to the day 0 reference time point (#) using the formula $2^{-\Delta\Delta CT}$.

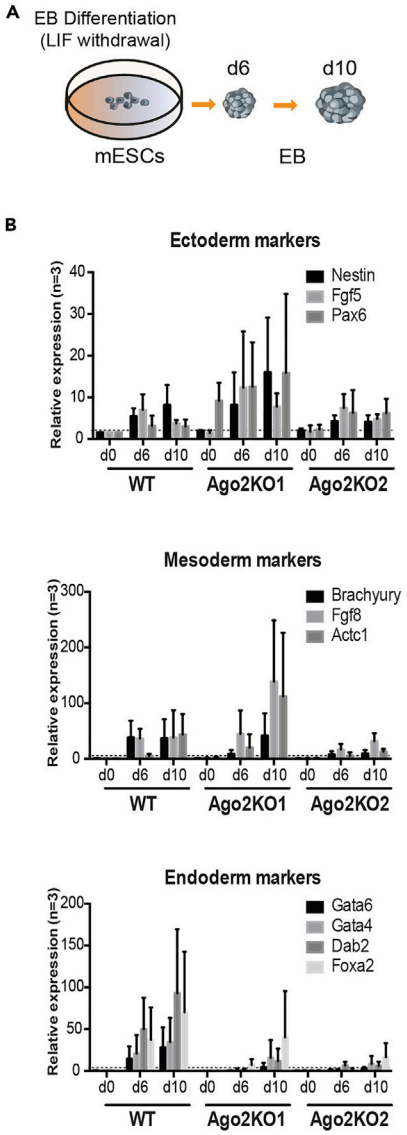


Figure 3. Molecular Characterization of EBs

(A) Schematic representation of EB differentiation protocol.

(B) Relative gene expression of ectoderm, mesoderm, and endoderm markers in WT, *Ago2*_KO1, and *Ago2*_KO2 mESCs during EB differentiation measured by RT-qPCR. The levels at day 6 (d6) and day 10 (d10) are represented relative to the levels WT cells at day 0 (d0). Error bars represent the standard deviation of three biological replicates. The dashed line shows levels at d0. Figure 2C reprinted with permission from Ngondo et al. (2018).

QUANTIFICATION AND STATISTICAL ANALYSIS

At least three replicate experiments are required in order to perform statistical analysis. More than 3 replicates are recommended. We analyze the qPCR data employing the $2^{-\Delta\Delta CT}$ method using Rrm2 as normalizer and "WT mESC at d0" as a reference sample. Here is an example of relative expression calculation.

Note: Other quantification method can be used.

Note: The qPCR primers should have an efficiency of 100%. Otherwise you should modify the calculation method accordingly (Pfaffl 2001).

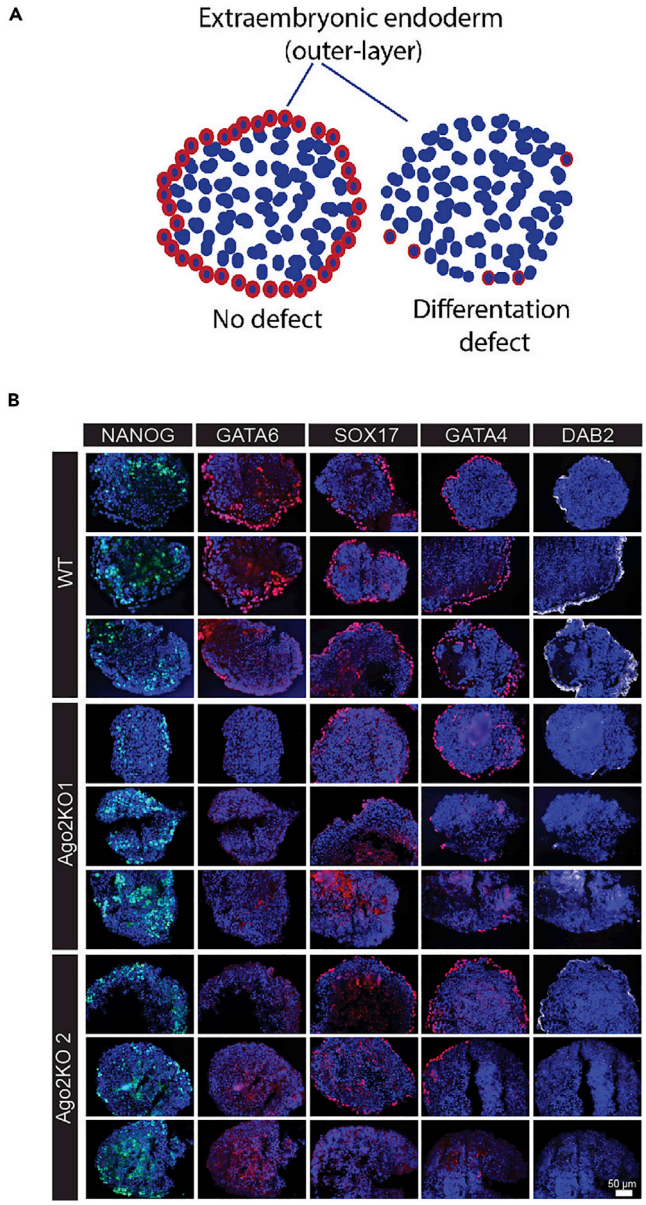


Figure 4. Cellular Characterization of EBs

(A) Schematic representation of typical image obtained after immunofluorescence staining of embryoid bodies sections using endodermal markers (GATA6, GATA4, or DAB2). The nuclei are represented in blue and the specific signal from the markers is shown in red.

(B) Immunofluorescence on EB sections after 10 days of EB differentiation of WT, *Ago2*_KO1, and *Ago2*_KO2 cells. The fluorescence signal of three different sections is shown for each of the proteins: NANOG, GATA6, SOX17, GATA4, and DAB2. The nuclei are stained with Hoescht 33342. The scale bar corresponds to 50 µm. Figure 2D reprinted with permission from Ngondo et al. (2018).

The statistical analysis can be performed using the software Prism Graphpad. For both EB and ExEn time-course differentiation, we perform a 2-way ANOVA analysis of the "mutant cells" versus "wild-type cells" over the time points to identify the statistically significant (p value < 0.05) factors responsible of the variation between the two samples (the nature of the sample or the time factor). We analyze each marker independently using relative expression data from all the replicate experiments.

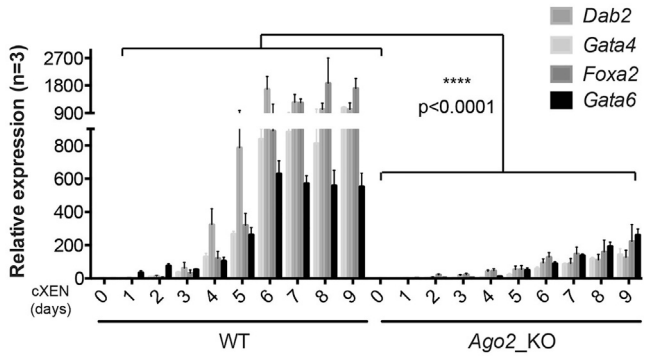


Figure 5. Molecular Characterization of ExEn

Relative expression of ExEn markers during time series of ExEn conversion of *Ago2*_KO and WT cells, measured by RT-qPCR. The error bar represents the range of three independent experiments. For each gene the statistical significance of the variation caused by the absence of *Ago2* over the time of differentiation (two factors) was assessed using a 2-way ANOVA test. The p values obtained for each gene are: $p < 0.0001$. Figure 4E reprinted with permission from Ngondo et al. (2018).

To compare the mean expressions of only two conditions, a student t test or a Wilcoxon Signed-rank test might be used to obtain a p value to validate significant (p value<0.05) or non-significant differences (p value >=0.05).

LIMITATIONS

This procedure is intended to point toward an extraembryonic differentiation defect. Its main limitation resides in the fact that it is an *in vitro* differentiation procedure and its inherent difficulty to discriminate between extraembryonic and embryonic lineages. If a defect is observed, it should be validated using an *in vivo* approach such as the analysis the contribution of mutant cells to embryonic/extraembryonic tissues using chimeric mice or conditional/constitutive knockout mice.

TROUBLESHOOTING

Problem 1

It could happen that the bacterial petri dish still allows the cells to attach to surface.

Potential Solution

Use ultra-low attachment commercial dishes or to coat the bacterial petri dish surface with poly-HEMA (Poly 2-hydroxyethyl methacrylate).

Problem 2

During EB differentiation, variations of expression between biological replicates could be very high, making it difficult to assess statistical significance.

Potential Solution

Use a higher number of replicates >3.

Problem 3

Depending on your cell line, you may need to adjust the number of cells (Table 3), since the proliferation rate may vary depending on the line/mutant tested.

Potential Solution

Start the experiment with enough cells for the first time points due to the massive cell death and at the same time avoiding reaching to fast confluency. Increasing or decreasing cell numbers by 100 000 cells is a good starting point. For the later time points reduce the number of cells in order to avoid confluency at

the day of harvest but be careful to keep at least 10%–20% of the cells surviving the initial massive cell death during the first three days. Increasing or decreasing the cell number by 50 000 is a good starting point.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Constance Ciaudo (cciaudo@ethz.ch).

Materials Availability

Mouse lines generated in the original study (Ngondo et al. 2018) are available upon request to cciaudo@ethz.ch.

Data and Code Availability

The published article (Ngondo et al., 2018) includes all data generated and analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.P.N. and C.C.; Methodology, R.P.N., M.C.-T., and C.C.; Formal Analysis, R.P.N.; Investigation, R.P.N. and M.C.-T.; Resources, M.C.-T. and C.C.; Writing, R.P.N., M.C.-T., and C.C.; Visualization, R.P.N. and C.C.; Supervision, C.C.; Project Administration, C.C.; Funding Acquisition, C.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Bodak, M., and Ciaudo, C. (2016). Monitoring long interspersed nuclear element 1 expression during mouse embryonic stem cell differentiation. Methods Mol. Biol. *1400*, 237–259.

Hamazaki, T., Oka, M., Yamanaka, S., and Terada, N. (2004). Aggregation of embryonic stem cells induces Nanog repression and primitive endoderm differentiation. J. Cell Sci. *117* (Pt 23), 5681–5686.

Keller, G.M. (1995). In vitro differentiation of embryonic stem cells. Curr. Opin. Cell Biol. *7*, 862–869.

Ngondo, R.P., Cirera-Salinas, D., Yu, J., Wischnewski, H., Bodak, M., Vandormael-Pournin, S., Geiselmann, A., Wettstein, R., Luitz, J., Cohen-Tannoudji, M., et al. (2018). Argonaute 2 is required for extra-embryonic endoderm differentiation of mouse embryonic stem cells. Stem Cell Reports *10*, 461–476.

Niakan, K.K., Schrode, N., Cho, L.T., and Hadjantonakis, A.K. (2013). Derivation of extraembryonic endoderm stem (XEN) cells from mouse embryos and embryonic stem cells. Nat. Protoc. *8*, 1028–1041.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, 45e–45.

Robertson, E.J. (1987). Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, First Edition, E.J. Robertson, ed. (IRL Press), pp. 71–112.