Derivation of mouse embryonic stem cells

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Here we describe a simple and efficient protocol for derivation of germline chimera-competent mouse embryonic stem cells (mESCs) from embryonic day 3.5 (E3.5) blastocysts. The protocol involves the use of early-passage mouse embryonic fibroblast feeders (MEF) and the alternation of fetal bovine serum– and serum replacement (SR)–containing media. As compared to other available protocols for mESCs derivation, our protocol differs in the combination of commercial availability of all reagents, technical simplicity and high efficiency. mESC lines are derived with approximately 50% efficiency (50 independent mESC lines derived from 96 blastocysts). We believe that this protocol could be a good starting point for (i) setting up the derivation of mESC lines in a laboratory and (ii) incorporating further steps to improve efficiency or adapt the protocol to other applications. The whole process (from blastocyst extraction to the freezing of mESC line) usually takes between 15 and 20 d.

INTRODUCTION

Embryonic stem cells (ESCs) were first isolated in 1981 from mouse blastocysts[1,2] and subsequently from human blastocysts[3,4]. Mouse ESCs (mESCs) originate from the inner cell mass (ICM) of a blastocyst[1,2], which represents a pluripotent cell population within a blastocyst giving rise to all embryonal tissues. ICM cells, as well as their *in vitro* counterparts—ESCs—have the unique capacity to differentiate into all the cell types that make up an individual, including germline cells[5]. mESCs self-renew in presence of leukemia inhibitory factor (LIF)[6,7] and grow indefinitely in culture without senescing[8]. These properties have classically made of ESCs a unique tool for gene targeting and generation of genetically modified mice. More recently mESCs have also become a tool for the study of development *in vitro*, for the development of stem cell–based therapies, and for drug assays. Nowadays, a large set of mouse mutant strains are available, but because current protocols for the derivation of mESCs are not very efficient and/or are difficult to perform, the potential of mESCs has not yet been fully and systematically exploited.

We hereby describe a modification of the protocol originally described by Liz Robertson[9]. When modifying this original experimental protocol, we decided to simplify it by using reagents that are commercially available and standard cell culture equipment, so that an inexperienced person with basic equipment could perform ESC derivations. The technique is used primarily for deriving mESCs for experimental purposes and is especially suited for experimental setups where larger numbers of new mESC lines must be derived. This includes situations where parental mouse mutants are lethal as homozygotes, where only some blastocysts carry the required allelic combination, or where it is necessary to generate several mESC lines from one pregnant female.

After the first derivation of mESC lines from blastocysts[1,2], several standard protocols were developed[9–12]. These protocols produce ESCs with low efficiency (10–30% in the most favorable strains)[12] and require specialized tools for blastocyst handling. In order to increase the efficiency, many improvements have been introduced, including among others the use of specifically conditioned medium[13] or genetically modified blastocysts[14] or microdissection of the blastocyst[15]. Such modifications improve efficiency (up to 100%) but introduce new, specialized techniques and/or require home-made reagents, all of which make it difficult for inexperienced labs to set up the technique. The protocol we present here for the derivation of ESCs from mouse blastocysts is optimized for simplicity (while retaining very high efficiency) and is easier to perform than standard procedures[10–12]. This protocol allows investigators to (i) efficiently derive new mouse ESC lines[16] even if they do not have previous experience in this area and (ii) introduce additional modifications[13–15] to make the process even more efficient.

We believe that the protocol presented here could serve as a starting point for the development of a wider range of applications, including the derivation of human ESCs. Additional improvements would include making the procedure fetal bovine serum (FBS)-free and feeder-free. Pulses of FBS can be potentially replaced by the use of trypsin inhibitors in combination with purified serum components such as fibronectin or BMP4 (ref. 17). A potential approach for removing MEFs can include the use of FBS-free MEF-conditioned medium in combination with various substrates and matrices for cell attachment.

MATERIALS

REAGENTS

- Phosphate-buffered saline (PBS), without calcium or magnesium
- 70% (v/v) ethanol
- MEF culture medium: D-MEM supplemented with 10% FBS (Gibco, cat. no. 10270-106), penicillin (100 U ml^{-1})/streptomycin (100 μg ml^{-1}) (Gibco, cat. no. 15140-122) and 2 mM L-glutamine (Gibco, cat. no. 25030-024)
- Mitomycin C (see REAGENT SETUP)
- Embryonic day 12.5 (E12.5) or E13.5 mouse embryos
- 0.1% gelatin: dissolve 1 g of gelatin (Sigma, cat. no. G1890) in MilliQ column–purified water and sterilize by autoclaving 30 min at 120 °C
- HEPES/DMEM, obtained by mixing Knockout D-MEM medium (Gibco, cat. no. 10829-018) with HEPES buffer solution (100×, Gibco, cat. no. 15630-056)
- SR-ES medium (see REAGENT SETUP)

- FBS-ES medium, a medium similar to SR-ES medium, in which SR was replaced by 20% ES cell-pretested FBS (PAA, cat. no. A15-080)
- 0.25% trypsin-EDTA (Gibco, cat. no. 25300-54)
- 2.5% trypsin (Gibco, cat. no. 15090-046)

EQUIPMENT

- 35-, 60- and 100-mm tissue culture plates
- 12-well plates (Falcon, multiwell 12-well, cat. no. 353043)
- 10 µl pipette tips (Gilson, Diamond D10, cat. no. F161632; Sarstedt, cat. no. 70.1130; or similar)
- 20 µl pipette
- 23-G needles (0.6 × 25 mm)
- 1-ml syringes
- Dissection forceps and scissors
- Inverted microscope with phase contrast
- Dissection scope

REAGENT SETUP

Mitomycin C Prepare 1 mg ml^{-1} stock solution by dissolving 2 mg of mitomycin C (Roche, cat. no. 107409) in 2 ml of culture medium, and store at 4 °C. ! CAUTION Mitomycin C is very toxic by inhalation and if swallowed, and poses a danger of cumulative effects.

SR-ES medium Knockout D-MEM supplemented with 20% Knockout Serum Replacement (SR, Gibco, cat. no. 10828-028), penicillin (100 U ml^{-1})/streptomycin (100 µg ml^{-1}) (Gibco, cat. no. 15140-122), 2 mM L-glutamine (Gibco, cat. no. 25030-024), 1× MEM non-essential amino acids (Gibco, cat. no. 11140-035), 100 µM β-mercaptoethanol (dissolve 7 µl of β-mercaptoethanol (Sigma, cat. no. M-7522) in 10 ml of PBS to prepare 100× stock solution) and recombinant mouse LIF (1,000 U ml^{-1} of ESGRO; Chemicon International, cat. no. ESG1107). ! CAUTION β-mercaptoethanol is toxic; avoid inhalation, ingestion or contact with eyes, skin or mucous membrane.

PROCEDURE

Preparation of mouse embryonic fibroblasts (MEFs) ● TIMING 2–3 d

1| Prepare and label 100-mm tissue culture plates. Usually the MEFs from two embryos provide a sufficient number of cells for one plate.

2| Kill pregnant female mouse between 12.5 and 13.5 days post coitum.
! CAUTION Handling of experimental animals must be done in accordance with relevant guidelines and regulations.

3| Dissect out the uterine horns with embryos and briefly rinse them in 70% ethanol. Put the horns into a 100-mm-diameter culture dish with 20 ml of PBS. Use a culture hood equipped with a dissection scope, and dissection forceps and scissors that have been autoclaved (or cleaned and submerged in 70% ethanol). Release the embryos and placenta from the uterine horns by cutting the walls of the horn, and separate each embryo from the placenta and surrounding membranes.
▲ CRITICAL STEP Perform Step 3 and later steps under sterile conditions.

4| Cut out the heads and remove internal abdominal and thoracic organs (lungs, heart, liver, intestine, stomach, etc.) from all embryos in a litter. Trypsinize the embryonal tissue by transferring all embryos to 35-mm-diameter dishes with 1 ml of 0.25% trypsin/EDTA (two embryos per plate). Trypsinize the tissue 2–4 min in the 37 °C incubator until tissues start to disintegrate. Quickly pipette all the tissue up and down with a 1-ml pipette to achieve efficient dissociation and proceed to Step 5. Continue immediately with the next dish to avoid unnecessary prolongation of the trypsinization time. In case when more than ten embryos are obtained, the extra embryos can be kept in ice-cold PBS for up to 15 min without affecting MEF viability.
▲ CRITICAL STEP Too short incubation may result in insufficient trypsinization and low yield, whereas too prolonged incubation may decrease the viability of MEFs. Do not use needle and syringe for dissociation.

5| Inactivate the cell suspension in trypsin by pipetting it into the MEF culture medium (1 ml of suspension per 10 ml of medium). Pool the embryos from one female in one 50-ml tube (or in two 50-ml tubes when more than ten embryos are obtained) and allow the large pieces of tissue to sediment.

6| Collect the supernatant and seed out a homogenous suspension, keeping a ratio of two embryos per 100-mm plate.

7| Change culture medium 4–5 h after seeding to remove unattached cells, and let the MEFs grow to ~90% confluence (it usually takes 2–3 d). MEFs at this stage ready to be frozen are considered to be passage 1.
■ PAUSE POINT The MEFs can be frozen for several weeks at −80 °C or for unlimited time in liquid nitrogen.

8| Proceed to the next step, or freeze the MEFs down (cells from one 100-mm plate per single vial) using standard cell culture techniques. When using frozen MEFs, the cells are usually ready for the next step 1 d after thawing (if thawed, 1 vial per 100-mm dish). Alternatively, when thawed MEFs are confluent, they can be split 1:3 to generate three plates of passage 2 MEFs.

Preparation of MEF feeder layer ● TIMING 1 d

9| Treat confluent MEFs with mitomycin C for 2 h (10 µg ml^{-1}) or 1 µg ml^{-1} overnight. Alternatively, cells may also be mitotically inactivated by exposure to 8,000 rad of X-ray irradiation or 3,000 rad of gamma irradiation.
▲ CRITICAL STEP Use passage 1 or 2 MEFs, because we have observed that later-passage MEFs (passages 3–5) do not support the ESC derivation to the same extent.

10| Gelatinize 12-well plates by covering the bottom of wells with 500 µl of 0.1% gelatin for a minimum of 5 min at 37 °C. Remove the excess gelatin solution just before next step.

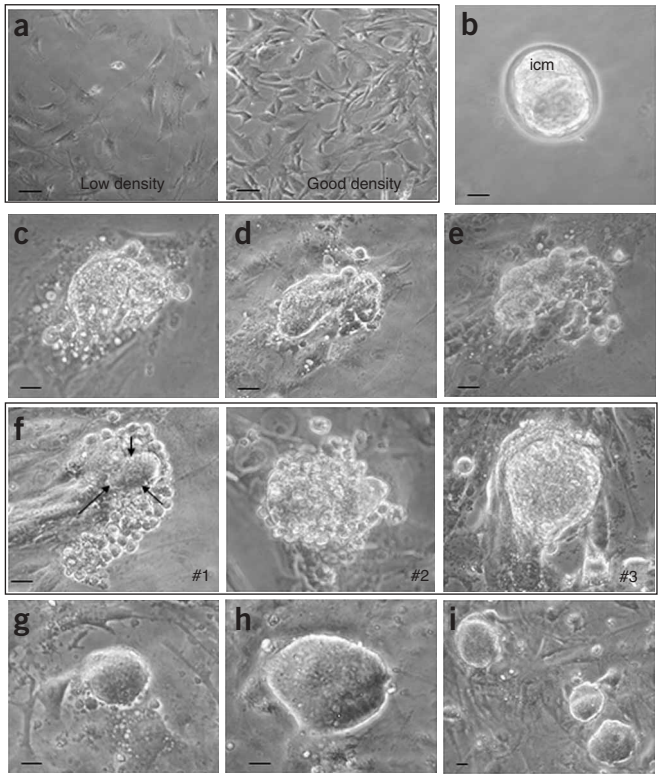

Figure 1 | Individual steps of mESC derivation. (**a**) MEF feeder layer at low (insufficient) and proper density. (**b**) Morphology of mouse E3.5 blastocyst with inner cell mass (icm) indicated. (**c**–**e**) Blastocyst hatched on MEF feeder 2 d (**c**), 3 d (**d**) and 4 d (**e**) after extraction. (**f**) Examples of hatched blastocysts with expanded ICM and defined ESC like population (arrows), ready for the first trypsinization at day 5 or 6 after extraction. The blastocyst shown gave rise to ESCs. (**g**) Morphology of mESC-like colony 2 d after the first trypsinization. (**h**) Typical appearance of mESC colony at the stage when it should be subjected to the second trypsinization (usually 4–5 d after the first trypsinization). (**i**) Established mESC line. The pictures were taken with an Olympus C7070 camera. Scale bars, 50 μm (**a**), 25 μm (**b**–**i**).

11| After mitomycin C inactivation, wash MEFs once with PBS, trypsinize, count and seed at a density of 20,000–25,000 cells per cm^2 (80,000–90,000 cells per well) onto gelatinized wells of 12-well plate. The feeder layer should cover the entire well (see **Fig. 1a** for an example of a good-quality MEF feeder layer). Normally confluent MEFs from a 100-mm plate are sufficient for two entire confluent 12-well plates.
▲ CRITICAL STEP Do not use wells in which the MEF feeder layer is not confluent.

Blastocyst extraction and ICM expansion ● TIMING 5–6 d

12| Replace MEF medium with SR-ES medium 1–3 h before blastocysts extraction.
▲ CRITICAL STEP Use only freshly prepared MEF feeder layers (prepared the same or previous day) to avoid the retraction/detachment of feeder, which could negatively influence ESC derivation.

13| Kill time-mated females at E3.5 and immediately dissect their uteri into 10 ml of HEPES/DMEM in 100-mm plate preheated to 37 °C in the incubator.

14| Transfer the uteri into 2 ml of fresh preheated HEPES/DMEM in 35-mm dish or as an alternative into an organ tissue culture dish (more expensive but more convenient).

15| Flush the blastocysts out of the uterine horn under a dissection scope using a 1-ml syringe with 23-G needle, as described earlier[10].

16| Collect blastocysts (**Fig. 1b**) under an inverted microscope using 10 μl pipette tips and a 20-μl pipette and transfer individually to wells of a 12-well plate containing a feeder layer of MEFs mitotically inactivated 1 d earlier.
▲ CRITICAL STEP For unexperienced eyes blastocysts might not be readily visible in dissection microscope. We suggest picking up the extracted blastocysts using an inverted microscope with phase contrast.

17| Allow blastocysts to attach to the MEF feeder layer. That usually takes 2 d. Avoid any interference during this period. After attachment, the blastocyst hatches (**Fig. 1c**). SR-ES medium can be then changed, and should subsequently be replaced every second day while the inner cell mass expands (**Fig. 1d,e**), until it adopts a morphology comparable to that shown in **Figure 1f** (usually on day 5 or 6).
▲ CRITICAL STEP Do not shake the plate or move the blastocyst during attachment.
? TROUBLESHOOTING

First trypsinization and ESC expansion ● TIMING 10–15 d

18| Prepare and label appropriate numbers of 12-well plates with confluent layers of mitotically inactivated MEFs 1 d in advance.

19| Two hours before trypsinization, replace the MEF medium with 1 ml per well of FBS-ES medium.

20| Make 20-μl drops of 2.5% trypsin on a 60-mm-diameter culture dish (for Steps 23 and 24).

21| Aspirate SR-ES medium from the wells containing expanded blastocysts on MEFs and add 200 μl of 0.25% trypsin-EDTA to the well.

22| After 15 s, aspirate the trypsin-EDTA, leaving only a wet surface. This procedure reduces stickiness of the cells and allows easier detachment. Proceed immediately to the next step.

23| To mechanically detach an expanded blastocyst and surrounding MEFs, use a 20-µl pipette and 10-µl pipette tip. Under a dissection microscope, pipette 10 µl of 2.5% trypsin from the 20-µl drops (prepared in Step 20) onto each blastocyst (one at the time). Make several circles with the pipette tip around the blastocyst to remove surrounding MEFs and separate the blastocyst (on the top of some MEFs) from the rest of the feeder layer. Aspirate the blastocyst back with the pipette and transfer into the remaining 10-µl drop of 2.5% trypsin.
▲ **CRITICAL STEP** MEFs cultured in SR-ES medium for 5–6 d produce large amounts of extracellular matrix resistant to trypsin, which increases their stickiness. Make sure that the blastocyst was successfully transferred by checking for its presence under the dissection microscope.

24| Trypsinize the cells in a drop for ~5 min in the 37 °C incubator and then dissociate into individual cells and small cell clumps, by pipetting up and down approximately ten times with a 10-µl pipette tip.
▲ **CRITICAL STEP** Monitor dissociation under the dissection microscope until the blastocyst is disintegrated into several smaller cell clumps.
? TROUBLESHOOTING

25| Pipette the cell suspension into a well of a 12-well plate containing MEFs and FBS-ES medium.
▲ **CRITICAL STEP** FBS medium functions as a trypsin inhibitor. One milliliter of FBS-ES medium can inactivate a maximum of 20 µl of 2.5% trypsin. Make sure that the ratio of 2.5% trypsin and FBS-ES medium is 1:50 or lower.

26| The next day, replace FBS-ES medium with SR-ES medium and let the cells continue growing, changing the medium every second day.

27| Several days after trypsinization, compact cell colonies with typical ESC colony morphology (see **Fig. 1g**) will be detected in some of the wells (the number of colonies usually varies from 1 to 20). Monitor the colonies daily, focusing especially on colony morphology and growth, and proceed to Step 28 accordingly.

28| Passage ESCs according to their needs. Trypsinize all cells in a well with 0.25% trypsin-EDTA when most colonies in the well reach the size and morphology shown in **Figure 1h**. Mix the resulting single-cell suspension with FBS-ES medium and seed the cells in a dish (well) of suitable size containing fresh feeder cells. The next day, replace the medium with SR-ES medium. Usually an additional trypsinization step is needed before the cells can be grown on 60-mm-diameter dishes to sub-confluency before being frozen as described[11]. For appropriate density and morphology of new ESCs ready to be frozen, see **Figure 1i**.

● **TIMING**
Steps 1–8 (preparation of MEFs): 2–3 d.
Steps 9–11 (preparation of MEF feeder layer): 1 d.
Steps 12–17 (blastocyst extraction and ICM expansion): 5–6 d.
Steps 18–28 (first trypsinization and ESC expansion): 10–15 d.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Step 17: Blastocysts are only poorly attached after 2 d.	Low density or quality of MEFs.	Transfer blastocysts to better feeder.
	Dead blastocysts (possible causes: initial bad blastocyst quality, embryotoxicity in the medium or overly slow extraction process).	Check the quality of blastocysts by assessing their morphology. Test the toxicity of used medium or next time perform blastocyst extraction more rapidly (one female at a time).
Step 17: Medium in blastocyst cultures turns yellow despite changing medium every 2 d.	High density of MEFs.	Change medium daily.
Step 17: Hatched blastocysts die after partial expansion.	Poor quality of MEFs.	Use confluent MEFs of passage 1 and 2.

TABLE 1 | Troubleshooting table (continued).

Problem	Possible reason	Solution
Step 24: No colonies are detected after first trypsinization.	Not all ICMs give rise to ESCs: usual frequency is ~50–60%. Expanded ICM was lost.	Perform first trypsinization under inverted microscope or, where applicable, use microdissection tools to provide better control of the trypsinized tissue.
	Expanded ICM died during trypsinization.	Perform first trypsinization faster (one expanded ICM per time).
Step 24: Following trypsinization, only a few big and differentiated colonies appeared.	The expanded ICM was not trypsinized properly.	Prolong incubation in 2.5% trypsin and perform physical disintegration of the blastocyst using the pipette tip.
Steps 17–28: Blastocyst did not attach; ICM did not outgrow; ESC-like colonies or ESCs did not expand and/or died.	The incubator is not set properly.	Adjust the incubator settings (5% CO_2, 37°C). Check water tray in the incubator.

ANTICIPATED RESULTS

The above described protocol was applied in our lab to blastocysts from several knockout mouse strains. In total we obtained 50 ESC lines out of 96 blastocysts (this summary does not include the experimental conditions where any of the protocol steps labeled as ▲ CRITICAL STEP were not fulfilled). In our hands, genetic background did not affect the efficiency—four different mouse strains on a predominantly C57Bl/6 background (at least ten backcrossings with C57Bl/6)[16] were used to isolate 38 ESC lines out of 71 blastocysts (53.5% efficiency), whereas 25 blastocysts carrying a *lox-Rbp-J-lox* allele (encoding RBP-J, also known as CSL, which is a crucial effector of the Notch signaling pathway)[18], with mixed genetic background (with contribution from *ROSA26*, 129 and C57Bl/6 strains), produced 12 independent ESC lines (48% efficiency). Because individual mouse strains are known to differ in their susceptibility to ESC derivation[13,15,19], it is difficult to predict the efficiency of this protocol for other mouse strains with lower (e.g., CBA, DBA, FVB/N or BALB/c) or higher derivation efficiencies (e.g., 129)[15,19,20]. However, studies using C57Bl/6-based strains[15,20,21] concluded that C57Bl/6 is not the most favorable strain for ESC derivation, and we therefore believe that our results represent a clear improvement compared to those of previous protocols.

Although mESCs are characterized by their typical morphology (see **Fig. 1**) and fast proliferation, we strongly recommend that a set of molecular and functional markers be used to characterize newly derived ESCs that are to be used for further experiments. The list of tested properties should include, as a minimum, high expression of Oct4, high activity of alkaline phosphatase, high and homogenous staining for the cell-surface antigen SSEA-1, and the ability to differentiate into lineages of all three germ layers *in vitro* (either using embryoid body formation or specific protocols). However, whenever applicable, we suggest testing the ultimate quality of the new ESCs line on the basis of its ability to generate germline-competent chimeras[5]. ESCs derived by the protocol described here contributed to germline chimeras. In our hands, two out of two male chimeras tested (generated by injection of ESCs from C57Bl/6 background heterozygous for *Wnt-5a* mutation into FVB/N morulas) were able to transmit the mutation and genetic background to the next generation very efficiently (**Fig. 2**). We found that 100% of the pups were agouti and that 16 of 29 pups (~55%) carried the *Wnt-5a* mutation.

We believe that the protocol presented here will help to generate ESCs from a plethora of available mouse mutants. Mutant ESCs derived by this method can be a unique tool for *in vitro* modeling of disease animal models, for analyses of the function of particular mutations in development, for biochemical studies of specific cell types derived from ESCs (after upscaling and/or cell sorting), or for high-throughput drug screenings using ESCs derived from reporter knock-in mice.

Figure 2 | Derivation of germline-competent chimeras from newly derived mESCs. The inset shows a chimeric male derived by injection of C57Bl/6 mESCs into FVB/N morulas and his progeny (agouti) after crossing with FVB/N female (white).

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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