Preface

Genomic imprinting is the process by which gene activity is regulated according to parent of origin. Usually, this means that either the maternally inherited or the paternally inherited allele of a gene is expressed while the opposite allele is repressed. The phenomenon is largely restricted to mammals and flowering plants and was first recognized at the level of whole genomes. Nuclear transplantation experiments carried out in mice in the late 1970s established the non-equivalence of the maternal and paternal genomes in mammals, and a similar conclusion was drawn from studies of interploidy crosses of flowering plants that extend back to at least the 1930s. Further mouse genetic studies, involving animals carrying balanced translocations (reviewed in Chapter 3), indicated that imprinted genes were likely to be widely scattered and would form a minority within the mammalian genome. The first imprinted genes were identified in the early 1990s; over forty are now known in mammals and the list continues steadily to expand.

Genomic Imprinting: Methods and Protocols aims to collect protocols that have been applied to the study of imprinting or imprinted genes. Many of the protocols are based on more widely used embryology or molecular biology techniques that have been adapted for imprinting research. All of the included methods remain gainfully employed in either (or both) the discovery or analysis of imprinted genes. Chapter 1 describes the nuclear transplantation methods, first used in the 1970s, for the generation of mouse embryos with genomes of entirely maternal or entirely paternal origin. The first five chapters are specific to the mouse, though some of the principles could be applied to other species. For instance, the techniques described in Chapters 4 and 5 for generating transgenic mice using large fragments of genomic DNA have resulted in several examples of the faithful reproduction of imprinted gene expression at ectopic loci. The first few imprinted genes have recently been identified in plants and it will be interesting to know whether the imprinting of these genes can be similarly reproduced within plant transgenes.

The majority of protocols describe molecular techniques and most of these allow examination of gene structure or expression in an allele-specific manner, which is an essential aspect of most imprinting studies. Protocols are

included for identifying imprinted genes (Chapters 6–8), for analyzing imprinted gene expression (Chapters 9–12), for the study of DNA methylation and methylation-sensitive DNA-binding proteins (Chapters 13–20), and for examining chromatin structure (Chapters 21–24). The final chapter is a review of genomic imprinting in plants. Although imprinting must have arisen independently in plants and animals, the available evidence suggests that the imprinting mechanisms in these species may share common features, such as the involvement of DNA methylation in distinguishing maternal and paternal alleles. Thus, the molecular methods that are already extensively used to study mammalian imprinted genes will surely find even wider employment as the genomic imprinting field continues to expand.

I thank all of the authors for their outstanding contributions to this volume. On behalf of us all I extend the hope that this effort to make these methods accessible will prove useful to genomic imprinting aficionados everywhere.

Andrew Ward

2

Deriving and Propagating Mouse Embryonic Stem Cell Lines for Studying Genomic Imprinting

Jeffrey R. Mann

1. Introduction

Embryonic stem (ES) cells are a cell culture derivative of the blastocyst inner cell mass (ICM), the latter giving rise to the embryo, the amnion, the yolk sac, and the chrorioallantoic portion of the placenta. Blastocyst injection chimera experiments show that ES cells are similar to early-stage ICM cells in that they contribute to the primitive ectoderm and endoderm derivatives ***(1)***. However, it is probably not posssible to equate these two cell types, as ES cells appear to be produced by the cell culture environment and have no exact counterpart in the blastocyst. Instead, ES cells could be thought of as being ICM cells that, instead of undergoing rapid differentiation as they would in vivo, are abnormally locked into continuing cycles of division in the undifferentiated state by virtue of the action of exogenous factors. Leukemia inhibitory factor, LIF, is one such factor ***(2,3)*** and is indispensable for the propagation of mouse ES cells at least when primary embryo fibroblasts (PEFs) are used as feeder layers ***(4)***.

A number of features of ES cells make them useful for studying genomic imprinting. They (1) are diploid and can be derived such that they contain only maternally and paternally derived genomes, termed parthenogenetic and androgenetic cell lines, respectively ***(5)***, or uniparental duplication of chromosome regions ***(6)***; (2) retain imprints as assessed by the developmental potential of chimeras ***(7–9)***; (3) offer a rudimentary in-vitro system of differentiation in the production of embryoid bodies ***(10)*** in addition to the the in-vivo system of chimera production; and (4) can provide large quantities of cellular material such as DNA for studies of chromatin structure ***(11,12)***. Nevertheless, it is

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important to realize that their derivation and unlimited capacity for division can result in epigenetic change. For example, methylation of the paternal *H19* allele in ES cells appears to resemble more the later somatic cell pattern rather than the pattern in the ICM, suggesting that this methylation is more a function of the number of cell cycles in development rather than the stage of differentiation ***(10–14)***. In addition, the allele-specific methylation and expression patterns of imprinted genes in ES cells are unstable with passage ***(10)***. While ES cells retain imprints as ascertained by the developmental potential of chimeras, they also appear to lose some. Parental-specific expression of imprinted genes is destabilized in ES cells and cannot be corrected upon differentation of the cells in chimeras ***(10,15)***, and this destabilization may contribute to the developmental abnormalities often observed ***(10,16)***. There is no substitute for the use of real embryonic material for studying genomic imprinting when possible.

2. Materials

1. 1× Dulbecco's phosphate-buffered saline; with and without calcium and magnesium ($DPBS^+$ and $DPBS^-$, respectively).
2. 1× trypsin-EDTA solution; 0.25% trypsin (1:250) and 1 m*M* ethylenediaminetetraacetic acid (EDTA) in Hank's balanced salt solution without calcium and magnesium or in $DPBS^-$. To 100 mL, add 1 mL of a 5% (w/v) solution of bovine serum albumin (BSA). To make BSA solution, dissolve 5 g of BSA (Miles, Inc., Diagnostics Division, cat. no. 82-047-3, Kankakee, IL, USA) in cell culture-grade water and filter-sterilize. Store 1-mL aliquots at –20°C.
3. 1000× 2-mercaptoethanol solution; add 70 µL of 2-mercaptoethanol to 10 mL of $DPBS^-$ to give 0.1 *M* solution. Mix, then filter-sterilize. Store at 4°C and discard after 3 wk.
4. 1× gelatin solution; add 0.5 g cell culture-grade gelatin to 500 mL $DPBS^-$ in a glass media bottle and sterilize by autoclaving. After cooling, swirl solution to mix dissolved gelatin.
5. ES cell media; to 500 mL of Dulbecco's modified Eagle's medium (DMEM) (with 4.5 g/L glucose, 2.2 g/L sodium bicarbonate, without L-glutamine, without pyruvate; *see* **Note 1**), add 75 mL fetal bovine serum (FBS), 0.6 mL of 1000× 2-mercaptoethanol, 6 mL of 100× penicillin/streptomycin solution, 6 mL of 100× L-glutamine (200 m*M*), and 6 mL of 100× nonessential amino acids solution. The latter three solutions are the standard formulations obtained from any supplier of cell culture reagents. Store media at 4°C, and if not used in 1 mo, add L-glutamine and 2-mercaptoethanol at half the initial proportion according to residual volume. Thawed FBS is also kept at 4°C and is stable for many months, although it can be refrozen if necessary.

6. Media for PEFs; as for ES cell media, except add 55 mL of FBS.
7. STO fibroblast media; as for ES cell media, except add 35 mL FBS.
8. Mineral oil (Sigma, cat. no. M 8410).
9. 100× G418 solution. Dissolve geneticin sulfate (Gibco BRL, cat. no. 11811-031) in 40 mL of $DPBS^-$ to achieve a concentration of 17.5 mg/mL active weight; proportion of active weight or microbiological potency is stated on bottle. Store as 1.5-mL aliquots in 2-mL sterile screw-capped tubes (Sarstedt, cat. no. 72.693.005) at –20°C. Thawed tubes are stable at 4°C for at least 1 mo.
10. 100× mitomycin C solution: dissolve 2 mg in 4.0 mL of $DPBS^-$. Store 0.12-mL aliquots in sterile 2-mL Sarstedt tubes. Thawed tubes are stable at 4°C for at least 2 wk.
11. Freezing solution I; Mix 12 mL of FBS with 18 mL of $DPBS^+$ in a 50-mL centrifuge tube and store at 4°C. Freezing solution II; Mix 5 mL of sterile cell culture-grade dimethyl sulfoxide (DMSO) with 20 mL of $DPBS^-$ in sterile 50-mL centrifuge tubes and store at –20°C. Thawed tubes can be kept at 4°C for a number of weeks while in use.
12. STO immortalized fibroblast cells for feeder layers. A line with suitable characteristics for ES cell culture can be obtained from the American Type Culture Collection, Mannasas, VA, USA (cat. no. CRL-2225). This line has been transfected with the *neo* and leukemia inhibitory factor, *LIF*, genes, hence is designated SNL.
13. For handling ova: Pasteur pipets pulled by hand over a flame to 0.2 and 0.3 mm in outer diameter (od) and flame polished at the tip.
14. For disaggregating blastocyst outgrowths: Borosilicate glass capillary tubing, 1 mm od and standard wall thickness, pulled and broken to 0.06 mm od and flame-polished at the tip with the aid of a pipet puller and microforge. These can be stored in aluminum blocks with holes drilled at slightly greater than 1 mm od Alternatively, Pasteur pipets can be hand-pulled.
15. For plating of disaggregated blastocyst outgrowths: 4-well tissue culture dishes (Nunc, cat. no. 176740).
16. 10× hyaluronidase solution for removal of cumulus cells from oocytes: 3 mg/mL (Sigma, cat. no. H3884) in medium M2. Stable at 4°C for months. Add 0.2 mL to approx. 1.8 mL medium M2 in a 3-cm Petri dish to give 1x solution.
17. Mouth-controlled aspiration device to control flow of medium in pipets described above ***(17)***. Thin-diameter tubing can be attached to accommodate 1-mm-od glass capillaries.
18. Ovum culture dish: Drops of medium CZB ***(18)*** of approx. 10 µL pipetted in rows onto a 3-cm Petri dish, then overlaid with mineral oil. These dishes will equilibrate in 30 min if mineral oil stock is kept permanently in an incubator. Incubator settings are 37.5°C and 6% CO_2 in air.
19. Cell digest buffer: 50 m*M* Tris-HCl pH 7.5, 20 m*M* EDTA, 0.45% each of the detergents Tween 80 and NP40, and 0.3 mg/mL proteinase K added from powder. Stable at 4°C for at least 2 mo. Do not freeze.

3. Methods

3.1. Preparation of Fibroblast Feeder Layers for ES Cells

STO cells or primary embryo fibroblasts (PEFs) are used as feeder layers for deriving and propagating ES cells (*see* **Notes 2** and **3**).

3.1.1. Propagating STO Cells

1. Thaw vial of frozen STO cells quickly in water bath and add contents directly to 10 mL of STO cell media in a 10-cm plate, then disperse the cells by crosswise agitation. Next day, replace with 10 mL of fresh medium and add 0.1 mL of 100X G418 solution.
2. Passage STO cells when they reach confluency. Rinse plate with 5 mL of DPBS$^-$, add 2 mL of trypsin-EDTA, leave in incubator for 5 min, add 3 mL of media, and pipet cells up and down in plate to produce single cell suspension. Add 4 drops of the cell suspension to a 10-cm plate containing 10 mL of media and 0.1 mL of 100× G418 solution, then disperse the cells by crosswise agitation. This is the propagating plate and is passaged for approx 50 d in this manner, at which time a new vial is plated. It is important to passage this line of plates regularly and to keep it under G418 selection so that cells retain desirable morphology and remain drug-resistant.
3. To obtain cells for making feeders, at each passage of the propagating plate, the unused portion of cells is plated at any density and grown without G418 selection for up to three passages according to the number of feeder cells that will be required. $10–15 \times 10^6$ cells can be obtained from one confluent 15-cm plate.

3.1.2. Deriving and Propagating PEFs

1. Some strains of PEFs grow better than others. One good combination is (C57BL × C3H)F_1 females mated to 129/Sv males.
2. Kill pregnant mouse at 12.5 d post coitum (dpc), saturate with disinfectant solution, and pull skin back with fingers to expose the body wall. With sterile instruments, remove both uterine horns containing the fetuses. Place immediately into a 10-cm plate.
3. Take plate to sterile hood. Slit uterus open and remove up to 10 fetuses. Place them into a fresh 10-cm plate with 10 mL DPBS$^+$. Using #5 watch maker's forceps, pinch off the head and remove liver from each fetus.
4. Transfer the carcasses to a fresh 10-cm plate containing 10 mL DPBS$^+$. Tilt the plate, then, with a 1-mL syringe fitted with an 18-gage needle, draw up each fetus individually into the syringe to shear it into small pieces, then expel into a fresh 10-cm plate.
5. Aspirate DPBS$^+$ from plate, add 10 mL trypsin-EDTA, then leave plate in incubator for 15 min.
6. With 5-mL pipet, transfer trypsin and fetus pieces to 50-mL centrifuge tube, then pipet vigorously up and down to create a cell suspension. Pieces should

Table 1
Plating STO and PEF Feeder Cells

	Volume required for one plate or well		
Plate type	Medium	Feeder stock[a]	Feeder stock/4[b]
3-cm	1 mL	0.5 mL	—
6-cm	2.5 mL	1 mL	—
10-cm	4 mL	3.5 mL	—
4-well	—	—	0.5 mL
12-well	—	—	1 mL
24-well	—	—	0.5 mL

[a] 1.5×10^6 cells/mL.
[b] Four times dilution of feeder stock.

dissociate almost completely. Pipette one embryo equivalent into 15 mL of PEF medium in a 15-cm plate—that is, 10 fetuses gives 10 plates.

7. After overnight culture, aspirate medium from plate and replace with 25 mL of fresh medium. Grow for 3–4 d until cells become confluent. Freeze cells (*see* **Subheading 3.1.4.**).
8. To propagate PEFs in making feeder layers, thaw one vial and transfer contents to 10 mL of PEF medium in a 10-cm plate. PEFs do not need to be grown in gelatinized plates.
9. The next day, change the medium. When the plate becomes confluent, passage the cells into up to three 15-cm plates and grow to confluency. PEFs can be passaged again, but their growth slows substantially in this and subsequent passages.

3.1.3. Making STO and PEF Feeder Layers

1. For one confluent 15-cm plate, aspirate some of the medium so that it just covers the plate. This will be approximately 12 mL. Add 0.12 mL of 1000× mitomycin C solution to achieve a concentration of approx. 5 μg/mL.
2. Leave plate for 1.5–4 h in incubator to mitotically inactivate cells, rinse plate three times with 10 mL of DPBS$^+$, add 5 mL trypsin-EDTA, then incubate plate for 5 min.
3. Add 7 mL medium, pipet up and down to disperse cells, then transfer cells to centrifuge tube. Take aliquot for cell counting, then centrifuge cells at 250*g* for 3 min.
4. Resuspend cell pellet to 1.5×10^6 cells per mL in STO or PEF medium to obtain a stock of feeder cells. Plate this feeder stock, again in STO or PEF medium, according to the guidelines given in **Table 1**. Plates must be thoroughly agitated

crosswise to achieve an even settling of cells. Feeder plates can be used from 4 h to 7 d after plating. Also, the unplated stock can be kept at 4°C for 4 d without significant loss of viability. Freezing feeder cells for plating later works well, although some viability is lost.

3.1.4. Cryopreservation

1. For one confluent 15-cm plate of STO cells or PEFs, trypsinize plate as in passaging (*see* **Subheading 3.1.1.**), transfer 12 mL of cells to centrifuge tube, then pellet cells at 250*g* for 3 min. For one confluent 6-cm plate of ES cells, trypsinize with 1 mL of trypsin-EDTA as in passaging (*see* **Subheading 3.3.6.**), then transfer cells to 3 mL of medium and pellet cells.
2. For STOs and PEFs, aspirate media, then resuspend cells in 1.25 mL of freezing solution I. Slowly add 1.25 mL of freezing solution II while gently swirling the cells, then dispense 0.5-mL aliquots of cells to five cryovials, approx. 2.5×10^6 cells per vial. Keep caps of cryovials with internal thread sterile by standing them on the inside of the 15-cm plate lid. For ES cells, aspirate media, resuspend in 2 mL solution I, then add 2 mL solution II and dispense 1-mL aliquots into four cryovials, approx. 2×10^6 cells per vial.
3. Place cryovials into a polystyrene 15-mL centrifuge tube rack "sandwich" and place at –70°C to –80°C overnight. Alternatively, use commercially available containers designed to cool at a more controlled rate. STO cells, PEFs, and ES cells are stable at –70°C for many months, but it is best to transfer to liquid nitrogen as soon as possible. In the liquid nitrogen tanks, plastic screw-capped cryovials should be stored in the vapor phase.
4. To thaw STOs and PEFs, hold the ampule in awater bath (set at 35°C) until completely thawed, then dispense contents immediately into a 10-cm plate containing 10 mL of media and agitate to disperse cells. For ES cells, thaw as above, then transfer 1 mL of cells to a 15-mL centrifuge tube, slowly add 4 mL of medium while gently swirling the tube, then pellet the cells. Resuspend in 4 mL of media and place into a 6-cm feeder plate. In this step, medium is not warmed prior to use.
5. For all types of cell, change media the next day to remove DMSO.

3.2. Obtaining Blastocysts for ES Cell Derivation

3.2.1. Parthenogenetic

1. Superovulate mice at 4–12 wk of age by injecting them in the peritoneum with 0.1 mL of saline containing 5 U of pregnant mare serum gonadotrophin followed approx. 48 h later with 5 U of human chorionic gonadotrophin (*see* **Note 4**). Eggs are ovulated approx. 12 h after the latter injection, thus 14 h after this injection, kill mice by cervical dislocation, then isolate the oviducts and place them in 2 mL of medium M2 ***(19)*** in a 3-cm Petri dish. For details on dissection, *see* **ref. *20***.

2. Transfer oviducts to 2 mL of 1× hyaluronidase solution in medium M2 and tear open the ampulla to release the "cumulus masses" of follicle cells and oocytes. Place the dish on a warm plate set at 35–37°C and leave for 10 min to release oocytes from follicle cells.
3. Activation of oocytes with ethanol ***(21)***: Using a Pasteur pipet pulled to 0.3 mm od and working with approx. 50 oocytes at a time, transfer them using the mouth-controlled aspiration device to 2 mL of 7% (v/v) ethanol in medium M2 without calcium and magnesium in a 3-cm Petri dish. Mix oocytes into medium by pipetting them up and down and around the dish.
4. After 3 min in the ethanol solution, transfer oocytes to 2 mL of standard medium M2 to wash out ethanol.
5. Transfer oocytes to culture drops of medium CZB ***(18)*** containing 5 μg/mL cytochalasin B or 1 μg/mL cytochalasin D. Culture for 4 h to inhibit extrusion of the second polar body, then transfer oocytes to standard medium CZB and culture for another 4 h.
6. Eight to 12 h after ethanol treatment, select 1-cell ova which have two pronuclei and no polar body. This should be done with a microscope under phase contrast or differential interference contrast optics. Desired ova can be set aside in a micromanipulation chamber using a holding pipet as used in micromanipulation experiments. A significant proportion of the oocytes will be unusable; some will look like 2-cell ova having undergone immediate cleavage, have fragmented—"raspberries," or may contain one pronucleus or micronuclei ***(22)***.
7. Selected ova are cultured to the blastocyst stage in medium CZB. If oocytes of inbred mice are used, it may be necessary to transfer the diploid ova into oviducts of pseudopregant recipients to enable further development.

3.2.2. Gynogenetic

1. Gynogenetic ova are produced from zygotes by pronuclear transplantation; the paternal pronucleus from a zygote is removed and replaced with the maternal pronucleus of another to produce a 1-cell ovum with two maternal pronuclei. Parthenogenetic ova are then cultured in medium CZB and should reach the blastocyst stage by 3.5 dpc or 3.5–4.5 dpc if the egg cytoplasm is from F_1 females or inbred females, respectively. For methods of pronuclear transplantation, *see* ***20***, ***23***, and ***24***. Parental-specific genetic markers are used to ensure that derived cell lines have only maternally derived genomes—for example, a convenient marker is the ubiquitously expressed glucose 6-phosphate isomerase-1 gene located on chromosome 7. Three alleles encode three electrophoretic variants detected in a simple assay ***(20)***: The A and B forms are common among laboratory strains, while the ferally derived C form is present in the 129/Sv//Tac strain (Taconic Farms, Germantown, NY).

3.2.3. Androgenetic

1. Androgenetic ova are produced by pronuclear transplantation as are gynogenetic ova, except that the maternal pronucleus from a zygote is removed and replaced

with the paternal pronucleus of another to produce a 1-cell ovum with two paternal pronuclei; zygotes for this manipulation are obtained by mating F_1 females to males of choice. Androgenetic ova are then cultured in medium CZB. Most should reach the blastocyst stage by 3.5 dpc, although some take an extra day. Parental-specific genetic markers are used to ensure that derived cell lines have only paternally derived genomes.

2. Androgenetic ova can also be produced by removing the maternal pronucleus from a zygote, then culturing the haploid ovum in cytochalasin B or D to inhibit first cleavage and make it diploid. However, only blastocysts of an XX sex-chromosome constitution can be obtained by this method, and for reasons discussed in **Note 5** it is probably best to utilize only XY cell lines.

3.2.4. Uniparental duplication

Due to the high frequencies of chromosomally unbalanced and inviable ova derived from intercrossing mice with reciprocal translocations (*see* **Note 6**), it is best to superovulate and mate females and flush out morulae at 2.5 dpc. Transfer all morula that form blastocysts to feeder layers by 4.5 dpc.

3.2.5. Wild Type

1. For inbred strains, it is best to obtain ova by natural mating. Many inbred strains do not respond well to superovulation. Often the problem lies in obtaining mating and fertilization of ovulated eggs.
2. At 2.5 dpc, dissect out the oviduct and flush out morulae in medium M2. Transfer to medium CZB and culture overnight to the blastocyst stage.

3.3. Deriving and Propagating ES Cell Lines

All culture conditions are 37.5°C and 5% CO_2 in air.

3.3.1. Culture of Blastocysts

1. Ova that have reached the blastocyst stage in medium CZB are treated with acidic Tyrode's solution to remove the zona pellucida ***(20)***. This is done as some blastocysts, especially androgenetic ones, fail to shed this coat and thereby fail to attach to the dish in subsequent steps.
2. Immediately after the zona is removed, 16 or fewer blastocysts are pipetted into one 3-cm feeder-layer plate with ES cell media and incubated.
3. 2 d after being placed in FBS culture media, the blastocysts begin to attach or "implant" to the bottom of the culture dish (**Fig. 1A**). Growth after attachment is rapid and some will already be forming outgrowths. By 3 d virtually all should be attached, and most will be forming outgrowths. By 4 d the outgrowths will be larger, and many are surrounded by trophectoderm giant cells (**Fig. 1B**).

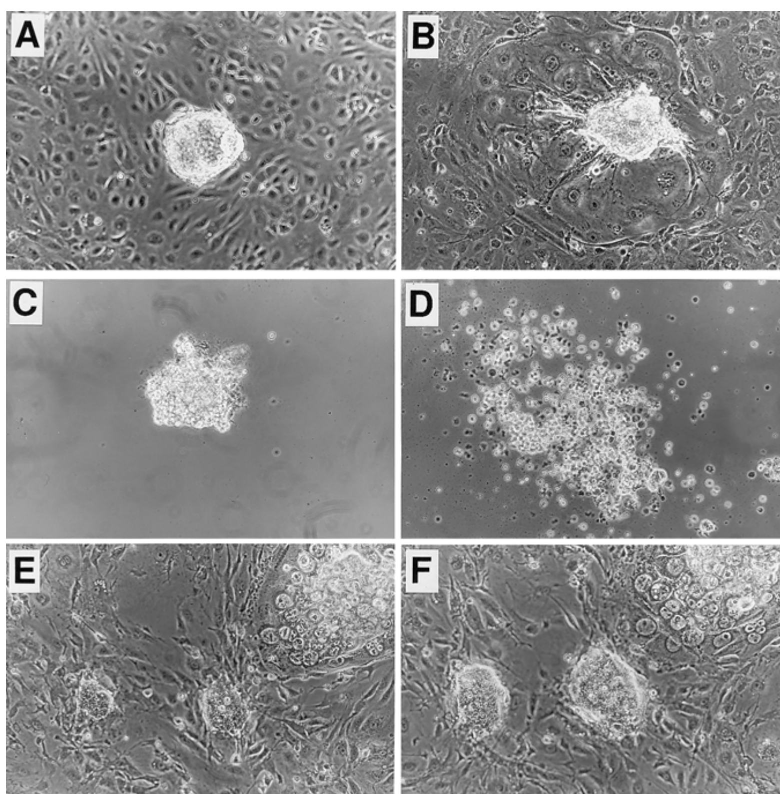

Fig. 1. Manipulation of blastocysts for deriving ES cell lines. **(A)** Blastocyst attached to STO feeder layer 3 d after transfer to feeder plate. ICM can be seen at "3-on-the-dial." **(B)** Blastocyst outgrowth 4 d after transfer to feeder plate. Note layer of trophectoderm cells surrounding outgrowth. **(C)** Picked outgrowth in trypsin-EDTA, **(D)** disaggregated outgrowth, **(E)** two 129/SvImJ ES cell colonies at passage 1 after 3 d of culture; note patch of trophectoderm cells at top right. These colonies were derived from a 4-well passaged in its entirety into a 12-well, and **(F)** colonies in **(E)** after 4 d of culture. All objects at 100× (original magnification) and under phase-contrast optics.

3.3.2. Picking and Seeding Blastocyst Outgrowths

1. In picking outgrowths, our rule of thumb is to pick no later than 1 d after attachment has begun, this attachment being depicted in **Fig. 1A**. This is usually at 4 d after 3.5-dpc blastocysts have been transferred to feeder dishes in FBS

culture media (**Fig. 1B**). Picking can be done at 2 d or more after attachment, but the frequency of cell line derivation may fall. It is difficult to obtain cell lines from outgrowths that have developed a layer of endoderm ***(25)***. Almost all blastocysts of an expected euploid chromosome constitution, that is, wild-type, parthenogenetic, gynogenetic, and androgenetic blastocysts, should attach and produce outgrowths that can be picked, although androgenetic outgrowths are often not vigorous.

2. Before picking outgrowths, a blastocyst outgrowth disaggregation dish is prepared consisting of approx. 5-μL drops of trypsin-EDTA pipetted onto a 6-cm Petri dish in *n* columns of four and overlaid with mineral oil. Also, 1–2 d in advance, 4-well feeder plates are prepared for plating of disaggregated outgrowths. The night before disaggregation, the wells are aspirated and 1 mL of ES medium is added. The next morning, half of this medium is replaced with fresh medim, and 1 drop of FBS is added from a 1-mL pipet to bring the serum concentration to approx. 16%.
3. To pick the outgrowths, rinse the dish with 2 mL of DPBS$^+$ containing 0.01% (w/v) BSA, then add 2 mL of this same solution to the dish.
4. Using a Pasteur pipet pulled to 0.2–0.3 mm od and with a stereomicroscope, nudge or pull off the outgrowth from the trophectoderm layer, then transfer it to a drop of trypsin-EDTA in the blastocyst outgrowth disaggregation dish (**Fig. 1C**). Proceed until all outgrowths in the dish are picked. Between each pick, ensure that there are no cells remaining in the pipet from the previous outgrowth by observing it briefly under the stereomicroscope and pipetting up and down to dislodge them if necessary.
5. Keep the picked outgrowths at RT, then approx. 15 min after the first outgrowth was picked, aspirate a small mount of medium into a glass capillary pulled to 0.06 mm od (**Fig. 1C**). Expel a small amount of medium over an outgrowth, aspirate it into the pipet, expel it back into the drop, then repeat this aspiration/ expulsion step. A near-single-cell suspension should result (**Fig. 1D**). Aspirate all of the cells then expel them into one well of a 4-well feeder plate. Use a new capillary for each disaggregation. After all outgrowths are seeded, place the 4-well plates into the incubator, labeling them "passage 0."
6. After 3 d of culture, aspirate two-thirds of the medium from each well, then add fresh medium to 1 mL. Culture for another 2 d, then examine the wells for growth of primary ES cell colonies.

3.3.3. Selecting and Picking Primary ES Cell Colonies

1. At passage 0, emerging primary ES cell colonies will appear exactly like colonies obtained when plating an existing cell line at very low density—for example, at 200 cells per 3-cm feeder plate. In plating C57BL/6J and CBA/CaJ outgrowths, it is usual that ES cell colonies are the only type of colony that grow vigorously, thus identification is generally straightforward. However, in seeding 129/SvImJ

outgrowths, other types of cell also grow vigorously and often the primary ES cell colonies are obscured or mixed with these cells. In seeding Swiss mouse outgrowths, many colonies that appear similar to ES cells proliferate at passage 0 (*see* **ref. *25*** for a detailed discussion). In any event, at the next passage, the presence of ES cells is revealed, as generally no other cell type continues to proliferate.

2. If putative primary ES cell colonies are observed, at passage 0 and at 6 d after seeding the disaggregated outgrowths, one of two paths can be followed. (1) Individual colonies are picked as described for blastocyst outgrowths except they are placed into 0.06 mL of trypsin-EDTA in a round-bottomed well of a 96-well plate. After 10 min, with a pipetman and barrier tip, 0.1 mL of medium is added, the colony is broken up into a single cell suspension by repeated pipetting, then the well contents dispensed into a well of a 24-well feeder plate and labeled passage 1. With this method there is a high probability that the cell line will be derived from one cell of the outgrowth. (2) Passage all of the contents of the well. This may be desirable if one is uncertain that ES cell colonies have been obtained. Rinse a well with DPBS$^-$, add 0.15 mL of trypsin-EDTA, incubate for 10 min, add 0.3 mL of medium (from a 12-well feeder plate well containing 2 mL of medium), break up cell colonies by repeated pipetting, then seed all 0.45 mL back into the 12-well plate and label it passage 1. With this method, there is a significant chance that the cell line will be derived from more than one cell, and for this reason it might be expected that later clonal derivatives of such a cell line, such as are obtained in gene targeting experiments, may be more heterogeneous in their characteristics than clones obtained from cell lines derived by method 1. In any event, cell lines can be cloned at passage 1 or later.
3. With either method 1 or 2 as described in the previous step, true ES cell colonies will be clearly visible at passage 1 after 3 d (**Fig. 1E,F**). If no colonies are observed after 5 d, it is very unlikely that any will appear and such wells can be discarded. *See* **Notes 7** and **8** for the frequency of cell line derivation per blastocyst.

3.3.4. Primary Expansion of New ES Cell Lines

1. Once ES cell colonies are obtained at passage 1, a new cell line has been derived and most of them should continue to proliferate. However, in a small number of cases, significant differentiation ensues in the next couple of passages and the cell line is lost.
2. To expand the cell line, the cells are passaged in their entirety from the 24-well plate (passage 1) to a 3-cm feeder plate (passage 2), then to a 6-cm feeder plate (passage 3). An extra passage may be required to obtain confluency at this last stage. The number of days at each passage depends on the density of cells, but 2–4 d is usually required. The cells in the confluent 6-cm plate are frozen in four vials and labeled passage 4 or 5, tier I. The cell line derivation schedule is depicted in **Fig. 2**.

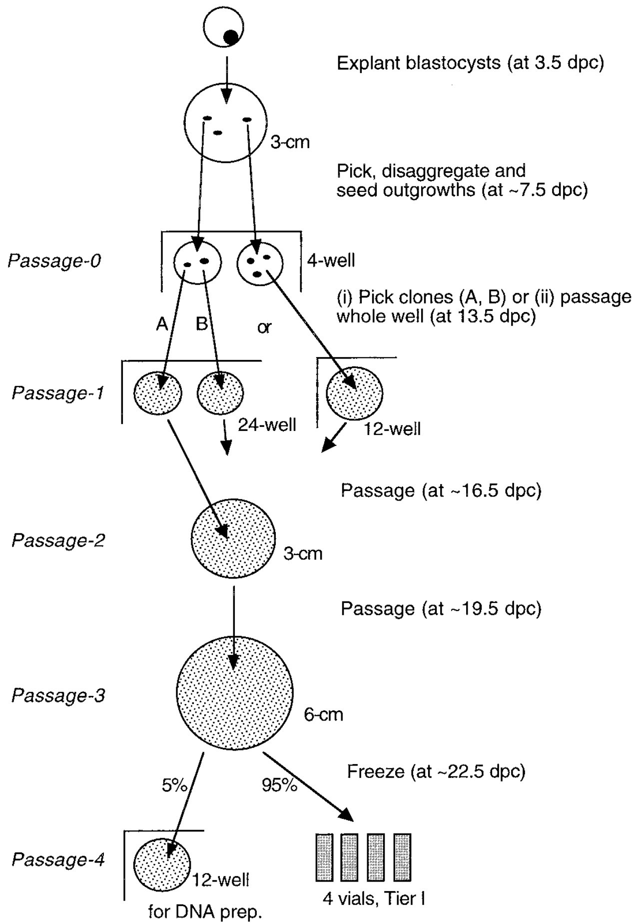

Fig. 2. Steps in the derivation of ES cell lines.

3.3.5. Characterizing New ES Cell Lines

1. Determine Y chromosome status. In freezing the cells for tier I, 5% of the cells are saved and plated into a gelatinized well of 12-well dish without feeder cells (**Fig. 2**). The ES cells should grow without differentiating and, when confluent, add 0.4 mL of cell digest buffer and incubate for at least 4 h at 37–55°C. Purify DNA and perform Southern blot. Probe pY353/B recognizes a repetitive element specific to the mouse Y chromosome ***(26)***. Cut plasmid pY353/B with EcoRI to yield a 1.5 kb-fragment for probe. Digestion of DNA with *Hin*dIII or *Eco*RI yields

strong hybridizing bands at 12 kb and at 1.5 kb, respectively. No hybridization at all is obtained with DNA from female mice. STO cells show relatively weak hybridization of a 7-kb band with *Hin*dIII digestion. Alternatively, metaphase spreads can be C-banded to reveal the Y ***(27,28)***. In deriving 129/SvImJ, C57BL/6J, and CBA/CaJ cell lines, we have obtained approximately equal numbers of pY353/B-positive and -negative lines. The remainder of the DNA can be used for further analysis of genotype.

2. Determine chromosome number. At least 80% of metaphase spreads with 40 chromosomes should be obtained with cell lines to be used for chimera production. Methods for making metaphase spreads are provided ***(25,27,28)***. There is no substitute for empirical observation in determining the efficacy of chimera formation and germ-line transmission, as a cell line or clone that is predominantly euploid may not necessarily form good chimeras.
3. Assess microbiological status ***(29)***.

3.3.6. Propagating ES Cell Lines

1. Plate a vial of cells at tier I—for example, passage 4—into one 6-cm feeder plate and grow to confluency.
2. Trypsinize cells and plate them at 0.5×10^6 cells per 6-cm feeder plate. When plates are confluent, freeze cells at four vials per plate and label them passage 6, tier II. At least 24 vials can be obtained.
3. Tier II vials can be used for further characterization of the cell line or in experiments, but it is desirable to passage tier II cells two or three more times to freeze them at passage 8 or 9, tier III, if the cell line is to be used extensively.
4. In propagating ES cells, plate them at 0.5×10^6 cells per 6-cm feeder plate in 3.5 mL of media (**Fig. 3A**). After 1 d (**Fig. 3B**), add 2 mL of fresh media, and after 2 d (**Fig. 3C**), replace all media with 6 mL of fresh media. After 3 d of growth, the plate is confluent, containing $8–10 \times 10^6$ cells (**Fig. 3D**) and is passaged again.
5. In passaging a confluent plate, if the medium is very acidic or yellow, it is replaced with 3 mL of fresh medium at least 3 h before trypsinization. This increases the viability of the cells upon passage or after cryopreservation. To passage, the plate is rinsed with 3 mL of DPBS$^-$, then 1 mL of trypsin-EDTA is added. After 10 min in the incubator, cells are broken up by vigorous pipetting in the plate with a plugged Pasteur pipet, then the 1 mL of disaggregated cells are dispensed into 4 mL of medium in a centrifuge tube and mixed immediately. Cells are counted, and 0.5×10^6 cells per 6-cm feeder plate are used for further growth (**Fig. 3A**). If the cells are plated at 1×10^6 cells or more per 6-cm plate, then passage may be required after 2 d of growth. Feeder cells and trypsin are carried over at each passage. A rule of thumb is that the trypsin-EDTA solution should not exceed 10% by volume in the culture media. If so, cells should be pelleted to remove it. At trypsinization, it is very important to create a single cell suspension, although it is impossible to avoid getting some doublets and triplets of cells (**Fig. 3A**). If undissociated clumps of ES cells are present, they

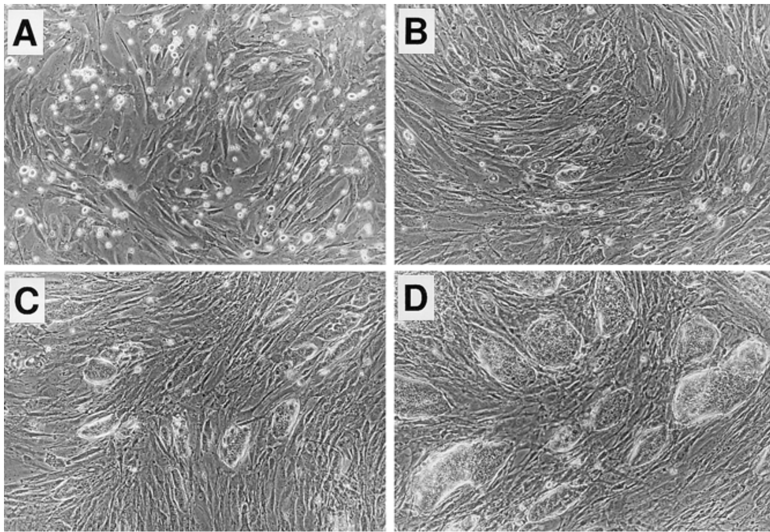

Fig. 3. One passage of a euploid XY C57BL/6J ES cell line derived from a normal blastocyst on a PEF feeder layer. **(A)** ES cells just seeded at 0.5×10^6 cells in a 6-cm plate; note single cell suspension. This same culture showing ES cell colonies at **(B)** 1 d, **(C)** 2 d, and **(D)** 3 d later. At 3 d, there are approx. 8×10^6 cells in the plate (cell doubling time approx. 18 h) and the culture is passaged again. All objects at 100X (original magnification) and under phase-contrast optics.

will become relatively large colonies and may begin to differentiate before the next passage. If this practice is continued, ultimately the cultures will deteriorate.

3.4. Testing Serum Lots

A number of commercial sources of FBS have been tested for growth of ES cells. Nevertheless, it is still a good idea to test different lots, as better or less expensive ones may often be found. To test three unknown against one control serum (control serum could be a sample of a commercial pretested lot):

1. Prepare two 12-well plates of feeder cells at least 1 d before plating ES cells.
2. Make up DMEM with all additives except serum, and dispense 1.35 mL into each well of one plate, and 1.2 mL into each well of the other plate. In the first plate, with a pipetman and barrier tip, for each serum dispense 0.15 mL into three wells (10% wells). Similarly, for the second plate, for each serum dispense 0.3 mL into three wells (20% wells).
3. Trypsinize a near-confluent 3-cm plate of ES cells, pellet cells, replate them into two nongelatinized 3-cm plates without feeders, then place in incubator.

4. After 45 min most feeder cells will have attached to the bottom of dish, while ES cells will be lightly attached at most. At this time, gently aspirate media from both dishes, then with a pipetman and barrier tip, vigorously pipet up and down over the bottom of the dishes with a total of 0.7 mL of media to dislodge ES cells, then dispense into a tube.
5. Count cells, then dilute in DPBS$^+$ to 100 cells per 0.05 mL in a total of 10 mL.
6. Dispense 0.05 mL of cell suspension, that is, 100 cells, into each well of the two 12-well plates, then incubate.
7. Change media 3 d and 6 d after plating, then score clones, or colonies, 8 d after plating.
8. In scoring clones, first check for differentiation: Most colonies should be completely or largely undifferentiated. After this examination, aspirate media, turn plates upside down, and count the number of colonies at the bottom of each well. Choose serum which passed the differentiation test and produced the highest number of clones. An acceptable serum will give approx. 20 clones in both 10% and 20% wells. Growth of clones should have been somewhat faster in the latter wells.

4. Notes

1. DMEM used in ES cell culture typically contains approx. 2.2 g/L rather than the standard 3.7 g/L of sodium bicarbonate. This lowers the osmotic pressure of the DMEM such that it approaches that of media used widely in culture of preimplantation stage ova ***(30,31)***. For this reason it may be better suited for culturing blastocyst-derived cells, although this is anecdotal and ES cells can be cultured successfully in standard DMEM.
2. For feeder layers, investigators generally have a preference for using either PEFs or STO cells. Each type of feeder has been used with equal success, and while the choice of one over the other depends mainly on tradition, these two cell types have different minor advantages. STO cells are immortal and grow rapidly. On the other hand, ES cells can be visualized more readily on PEFs and to many the cultures are more esthetic. If one intends to use STO cells, it is very important to obtain a subline of suitable morphology and that will form a durable feeder layer—for example, the source given in the Materials section. Unsuitable batches of STOs can be subcloned by manually dispensing single cells into wells using a pulled Pasteur pipet or capillary or by seeding at very low density. The morphology of clones is examined 7 d later and desirable ones isolated and expanded. To be useful, newly derived STO cell sublines must be able to support the growth of ES cells plated at very low density—for example, 200 cells per 3-cm feeder plate.
3. Adding LIF to ES cell culture media is generally unnecessary, and this is certainly true if one is using STO feeder cells that are transfected with the *LIF* gene. Some lines of PEF feeder cells expressing NEO are not fully resistant to G418, and it may be necessary to add LIF when ES cells are placed under selection.
4. Oocytes of hybrid females, such as (C57BL female × C3H, CBA, or SLJ male)F_1 are the best for parthenogenetic activation. For inbred strains, C57BL are good,

while 129/Sv are poor. Usually the problems lie not in activation but in the viability of the ovum following the experimental procedure. A potentially gentler method than ethanol treatment for activating oocytes is to culture them for 8 h in medium CZB containing 10 m*M* strontium chloride instead of calcium chloride. Again, 5 μg/mL cytochalasin B or 1 μg/mL cytochalasin D is used to inhibit polar body extrusion ***(32)***. Alternatively, the problem can be circumvented by transplanting the two pronuclei into enucleated parthenogenetically activated haploid ova of hybrid mice ***(23)***. The parthenogenetic ova then develop to the blastocyst stage at high frequency.

5. Both X chromosomes remain active in XX ES cells, and with passage the cells appear to compensate chromosomally for an X:autosome activity ratio that is probably not compatible with long-term survival. In XX parthenogenetic lines, one X often has deletions in the distal region ***(33)***. Of 10 pY353/B-negative lines derived from wild-type 129/SvImJ blastocysts, two were XO and five had only one normal X (K. Fowler and J. Mann, unpublished data). Also, XX embryonal carcinoma cells can possess supernumerary autosomes ***(34,35)***. This chromosomal instability of XX ES cell lines obviously has implications for the production and analysis of chimeras, and careful analysis of karyotype should be carried out before they are used.
6. Mice with maternal or paternal duplication of autosome regions are produced by intercrossing mice heterozygous for reciprocal translocations (*see* Chapter 3). In deriving ES cell lines, it is important to keep in mind that in these intercrosses (1) a high frequency of chromosomally unbalanced zygotes are obtained, such that the frequency of normal postimplantation development is one-third of normal; and (2) of the mice obtained, for the chromosome regions proximal and distal to the translocation breakpoint, one-sixth and one-tenth, respectively, possess the desired maternal or paternal duplication ***(36)***. Thus, one-eighteenth and one-thirtieth of all zygotes, respectively, have the desired genotype. Because of the high frequency of chromosomal imbalance, not all morulae will develop to the blastocyst stage, and not all blastocysts will produce outgrowths. In intercrosses involving the T(7; 15)9H translocation, the frequency of outgrowths per blastocyst was similar to the frequency of 9.5 dpc embryos per implantation site ***(37)***, thus the failure of blastocysts to develop in vivo occurs also in vitro. This is an advantage, as only euploid blastocysts are likely to form new cell lines in this system. The inviability might be expected, as all noneuploid zygotes resulting from intercrosses of reciprocal translocations are partially monosomic or nullisomic for one of the two chromosomes involved. For every mouse chromosome, monosomy is lethal at the peri-implantation stage ***(38)***.
7. Using the conventional means of derivation as described in this chapter, the frequency of ES cell line derivation (per 3.5–4.5 dpc blastocyst not subjected to implantation delay) that we have obtained has varied according to the mouse strain used. With wild-type, parthenogenetic and androgenetic blastocysts of the 129/SvImJ strain, or blastocysts containing significant amounts of this genetic background, a frequency of 50% has generally been obtained. With wild-type

C57BL/6J and CBA/CaJ blastocysts, frequencies have been 10%. CBA strains have been described as resistant to ES cell line derivation when similar methods to those described here have been used ***(39,40)***. Thus, differences between CBA substrains or other undefined experimental variables determine the success of derivation, and the same may be true for many mouse strains. With hybrid wild-type blastocysts derived from crosses of the mouse subspecies *Mus musculus castaneus*, CAST/Ei, that is, (C57BL/6J or 129/SvImJ × CAST/Ei)F_1 and the reciprocal, the frequency has been 90%.

8. Regarding the type of feeder cell used at passage 0, a consistent finding has been that SNL STO cells give approximately double the frequency of cell-line derivation than standard STO cells and PEFs, and a similar phenomenon has been reported previously ***(41)***. The frequency of derivation when using these latter cell types as feeders might therefore be increased by adding LIF to the medium at passage 0.

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